Interaction between the Type II Secretion Components EpsG and EpsL in *Vibrio cholerae*

by

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"And by the way, everything in life is writable about if you have the outgoing guts to do it, and the imagination to improvise. The worst enemy to creativity is self-doubt."

~Sylvia Plath

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Abstract

Gram-negative bacteria utilize the Type II Secretion (T2S) system to transport proteins across the outer membrane. In *Vibrio cholerae*, the T2S apparatus is encoded by 12 different genes in the extracellular protein secretion (*eps*) operon, and promotes secretion of the main virulence factor, cholera toxin. The assembled T2S complex consists of a cytoplasmic ATPase, an inner membrane platform, an outer membrane pore, and five proteins termed pseudopilins that are structurally homologous to the type IV pilins. The pseudopilins also require the prepilin peptidase PilD, which is responsible for cleavage and methylation of the newly generated N-terminus. The pseudopilins are predicted to form a periplasmic pilus-like structure that may act as a piston to promote secretion. Polymerization of the major pseudopilin EpsG is proposed to occur through an energy-dependent process that requires the ATPase, EpsE; however, a key question is how the membrane-bound EpsG interacts with the cytoplasmic ATPase. Studies have shown that EpsE interacts with the bitopic inner membrane protein EpsL; therefore, we hypothesized that EpsG may interact with EpsL in order to be linked to EpsE. In this study we used *in vivo* cross-linking and co-immunoprecipitation to establish an interaction between EpsG and EpsL. PilD was the only T2S component required for the interaction to occur, and both the cleavage and methylation activities of PilD were necessary for EpsG to cross-link with EpsL. Additionally, analysis of mutants generated by site-directed mutagenesis of *epsG* revealed structural domains involved in the

interaction. Replacement of residues in the αβ loop of EpsG reduced the cross-linking with EpsL and may represent a site of direct interaction. Substitution to residues in the C-terminus exhibited either a decrease or increase in cross-linking with EpsL, and indicated that this region may be important for protein stability. All mutations examined that altered the cross-linking between EpsG and EpsL also prevented secretion, demonstrating the importance of their native association within the assembled complex. These findings suggest that EpsL may act as a scaffold between EpsG and EpsE, and possibly function to transduce the energy generated during ATP hydrolysis in order to support polymerization and/or depolymerization of EpsG.

CHAPTER I

Introduction

Significance

The gram-negative bacterium *Vibrio cholerae* is a natural inhabitant of salt and fresh water; however, in humans it is an intestinal pathogen that is responsible for the diarrheal disease cholera (Sack *et al.*, 2004). The bacteria are spread through contaminated food and water, therefore, the disease is highly problematic in countries that have poor water sanitation efforts (Sack *et al.*, 2006). A large infectious dose of *V. cholerae* is required to result in disease, largely due to low bacterial survival during passage through the acidic environment of the stomach (Sack *et al.*, 1972). However, the bacteria that do survive are then able to colonize the small intestine through the production of the toxin co-regulated pilus, or TCP (Taylor *et al.*, 1987, Herrington *et al.*, 1988). Following colonization the bacteria secrete cholera toxin, the major virulence factor contributing to the disease, through the type II secretion (T2S) pathway (Sandkvist *et al.*, 1993). Cholera toxin is an AB_5-subunit toxin whose activity results in constitutive activation of adenylyl cyclase which then leads to an influx of water into the small intestine, thus resulting in the profuse diarrheal nature of the disease (Gill, 1977, Merritt & Hol, 1995). In 2008 the World Health Organization reported 190,130 cases of cholera from 56 countries including 5,143 deaths. However, many cases go unaccounted for and

the true impact of the disease is estimated to be 3-5 million cases per year resulting in 100,000-200,000 deaths (www.who.int; Fact sheet N°107, June 2010).

Type II Secretion

The gram-negative cell envelope is a complex environment consisting of an inner and outer membrane that is separated by the periplasmic space (Silhavy *et al.*, 2010). Gram-negative bacteria have evolved at least six different pathways to transport proteins from the bacterial cytosol across the cell envelope and into the extracellular milieu (Gerlach & Hensel, 2007). One such mechanism, the T2S pathway, involves a two-step process. Proteins to be secreted through the T2S system contain an N-terminal signal sequence that allows them to first be transported across the cytoplasmic membrane by either the Tat or Sec machinery (Voulhoux *et al.*, 2001, de Keyzer *et al.*, 2003). Following cleavage of the signal peptide, the proteins are released into the periplasm and adopt their tertiary conformation before being transported across the outer membrane through the T2S complex (Filloux, 2004). Bacteria utilize the T2S pathway to secrete a wide array of substrates, including virulence factors, which are required for their survival in the environment and/or host; therefore, the T2S system is considered a primary virulence system (Sandkvist, 2001b, Cianciotto, 2009). Although many different types of substrates have been identified as being transported through the complex, the secretion signal for these proteins has yet to be elucidated (DebRoy *et al.*, 2006, Sikora *et al.*, 2011).

In *V. cholerae*, the pathway is required for secretion of the major virulence factor, cholera toxin, as well as lipase, chitinase, chitin-binding protein, hemagluttinin-protease,

the serine proteases VesA, B, and C, and many others (Sandkvist *et al*., 1993, Overbye *et al.*, 1993, Connell *et al.*, 1998, Kirn *et al.*, 2005, Sikora *et al*., 2011). The *V. cholerae* T2S system requires the gene products from the extracellular protease secretion (*eps*) operon, *epsC-N*, and the prepilin peptidase *pilD*, also referred to as *vcpD* or *epsO* (Figure 1.1A) to support secretion (Sandkvist *et al.*, 1997, Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). The assembled components form a cell envelope spanning apparatus consisting of four major parts: (1) a cytoplasmic ATPase, (2) an inner membrane platform, (3) an outer membrane pore, and (4) a periplasmic pilus-like structure termed the pseudopilus (Johnson *et al.*, 2006, Korotkov *et al.*, 2011). The proposed model for how the complex supports secretion is illustrated in Figure 1.1B. Briefly, the cytoplasmic ATPase, EpsE, is proposed to provide the energy for either the assembly or disassembly of the pseudopilus which could then act as a piston to push secreted proteins across the outer membrane pore, or conversely, act as a plug of the outer membrane pore and retract to allow for secretion (Hobbs & Mattick, 1993, Sandkvist, 2001a, Filloux, 2004). The hypothesized model is based upon structural, biochemical, and genetic studies on each of the components. These findings will be elaborated upon for each of the four groups listed above within the following sections.

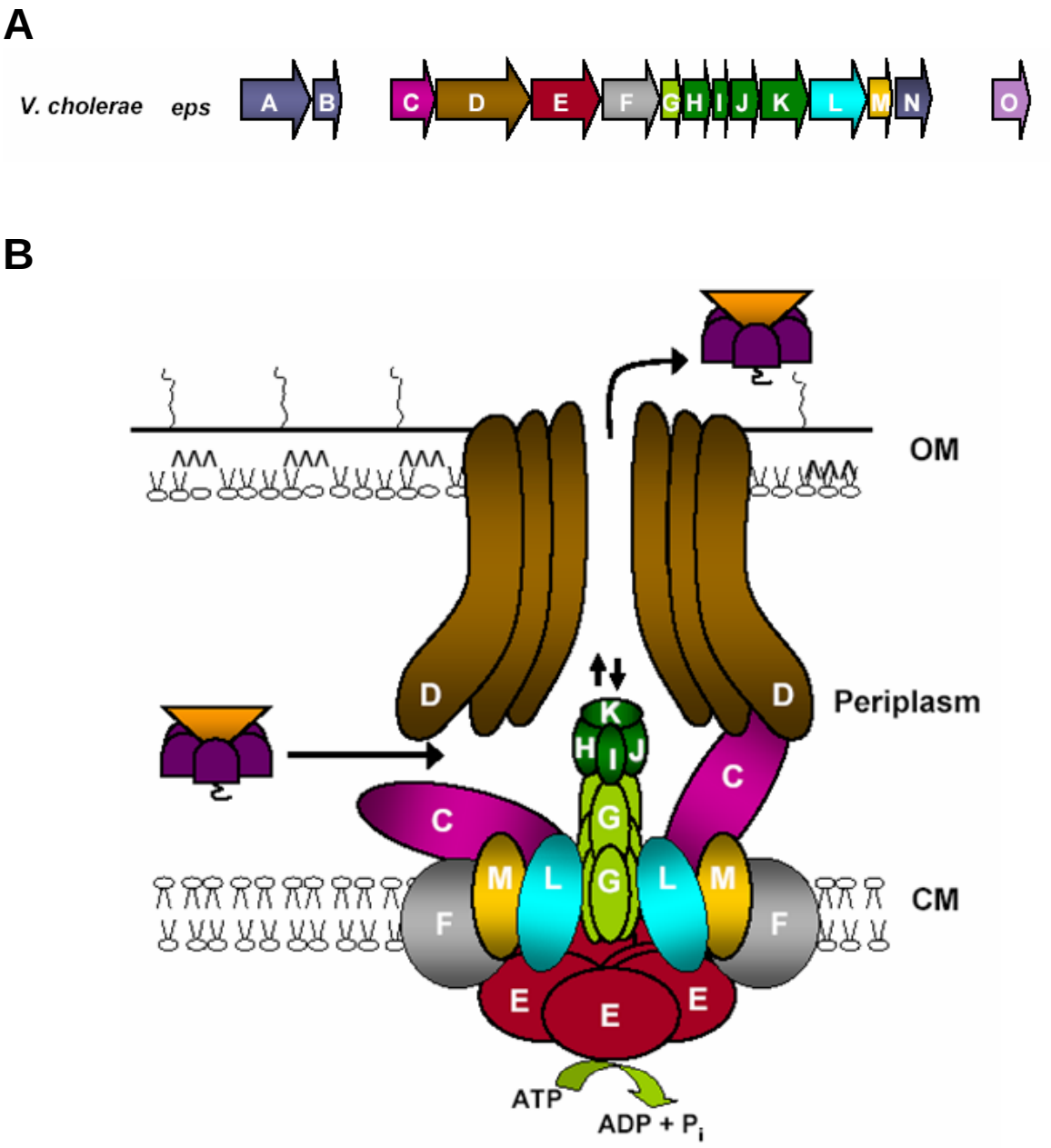

Figure 1.1. A model for the Type II Secretion complex.
A. The *V. cholerae* extracellular protein secretion (*eps*) operon. *epsO* is the prepilin peptidase, more commonly referred to as *pilD* or *vcpD*.
B. The T2S complex is composed of the cytoplamic ATPase, EpsE, the inner membrane complex EpsC, F, L and M, a pilus-like structure referred to as the pseudopilus comprised of EpsG, H, I, J, and K in the periplasmic compartment, and the outer membrane secretin EpsD. A protein to be secreted engages the complex through an unknown mechanism and exits the secretin, possibly promoted by the assembly or disassembly of the pseudopilus. Stochiometry of the components is unknown.

The ATPase

The ATPase, EpsE, belongs to a large superfamily of typeII/IV secretion NTPases. This family of NTPases includes molecular motors involved in a wide array of functions including type IV pilus assembly and disassembly, archael flagella assembly, DNA uptake, and protein secretion (Planet *et al.*, 2001, Robien *et al.*, 2003, Savvides *et al.*, 2003, Satyshur *et al.*, 2007, Yamagata & Tainer, 2007). Structures of these ATPases indicate that they are composed of a C-terminal domain containing a typical ATP-binding Walker A box and a less conserved Walker B box, and an N-terminal domain that is separated from the C-terminus by a short flexible linker (Possot & Pugsley, 1994, Robien *et al*., 2003, Thomsen & Berger, 2008). The subunits are proposed to form a hexameric ring as several members of this family were crystallized as hexamers (Satyshur *et al*., 2007, Yamagata & Tainer, 2007, Misic *et al.*, 2010). Although full length EpsE has not been crystallized, the monomeric structure for an N-terminally deleted form of EpsE has been determined and it crystallized in a helical arrangement, an occurrence that has been reported for other hexameric ATPases (Story *et al.*, 1992, Sawaya *et al.*, 1999, Robien *et al*., 2003). Furthermore, a small fraction of purified EpsE eluted as a hexamer and the hexameric form was capable of hydrolyzing ATP (Camberg *et al.*, 2007), suggesting that oligomerization is required for activity. Interestingly, the hexameric structures from related ATPases were found in two different conformations, with subunits in open and closed conformations, dependent upon if ATP or ADP was bound (Satyshur *et al*., 2007, Yamagata & Tainer, 2007, Misic *et al*., 2010). This indicates that EpsE undergoes a dynamic conformational change as ATP is hydrolyzed. Recently, EpsE was modeled as a hexameric ring and substitutions to residues within the subunit interfaces abolished T2S

as well as ATPase activity, further strengthening the hypothesis that EpsE function is dependent upon oligomerization (Patrick *et al.*, 2011).

EpsE has been shown to be an Mg^{2+}-dependent ATPase and activity was stimulated in the presence of cardiolipin and the cytoplasmic domain of the bitopic inner membrane protein EpsL (Camberg & Sandkvist, 2005, Camberg *et al.*, 2007). Mutational studies determined that the first 90 residues of EpsE are involved in the interaction with EpsL (Sandkvist *et al.*, 1995), and this finding was confirmed by co-crystallization of the EpsE N-terminus with the cytoplasmic domain of EpsL (Abendroth *et al.*, 2005). Furthermore, the interaction between EpsE and EpsL are required for EpsE to associate with the inner membrane and thereby be recruited to the rest of the T2S complex (Sandkvist *et al.*, 1995). Interestingly, the structure of EpsL's cytoplasmic domain revealed a unique actin-like domain (Abendroth *et al.*, 2004a), although no ATPase activity has ever been observed with EpsL. Studies from the EpsE and EpsL homologs from *Dickeya dadantii* (previously classified as *Erwinia chrysanthemi*) have suggested that movement in the N-terminus of EpsE due to ATP hydrolysis may cause a conformational change within the periplasmic domain of EpsL (Py *et al.*, 2001). Thus, it is likely that the interaction between EpsE and EpsL allows for transduction of the energy produced by ATP hydrolysis to support secretion through the T2S complex.

The Inner Membrane Platform

EpsC, F, L and M compose the inner membrane platform (Johnson *et al.*, 2006). As mentioned above, EpsL is known to interact with EpsE; however, it has also been shown to associate with other proteins of the inner membrane platform, with the EpsL-

EpsM complex being the best characterized (Sandkvist *et al.*, 1999, Sandkvist *et al.*, 2000b, Johnson *et al.*, 2007). EpsM is a bitopic membrane protein, although it has a much smaller cytoplasmic region in comparison to EpsL (Abendroth *et al.*, 2004b). However, the periplasmic domains of EpsL and EpsM are of similar sizes and structurally homologous (Abendroth *et al*., 2004b, Abendroth *et al.*, 2009a). The periplasmic domains of EpsL and EpsM form a stable complex that protect each other from proteolytic degradation (Sandkvist *et al*., 1999, Possot *et al.*, 2000, Sandkvist *et al.*, 2000a), and fluorescence microscopy has further illustrated the importance of their interaction. A chromosomally encoded *gfp*-tagged construct of *epsM* was shown to have a punctuate distribution within the cell envelope of *V. cholerae* and this localization was dependent on the presence of EpsL (Lybarger *et al.*, 2009). While the exact stochiometry of the complex is unknown both EpsL and EpsM have been shown to form dimers, although the arrangement of the dimers formed by each is unique (Abendroth *et al*., 2004b, Abendroth *et al*., 2009a); therefore, it is possible that they form heterodimers within the inner membrane (Sampaleanu *et al.*, 2009).

An EpsE-EpsL-EpsM trimolecular complex has been identified in *V. cholerae* (Sandkvist *et al*., 2000a), likely due to the direct interaction between EpsE and EpsL as EpsM does not interact with EpsE in the absence of EpsL. In *D. dadantii* and *Pseudomonas aeruginosa* a complex composed of the homologs for EpsE, L, M and F has also been demonstrated (Py *et al*., 2001, Robert *et al.*, 2005b). The incorporation and stability of the *P. aeruginosa* EpsF homolog required the presence of the EpsE, L and M homologs (Robert *et al*., 2005b). Analysis of EpsF homologs indicated that it crosses the inner membrane twice and has two large cytoplasmic domains (Thomas *et al.*, 1997, Arts

et al., 2007a). The recently solved structure of the first cytoplasmic domain revealed that it adopts a novel fold and sequence similarity suggests that the second cytoplasmic domain also exhibits this unique structure (Abendroth *et al.*, 2009b). The second cytoplasmic domain of the *P. aeruginosa* EpsF homolog was indicated to be involved in the interaction with the EpsE, L, and M homologs (Arts *et al*., 2007a). Although the function of EpsF is unknown, it has been speculated that its interaction with the other members of the inner membrane platform may help to modulate the ATPase activity of EpsE (Craig & Li, 2008).

The fourth component of the inner membrane platform is EpsC. EpsC homologs from *P. aeruginosa, Klebsiella oxytoca* and *Xanthomonas campestris* have been shown to interact with the homologs for EpsL and M (Bleves *et al.*, 1999, Possot *et al.*, 1999, Gerard-Vincent *et al.*, 2002, Lee *et al.*, 2004). This interaction protected all three components from proteolytic degradation and may aid in stabilizing the complex within the inner membrane. Additionally, in *K. oxytoca* the EpsC homolog was found in a larger complex with the homologs for EpsE, L, and M (Possot *et al.*, 2000). Structurally, EpsC is a bitopic inner membrane with a short cytosolic extension and a periplasmic domain containing a high homology region, or HR domain, and a C-terminal PDZ domain (Korotkov *et al.*, 2006). PDZ domains are typically involved in protein-protein interactions (van Ham & Hendriks, 2003). Although the exact function of EpsC's PDZ domain is unknown, a study from *D. dadantii* suggested that this region may play a role in the recognition of T2S substrates (Bouley *et al.*, 2001). The HR domain of EpsC has been shown to interact with EpsD (Bleves *et al*., 1999, Possot *et al*., 1999, Robert *et al.*, 2005a, Korotkov *et al*., 2006), the component of the outer membrane pore, and is thought

to act as a bridge between the inner and outer membranes. This interaction will be further discussed below.

The Outer Membrane Pore

EpsD is the only component of the outer membrane pore in *V. cholerae.* EpsA and EpsB have been implied to play a role in the stabilization of EpsD oligomers (Strozen *et al.*, 2011), and other bacterial species also require the presence of a lipoprotein, called a pilotin, to be effectively transported to the outer membrane (Korotkov *et al*., 2011). EpsD belongs to a large superfamily of proteins referred to as secretins that form outer membrane complexes that are involved in T2S, type III secretion, type IV pilus biogenesis, and filamentous phage extrusion (Korotkov *et al.*, 2011). Once assembled in the outer membrane, secretins form a large complex consisting of 12-14 subunits that are characterized by being resistant to heat and detergent (Bitter *et al.*, 1998, Nouwen *et al.*, 2000, Chami *et al.*, 2005).

Stucturally, the EpsD monomer consists of five domains, a C-terminal beta-rich domain that anchors the protein in the outer membrane and four N-terminal domains that extend deep into the periplasm (Reichow *et al.*, 2010). 3D cryo-EM studies of the assembled secretin have illustrated a cylindrical arrangement of the subunits that contain a narrow constriction site within the periplasm termed the periplasmic gate, and a wider gate structure near the extracellular surface (Chami *et al*., 2005, Reichow *et al*., 2010). These gates are proposed to open to allow for secretion and then close to preserve the integrity of the cell envelope (Korotkov *et al*., 2011). When constricted, the periplasmic vestibule is proposed to be 55 Å wide and then expand to a diameter of 70-90 Å in order

to support secretion (Reichow *et al*., 2010). These measurements would accommodate the passage of the 65 Å wide assembled cholera toxin from *V. cholerae*. Furthermore, surface plasmon resonance has shown that the periplasmic domain of the ETEC EpsD homolog can bind to the B-subunit of heat-labile enterotoxin (Reichow *et al*., 2010), and electron microscopy has identified *V. cholerae* EpsD in a complex with the cholera toxin B-pentamer (Reichow *et al.*, 2011).

As mentioned above EpsC has been shown to interact with EpsD and therefore connect the inner and outer membrane components. The first indication that EpsC and EpsD may interact came from findings in *P. aeruginosa* and *K. oxytoca* that demonstrated that the EpsC homolog was less stable in the absence of the EpsD homolog (Bleves *et al*., 1999, Possot *et al*., 1999). More recently, an *in vitro* study from *V. cholerae* used a truncated form of EpsC containing only the HR domain to establish that this was the region of EpsC involved in the interaction (Korotkov *et al*., 2006). Furthermore, fluorescent microscopy using a chromosomal *gfp*-tagged construct of *epsC* illustrated that EpsD was required for GFp-EpsC to form discrete foci within the cell envelope (Lybarger *et al*., 2009). Both EpsC and EpsD were also essential for Gfp-EpsM to localize in a similar focal pattern. These findings have suggested that the assembly of the secretin in the outer membrane might be a prerequisite for the inner membrane components to be recruited to the T2S machinery.

The Pseudopilus

EpsG, H, I, J and K have been termed the "pseudopilins" due to their N-terminal sequence similarity with the type IV pilins (Nunn, 1999). The relative abundance of

EpsG compared to the other pseudopilins has led to it being termed the major pseudopilin, whereas EpsH, I, J, and K have been termed the minor pseudopilins (Sandkvist *et al*., 1997, Bleves *et al.*, 1998, Nunn, 1999). Both T2S pseudopilins and the type IV pilins contain a conserved N-terminal leader peptide that is required for transport across the cytoplasmic membrane (Arts *et al.*, 2007b, Francetic *et al.*, 2007). Following insertion into the cytoplasmic membrane the N-terminus is cleaved off and the newly generated N-terminus is methylated by the prepilin peptidase, PilD (Pugsley & Dupuy, 1992, Nunn & Lory, 1993, Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). Interestingly, for bacteria that contain a T2S system and type IV pilus the same prepilin peptidase is used for processing each type of subunit.

Reports from *K. oxytoca* and *X. campestris* have shown that the homolog for EpsG can assemble into multimers, indicating that these proteins may be able to form a pilus-like structure, or "pseudopilus" (Pugsley, 1996, Hu *et al.*, 2002). More recent studies using *K. oxytoca* and *P. aeruginosa* confirmed the assembly of the pseudopilus by demonstrating that over-expression of the *epsG* homolog resulted in the presence of bundled surface pseudopili (Sauvonnet *et al.*, 2000, Durand *et al.*, 2003, Vignon *et al.*, 2003). Immunogold-labeling of the surface pseudopili suggested that the structures were composed predominately of the EpsG homolog (Sauvonnet *et al*., 2000, Durand *et al*., 2003), strengthening the hypothesis that EpsG is indeed the major subunit of the pseudopilus. In addition, the over-production of these EpsG homologs inhibited secretion through the T2S pathway (Durand *et al*., 2003, Vignon *et al*., 2003). This result suggests that surface pseudopili may be going through the outer membrane pore and prevented the export of proteins by occluding the secretin. These findings have led to a

model where the pseudopilin components polymerize a pseudopilus, which could either act as a piston to push secreted proteins through the outer membrane pore, or as a plug within the secretin that could depolymerize in order to allow for proteins to be secreted (Hobbs & Mattick, 1993, Filloux, 2004, Johnson *et al*., 2006). Surface pseudopili are not detected during native expression of the *epsG* homologs; therefore, the assembled pseudopilus is hypothesized to only span the periplasm.

Some insight into the role of the minor pseudopilins has come from studies on *P. aeruginosa* and *K. oxytoca* using the methodology of over-expressing the *epsG* homolog and monitoring the production of surface pseudopili. The EpsI homolog was the only minor pseudopilin found to be required for the production of surface pseudopili (Sauvonnet *et al*., 2000, Durand *et al.*, 2005). Conversely, deletion of the *epsK* homolog increased the length and number of surface pseudopili, and over-production of the EpsK homolog abolished the presence of surface pseudopili (Vignon *et al*., 2003, Durand *et al*., 2005). These results suggest that EpsI may be an initiation factor required for the pseudopilus to form, and that EpsK may be a termination factor that stops polymerization of the pseudopilus. Neither the EpsH nor EpsJ homologs had a visible effect on the production of surface pseudopili (Vignon *et al*., 2003, Durand *et al*., 2005).

In recent years the structures for all 5 pseudopilins have been determined for several organisms (Kohler *et al.*, 2004, Korotkov & Hol, 2008, Yanez *et al.*, 2008b, Korotkov *et al.*, 2009, Alphonse *et al.*, 2010), allowing a better view into what role these proteins may be playing in T2S. The structures for the pseudopilins further confirmed the homology with the type IV pilins (Hansen & Forest, 2006), with both groups containing an N-terminal alpha helix, a variable domain, and a C-terminal anti-parallel

beta sheet that completes the pilin fold (Figure 1.2). The structure for EpsG has been resolved for *V. cholerae* and *Vibrio vulnificus*, as well as the EpsG homologs in *K. oxytoca*, *P. aeruginosa*, and EHEC; however, there was slight variation amongst the structure from these species. *K. oxytoca* illustrated a C-terminal beta strand which was observed to be a helical conformation in the other species (Figure 1.2B) (Kohler *et al*., 2004, Korotkov *et al*., 2009). This difference is believed to be an artifact caused by a beta strand swap during the *K. oxytoca* crystallization process. Interestingly, Korotkov *et al*. found a calcium binding loop within the C-terminus of three separate organisms: *V. cholerae* (Figure 1.2A), *V. vulnificus*, and EHEC; and the sequence of the calcium coordinating side chains is highly conserved among all EpsG homologs (Korotkov *et al*., 2009). Disruption of the amino acids coordinating the calcium ion inhibited T2S illustrating that the calcium is playing an important role during secretion, perhaps acting as a stabilizing factor for EpsG (Korotkov *et al*., 2009). Although this is the first observation of metal coordination as a stabilizing factor for the major pseudopilin, other forms of stabilization had previously been observed for other bacterial fibrous arrangements: the type IV pilins utilize a disulfide bridge, and the thin fibers of gram positive organisms are stabilized by isopeptide bonds (Hansen & Forest, 2006, Kang *et al.*, 2007).

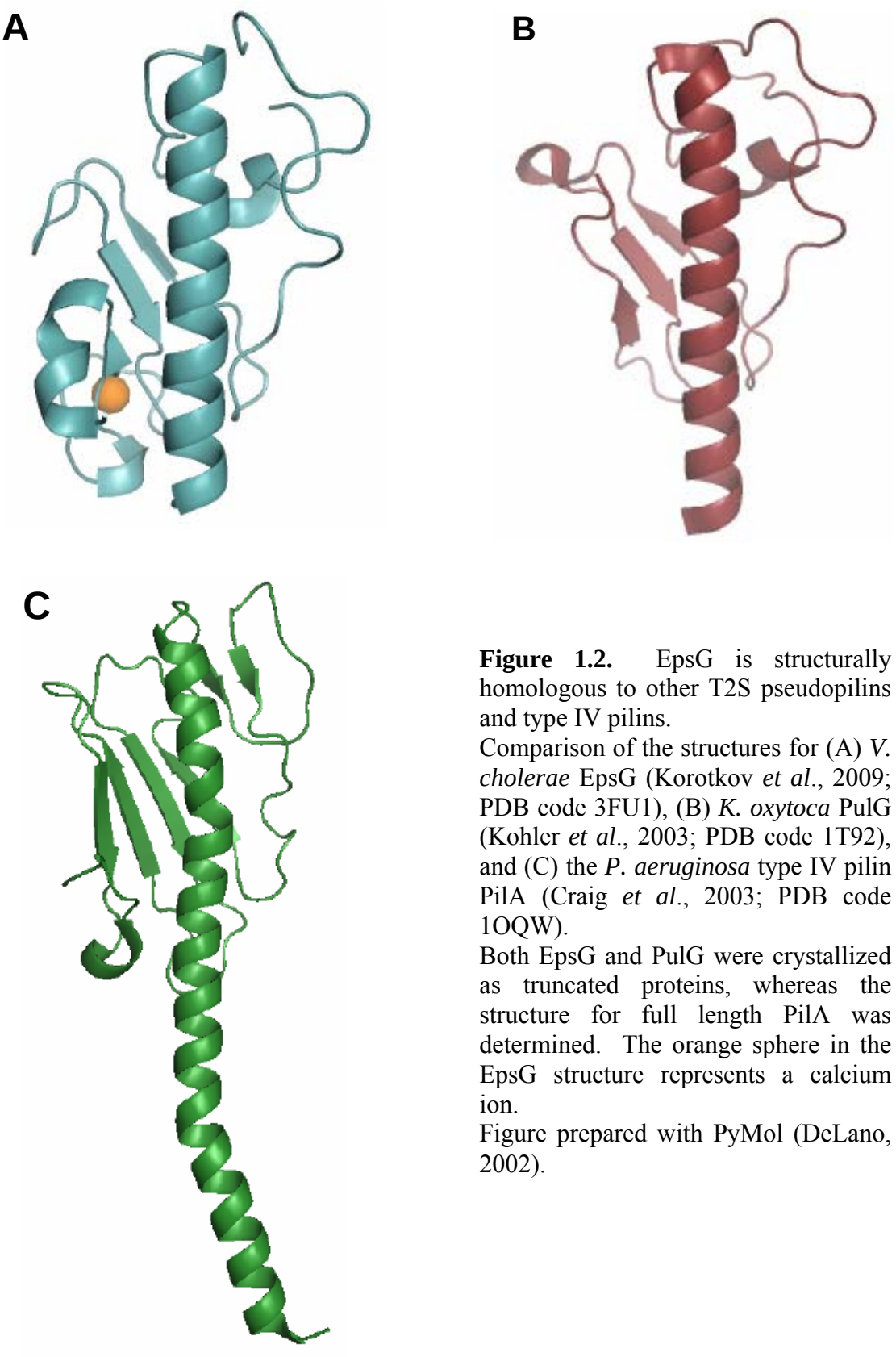

Figure 1.2. EpsG is structurally homologous to other T2S pseudopilins and type IV pilins.
Comparison of the structures for (A) *V. cholerae* EpsG (Korotkov *et al*., 2009; PDB code 3FU1), (B) *K. oxytoca* PulG (Kohler *et al*., 2003; PDB code 1T92), and (C) the *P. aeruginosa* type IV pilin PilA (Craig *et al*., 2003; PDB code 1OQW).
Both EpsG and PulG were crystallized as truncated proteins, whereas the structure for full length PilA was determined. The orange sphere in the EpsG structure represents a calcium ion.
Figure prepared with PyMol (DeLano, 2002).

Despite exhibiting the typical pilin fold, a great degree of dissimilarity was found within the variable domain amongst the minor pseudopilins. Both EpsH and EpsJ have large, elaborate variable domains, whereas EpsI and EpsK have small variable domains (Korotkov & Hol, 2008, Yanez *et al.*, 2008a, Yanez *et al*., 2008b). In addition, EpsK is the largest of the pseudopilins and contains a unique alpha domain that is absent in all other pseudopilins (Korotkov & Hol, 2008). The structures for the minor pseudopilins also gave insight into their interactions with each other. EpsJ and EpsI initially crystallized as a heterodimer and were subsequently shown to form a ternary complex with EpsK (Korotkov & Hol, 2008, Yanez *et al*., 2008a). It has been suggested that the EpsI, J, K complex may be positioned at the tip of the pseudopilus and therefore, serve as a cap to the pseudopilus. This hypothesis further supports the previous finding that EpsK may act as a termination factor for pseudopilus growth; the bulky alpha domain of EpsK would prevent the pseudopilus from exiting the secretin pore.

In addition to the structural data, both genetic and biochemical approaches have been utilized to illustrate interactions between the pseudopilins. Biochemical anaylsis of the *P. aeruginosa* EpsI, J, and K homologs confirmed the presence of a trimolecular complex identified through crystallization, and also suggested that the EpsH homolog can be incorporated to form a quaternary complex (Douzi *et al.*, 2009). In *X. campestris*, the EpsG homolog was found in heterodimers with the EpsH, I, and J homologs (Kuo *et al.*, 2005). The EpsH homolog was found to be associated with the EpsG, I, and J homologs, however, the absence of H disrupted the G-J and I-J interactions (Kuo *et al*., 2005). Additionally, a yeast two hybrid assay from *D. dadantii* showed that both the EpsJ and EpsH homologs can form homodimers, and a J-I interaction was observed (Douet *et al.*,

2004). These findings suggest that the interactions between the pseudopilins are complex and each individual pseudopilin is playing an important role in the formation of the pseudopilus; however, less is known about the interactions between the pseudopilins and other components of the T2S complex. Surface plasmon resonance has indicated that a trimolecular complex of the ETEC EpsI-J-K homologs can bind to the periplasmic domain of the EpsD homolog (Reichow *et al*., 2010). The *D. dadantii* yeast two hybrid analysis also suggested an interaction between the EpsJ and EpsD homologs, as well as between the EpsJ and EpsL homologs (Douet *et al*., 2004). Additionally, a point mutation in the gene for the *P. aeruginosa* EpsG homolog that inhibited secretion could be suppressed by a secondary point mutation in the *epsE* homolog (Kagami *et al.*, 1998). Taken together, these data support a model where the pseudopilins are polymerized within the inner membrane, presumably due to the energy provided by the ATPase, and that EpsJ is part of a complex with EpsI and EpsK at the pseudopilus tip, which then interacts with the secretin (Figure 1.1B).

The T2S system is homologous to the Type IV Pilus biogenesis machinery

The type IV pilus is an extracellular appendage involved in a multitude of functions including twitching motility, adhesion, biofilm formation, secretion, DNA uptake, and host cell invasion (Craig & Li, 2008). Two subclasses of type IV pili have been identified based upon the length of the leader peptide sequence as well as the overall amino acid length (Strom & Lory, 1993, Pelicic, 2008). Type IVa pilins have a shorter leader sequence and are typically 150-160 amino acids, whereas type IVb pilins have a longer leader sequence, different +1 residues, and can range from 40-200 amino acids

(Craig *et al.*, 2003, Craig *et al.*, 2006). Although both subclasses adopt a typical pilin fold, the T2S pseudopilins are more homologous to the type IVa pilins, such as PilA from *P. aeruginosa* (Figure 1.2C).

In addition to the structural homology between the type IV pilins and T2S pseudopilins, the overall architecture of the type IV pilus complex is highly similar to the T2S system (Ayers *et al.*, 2010). Assembly of the type IV pilus requires a cytoplasmic ATPase(s) that is within the same superfamily of proteins as EpsE, an inner membrane platform, and an outer membrane secretin that is in the superfamily including EpsD (Figure 1.3) (Carbonnelle *et al.*, 2006). Although the components between the two systems share low sequence similarity, crystallization studies have demonstrated that the components of the type IV pilus machinery are structurally homologous to their predicted T2S functional equivalent (Collins *et al.*, 2004, Collins *et al.*, 2007, Satyshur *et al*., 2007, Sampaleanu *et al.*, 2009, Karuppiah & Karuppiah *et al.*, 2010, Misic *et al*., 2010, Derrick, 2011). Table 1.1 lists the different functions and protein names for the *V. cholerae* T2S and *P. aeruginosa* type IV pilus complexes.

Besides the difference in the production of an extracellular pilus by the type IV machinery in contrast to the T2S periplasmic pseudopilus, several other differences exist between the two systems. The equivalent of T2S EpsL is broken down into two proteins for the type IVa pilus: PilM, an inner membrane protein displayed on the cytoplasmic face that is structurally homologous to the cytoplasmic domain of EpsL (Karuppiah & Derrick, 2011), and PilN, an inner membrane protein displayed within the periplasm whose modeling indicated that it is structurally homologous to the periplasmic domain of

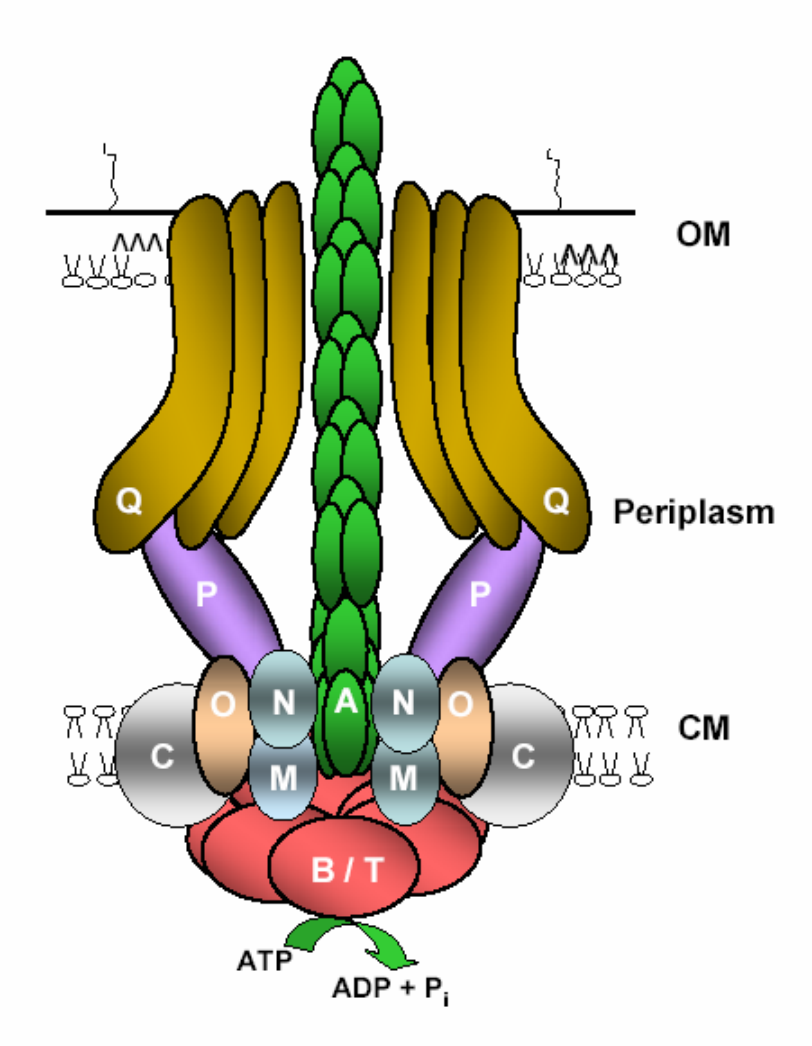

Figure 1.3. Model for the *P. aeruginosa* type IVa pilus biogenesis machinery.
The type IV pilus complex contains many components that are structurally and/or functionally related to the T2S machinery (compare model with Figure 1.1B). Key differences between the T2S and type IV pilus complexes include the number of ATPases (PilB and PilT for the assembly and disassembly of the type IV pilus), the functional equivalent of EpsL is split into PilM and N, and the production of a surface pilus in comparison to the T2S periplasmic pseudopilus. Stochiometry of the components is not known.

Table 1.1. Similarity between the T2S system and type IV pilus machinery.

Function	***V. cholerae* T2S**	***P. aeruginosa* type IV pilus**	**Structural similarity**
(Pseudo)pilus	EpsG	PilA	Yes
ATPase	EpsE	PilT[1]	Yes
		PilB[2]	ND
Inner Membrane Platform	EpsC	PilP	Yes
	EpsF	PilC	Yes
	EpsL	PilM[3]	Yes
		PilN[4]	Yes
	EpsM	PilO	Yes
Secretin	EpsD	PilQ	Yes
Prepilin Peptidase	PilD	PilD	ND

ND indicates not determined.
[1]PilT is the retraction ATPase.
[2]PilB is the assembly ATPase.
[3]PilM is homolgous to the cytoplasmic domain of EpsL.
[4]PilN is homolgous to the periplasmic domain of EpsL.

EpsL (Sampaleanu *et al*., 2009). Although the structure for PilN has yet to be resolved, the structure for the EpsM homolog, PilO, has been determined and PilN was modeled based upon sequence homology with PilO (Sampaleanu *et al*., 2009). This further highlights the similarities between the T2S system and type IV pilus complex as the periplasmic domain of EpsL is structurally homologous to EpsM (Abendroth *et al*., 2009a). Additionally, like the EpsL-EpsM complex, PilN and PilO were show to form a stable heterodimer that protected each other from proteolytic degradation (Sampaleanu *et al*., 2009).

The component for the type IVb pilus that is proposed to be functionally equivalent to EpsL is a bitopic membrane protein (Tripathi & Taylor, 2007). One explanation for the difference in EpsL's homologs for the type IV pilus systems may be related to the diverse functions and energy requirements for type IVa and type IVb pili. Type IVa pili are capable of retracting in order to promote twitching motility and studies of this subclass have shown that they encode at lease two different ATPases, one specifically for assembly of the pilus and one for disassembly (Turner *et al.*, 1993, Merz *et al.*, 2000, Carbonnelle *et al*., 2006). Conversely, type IVb pili only encode one ATPase specifically for assembly and do not retract once formed (Iredell & Manning, 1997, Pelicic, 2008), possibly due to their predominant role in aggregation and adhesion. Thus, the difference in EpsL homologs for the type IVa and type IVb pili may be related to the number of ATPases required to support each complex. Interestingly, the structure of PilM from the type IVa pilus complex of *Thermus thermophilus* found a unique fold that was capable of binding ATP (Karuppiah & Derrick, 2011); although ATP hydrolysis by PilM was not observed this finding may also account for the different ATPase

requirements for the type IV pilus subclasses. Furthermore, this domain is absent from the cyto-EpsL structure, and may represent a key difference between forming a pseudopilus or type IV pilus.

The requirement for the type IV pilus ATPases were discovered through chromosomal deletion of the genes encoding them and then monitoring the production and/or retraction of the pilus (Iredell & Manning, 1997, Merz *et al*., 2000, Carbonnelle *et al*., 2006). As the T2S system only encodes one ATPase and the pseudopilus does not extend past the outer membrane during native conditions, determining the function has been more difficult. However, over-production of the EpsG homolog in *K. oxytoca* or *P. aeruginosa* illustrated that the respective EpsE homolog was required for the production of surface pseudopili (Sauvonnet *et al*., 2000, Durand *et al*., 2005), indicating that the T2S ATPase is likely involved in assembly of the pseudopilus. One key question for both the T2S and type IV pilus machineries is how the (pseudo)pilus and ATPase are able to interact since they reside in different compartments of the bacteria, and moreover, if this is a direct or indirect interaction. The purpose of this study was to address that question for the *V. cholerae* T2S complex and the rationale and findings will be discussed in detail below.

Purpose of this Study

It has been suggested that the ATPase may directly interact with the major pseudopilin. A report from *P. aeruginosa* demonstrated that a point mutation in the *epsG* homolog which inhibited secretion could be suppressed by an additional point mutation within the *epsE* homolog (Kagami *et al*., 1998). A direct interaction between EpsE and

EpsG would involve the ATPase being able to insert into the cytoplasmic membrane since EpsE is a cytoplasmic protein and EpsG is located within the inner membrane prior to assembly. It is also possible that an additional T2S member found within the inner membrane may act as a facilitator to connect EpsG and EpsE. In support of this likelihood, the location of the EpsE suppressor in the *P. aeruginosa* homolog occurs within the N-terminal domain that is known to interact with the inner membrane protein EpsL (Abendroth *et al*., 2005). Furthermore, over-expression of the *K. oxytoca epsG* homolog in a strain deficient for the *epsL* homolog did not produce surface pseudopili, indicating that the EpsL homolog is also required for assembly (Sauvonnet *et al*., 2000). From these findings we hypothesized that EpsL is directly interacting with EpsG to provide a link between the ATPase and pseudopilus.

In the current study I sought to address this hypothesis through the following aims:

I. Identify the interaction between EpsG and EpsL, and determine which other T2S components are required for the interaction occur.

II. Investigate the structural domains of EpsG that are involved in the interaction with EpsL.

Chapter II addresses the first aim by using *in vivo* cross-linking and co-immunoprecipitation to identify an interaction between EpsG and EpsL. The interaction was observed in the absence of the other genes of the *eps* operon, *epsC-N*; however, the prepilin peptidase PilD was required for EpsG and EpsL to associate. Additionally, in

Chapter II I introduced the equivalent point mutation that was previously isolated in the *P. aeruginosa* study that suggested a direct interaction between the EpsE and EpsG homologs. Substitution to this residue in *V. cholerae* EpsG drastically reduced the cross-linking with EpsL. Overall this chapter confirms an interaction between EpsG and EpsL, and suggests that this interaction may occur to link EpsG to the ATPase.

In Chapter III I wanted to further investigate the requirement for the prepilin peptidase in allowing EpsG and EpsL to interact. PilD is a bifunctional enzyme whose activity results in both cleavage and methylation of the EpsG N-terminus. As Chapter II established that cleavage was a pre-requisite for their interaction, in this Chapter I wanted to determine if methylation was also necessary. Amino acid substitution within the EpsG N-terminus that prevents the N-terminus from being methylated also prevented EpsG from cross-linking with EpsL. This chapter indicates that both functions of the prepilin peptidase are needed in order for EpsG to interact with EpsL.

Finally, in Chapter IV I investigated EpsG residues within the C-terminus or the variable region in order to determine what role these structural domains have on cross-linking with EpsL. Substitution to residues in the C-terminus that are involved in coordinating the calcium ion altered the cross-linking with EpsL, possibly due to localized misfolding of EpsG. Substitution to residues in the variable region decreased the amount of cross-linking with EpsL and indicated that this region may be involved in directly interacting with EpsL. Overall, Chapters II-IV have addressed my second aim by examining substitutions within the three structural domains of EpsG and determining their effect on cross-linking with EpsL as well as their ability to support secretion.

This study has furthered the field of T2S by identifying a critical interaction within the assembled complex and providing evidence for how the pseudopilus may be linked to the ATPase in order to power assembly. Importantly, every EpsG substitution examined that grossly altered the cross-linking with EpsL also prevented secretion, validating the importance of their native association. Because of the similarities between the T2S system and type IV pilus machinery, an analogous interaction may occur between the type IV pilin and inner membrane platform. Thus, not only does this study advance the field of T2S but it also has important implications for the architecture of the type IV pilus complex.

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CHAPTER II

In vivo cross-linking of EpsG to EpsL suggests a role for EpsL as an ATPase pseudopilin coupling protein in the Type II secretion system of *Vibrio cholerae*

Note: A modified version of this chapter was previously published in *Molecular Microbiology* (Gray *et al*., 2011).

Summary

The type II secretion system is a multi-protein complex that spans the cell envelope of gram-negative bacteria and promotes the secretion of proteins, including several virulence factors. This system is homologous to the type IV pilus biogenesis machinery and contains five proteins, EpsG-K, termed the pseudopilins that are structurally homologous to the type IV pilins. The major pseudopilin EpsG has been proposed to form a pilus-like structure in an energy-dependent process that requires the ATPase, EpsE. A key remaining question is how the membrane-bound EpsG interacts with the cytoplasmic ATPase, and if this is a direct or indirect interaction. Previous studies have established an interaction between the bitopic inner membrane protein EpsL and EpsE; therefore, in this study we used *in vivo* cross-linking to test the hypothesis that EpsG interacts with EpsL. Our findings suggest that EpsL may function as a scaffold to link EpsG and EpsE and thereby transduce the energy generated by ATP hydrolysis to support secretion. The recent discovery of structural homology between EpsL and a

protein in the type IV pilus system implies that this interaction may be conserved and represent an important functional interaction for both the type II secretion and type IV pilus systems.

Introduction

The type II secretion (T2S) system is found widely distributed among gram-negative bacteria and is considered a primary virulence system (Sandkvist, 2001b , Cianciotto, 2005). In *Vibrio cholerae*, this system is encoded by the extracellular protein secretion (*eps*) operon and is used to secrete several proteins, including the main virulence factor cholera toxin, across the cell envelope (Sandkvist *et al.*, 1997). The complex is composed of at least 12 different proteins, each individually essential for secretion to occur, which form a structure that spans the entire cell envelope (Filloux, 2004). The T2S apparatus is highly homologous to the type IV pilus biogenesis machinery which produces cell surface filaments that are involved in multiple processes, including attachment and colonization, biofilm formation, DNA uptake, twitching motility, and virulence (Craig & Li, 2008).

Specifically, the assembled T2S system consists of the cytoplasmic ATPase, EpsE, an inner membrane platform composed of EpsC, F, L, and M, and an outer membrane secretin pore formed by EpsD (Johnson *et al*., 2006). The T2S machinery also contains five additional gene products termed the pseudopilins, EpsG, H, I, J and K, that are structurally homologous to the type IV pilins (Kohler *et al.*, 2004, Korotkov & Hol, 2008, Yanez *et al.*, 2008, Korotkov *et al.*, 2009, Alphonse *et al.*, 2010). Additionally, both the T2S pseudopilins and the type IV pilins contain a conserved N-terminus which is

recognized and processed by the prepilin peptidase PilD, also referred to as VcpD (Strom *et al.*, 1993, Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). Processing of the pilin subunits is a prerequisite for their incorporation into functional pili (Nunn *et al.*, 1990, Marsh & Taylor, 1998, Fullner & Mekalanos, 1999).

As a result of the similarities between the pseudopilins and the type IV pilins, EpsG-K have been suggested to form a pilus-like structure, or pseudopilus, where EpsG is the major and EpsH-K are minor pseudopilins (Johnson *et al.*, 2006, Campos *et al.*, 2010). Over-expression of the EpsG homologs XcpT and PulG in *Pseudomonas aeruginosa* and *Klebsiella oxytoca*, respectively, confirmed that the major pseudopilin is indeed capable of assembling into a pilus-like structure that can be detected on the surface of the bacteria (Durand *et al.*, 2003, Vignon *et al.*, 2003). Detection of surface pseudopili required the ATPase and inner membrane platform proteins, and was only observed under over-expression conditions when all other components of the T2S system were held constant. However, during native expression of the complex surface appendages were not observed. Instead, the pseudopilus is hypothesized to span the periplasm and promote secretion by functioning as a piston that can push secreted proteins through the outer membrane pore, or alternatively, by acting as a retractable plug (Hobbs & Mattick, 1993, Sandkvist, 2001a, Filloux, 2004).

The type IV pilins and T2S pseudopilins require the function of an ATPase to polymerize (Camberg & Sandkvist, 2005, Durand *et al.*, 2005, Pelicic, 2008); however, a key question is how the cytoplasmic ATPase of these systems is able to interact with the pilin or pseudopilin subunits that are located within the inner membrane and exposed to the periplasm. A study using *P. aeruginosa* identified a point mutation in the EpsG

homolog, XcpT, which inhibited secretion and could be suppressed by a second point mutation in the EpsE homolog, XcpR (Kagami *et al.*, 1998). Based on this result it has been proposed that EpsG and EpsE are capable of interacting directly, however, molecular mapping of EpsE using subcellular fractionation, domain swapping, and x-ray crystallography have strongly suggested that the region of the suppressor mutation in the EpsE homolog is involved in an interaction with the bitopic inner membrane protein XcpY, the EpsL homolog (Sandkvist *et al.*, 1995, Abendroth *et al.*, 2005). Therefore, EpsL is a plausible candidate to bridge EpsG and EpsE and transduce the energy provided by ATP hydrolysis in order to promote secretion.

In this study we used *in vivo* cross-linking and co-immunoprecipitation to establish an interaction between the major pseudopilin subunit, EpsG, and EpsL. This interaction occurs in the absence of all other structural components of the apparatus but does require the prepilin peptidase, PilD. Furthermore, when the point mutation originally isolated in XcpT was made in EpsG it severely reduced the amount of cross-linked EpsG-EpsL complex. Based on our findings we propose that EpsL is the linker that allows for association of the ATPase with EpsG, and may function as a mechanical lever to transfer the energy required to support either assembly or disassembly of the pseudopilus.

Results

EpsG and EpsL interact in V. cholerae

Based on our previous work detailing the interaction between EpsE and the cytoplasmic domain of the membrane protein EpsL (Sandkvist *et al.*, 1995, Sandkvist *et*

al., 2000, Abendroth *et al*., 2005), we sought to determine if EpsL interacts with EpsG and thus provides a link between the pseudopilus and the ATPase. Previous reports on EpsG homologs have focused on the interactions amongst the five pseudopilins (Hu *et al.*, 2002, Kuo *et al.*, 2005, Douzi *et al.*, 2009); however, a definitive interaction between the major pseudopilin and other components of the T2S apparatus has yet to be revealed. This is likely due to the dynamic nature of the secretion machinery in which transient or weak interactions may be difficult to capture; therefore, we chose to use the membrane permeable cross-linker dithiobis (succinimidyl propionate), DSP, to preserve potential interactions that involve EpsG. Specifically, whole cells were treated with several concentrations of DSP, lysed, and subjected to SDS-PAGE and immunoblotting with anti-EpsG antiserum. Treatment with DSP produced higher molecular weight species in a concentration dependent manner in a wild type strain that were specific for EpsG, as these complexes were absent in a Δ*epsG* mutant strain (Figure 2.1A, compare lanes 4-6 with 7-8). The two most prominent cross-linked species were approximately 35 and 60 kDa. The 60 kDa complex was of the appropriate molecular weight to represent a heterodimer of EpsG and EpsL, whose molecular masses are 15 kDa and 45 kDa, respectively. Furthermore, this species was shown to be absent in a Δ*epsL* mutant strain (Figure 2.1A). The complex at 35 kDa is presumed to be a dimer of EpsG as it has been well documented that EpsG and its homologs can form dimers even in the absence of other T2S components (Kohler *et al*., 2004, Korotkov *et al*., 2009).

In order to increase the detection of the specific EpsG complexes we also examined the cross-linking profile in a strain where the native *eps* promoter has been replaced with the P_{BAD} arabinose-inducible promoter, referred to as P_{BAD}::*eps* (Sikora *et*

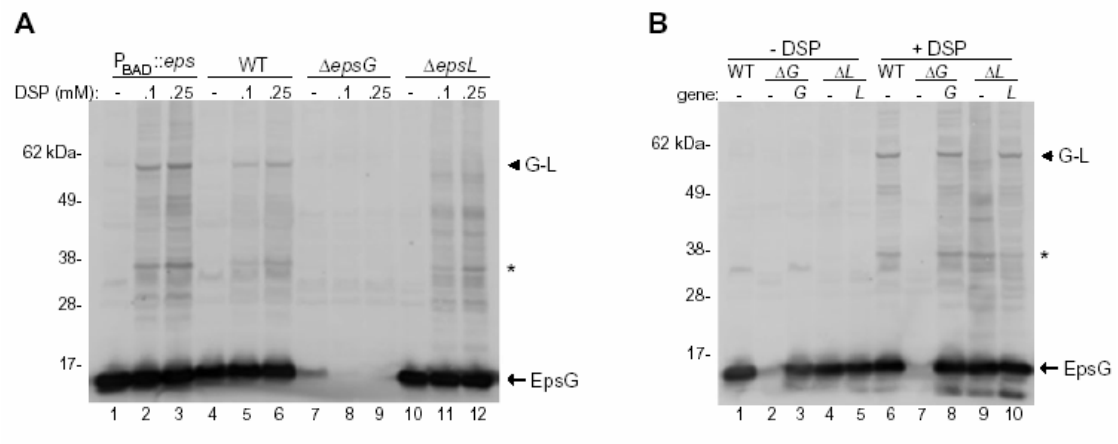

Figure 2.1. *In vivo* cross-linking of EpsG and EpsL. (A) Whole cells of *V. cholerae* TRH7000 wild-type, Δ*epsG*, Δ*epsL*, and P_{BAD}::*eps* (native *eps* promoter replaced by arabinose inducible promoter and grown in the presence of 0.01% arabinose) were cross-linked using the indicated concentrations of DSP as described in the experimental procedures, and immunoblotted for EpsG. (B) Whole cells of P_{BAD}::*eps* wild-type containing pMMB67 vector, P_{BAD}::Δ*epsG* with empty vector or vector encoded *epsG*, and P_{BAD}::Δ*epsL* with empty vector or vector encoded *epsL* were cross-linked with 0.1 mM DSP and immunoblotted for EpsG. The position of EpsG is indicated. An arrow head designates the EpsG-EpsL complex, and an asterisk represents the putative EpsG dimer. The band directly below the putative EpsG dimer is presumed to be cross-reactive as it is also observed in the absence of DSP. The molecular weight markers are shown in kilo Daltons and lane numbers are indicated.

al., 2007). This strain allows for specific upregulation of the entire *eps* operon, resulting in increased levels of all T2S components and thereby maintaining the stochiometry of the secretion complex. Growth of the P_{BAD}::*eps* strain in the presence of 0.01% arabinose enhanced the detection of cross-linked EpsG complexes while preserving the cross-linking profile that was observed in wild type cells (Figure 2.1A lanes 1-3); thus, we chose to use the P_{BAD} promoter background to further elucidate the EpsG-EpsL interaction. First, we confirmed that deletions of *epsG* and *epsL* in the P_{BAD}::*eps* background inhibited secretion and complementation with plasmid-encoded *epsG* and *epsL* restored secretion in the deletion mutants to near wild-type levels (Figure 2.2). As expected, we found that deletion of either the *epsG* or *epsL* genes in the P_{BAD}::*eps* strain prevented detection of the 60 kDa cross-linked species with anti-EpsG antibodies (Figure 1B lanes 7 and 9). Furthermore, complementation of the deletion strains with *epsG* or *epsL* encoded upon a plasmid restored the detection of the 60 kDa complex (Figure 2.1B lanes 8 and 10).

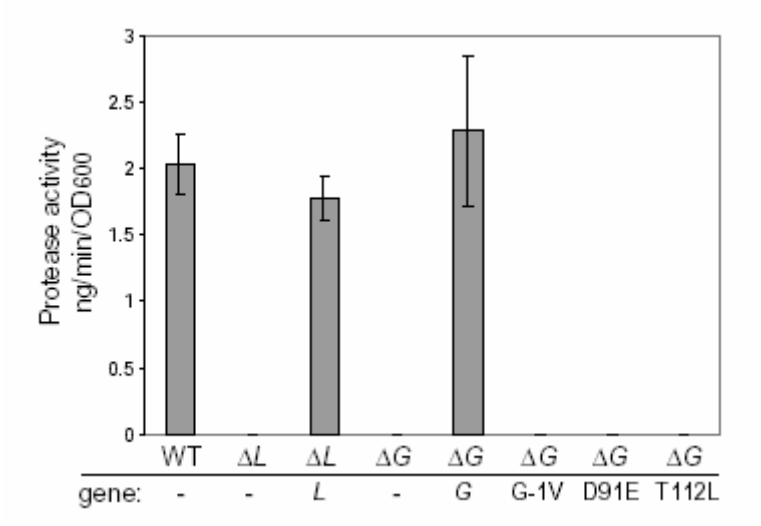

Figure 2.2. Mutations in EpsG prevent secretion. Supernatants of over-night cultures of *V. cholerae* TRH7000 P_{BAD}::*eps* wild-type, P_{BAD}::Δ*epsL* containing empty vector or vector encoding wild-type *eps*L, P_{BAD}::Δ*epsG* with empty vector, and vector encoded wild-type *epsG* or mutants were grown over-night at 37°C. Supernatants were separated from the cells and analyzed for the presence of an extracellular protease using the cleavable fluorogenic substrate *N*-tert-butoxy-canbonyl-Gln-Ala-Arg-7-amido-4-methyl-coumarin as described in the materials and methods. Each sample was assayed in triplicate and the standard error is indicated.

To further verify that the 60 kDa species corresponds to an EpsG-EpsL complex we subjected cross-linked samples to co-immunoprecipitation. Whole cells of P_{BAD}::*eps*, P_{BAD}::Δ*epsG,* P_{BAD}::Δ*epsL*, and their respective complemented strains were cross-linked with 0.25 mM DSP, and cell lysates were prepared and incubated with antibodies specific for either EpsG or EpsL bound to protein G sepharose beads. Following extensive washing the precipitated material was analyzed by SDS-PAGE, and immunoblotted with biotinylated anti- EpsG or anti-EpsL antibodies. The results from the co-immunoprecipitation supported the whole cell cross-linking data by demonstrating that the 60 kDa band was only precipitated from the P_{BAD}::*eps* and complemented strains. The 60 kDa complex was not pulled down from the *epsG* and *epsL* deletion strains when the cell extracts were precipitated with either EpsG or EpsL anti-sera (Figure 2.3). Precipitating cell extracts with anti-EpsG antibodies followed by immunoblotting for EpsG enriched for monomeric EpsG as well as cross-linked EpsG species including the 60 kDa complex (Figure 2.3A lanes 4 and 6; Figure 2.3D lanes 4-6). Likewise, precipitating samples with anti-EpsL antibodies followed by immunoblotting for EpsL enriched for the EpsL monomer and the 60 kDa protein species (Figure 2.3B lanes 7-9; Figure 2.3C, lanes 7 and 9). Most intriguingly, when samples were pulled down with anti-EpsG antibodies and immunoblotted for EpsL the only band that was detected was the 60 kDa species (Figure 2.3B lanes 4 and 6; Figure 2.3C lanes 4 and 6). Similarly, when samples were immunoprecipitated with anti-EpsL antibodies and immunoblotted for EpsG (Figure 2.3A lanes 7 and 9; Figure 2.3D lanes 7 and 9), the only band that was detected was the 60 kDa species. These results confirm that the 60 kDa band represents a cross-linked complex between EpsG and EpsL.

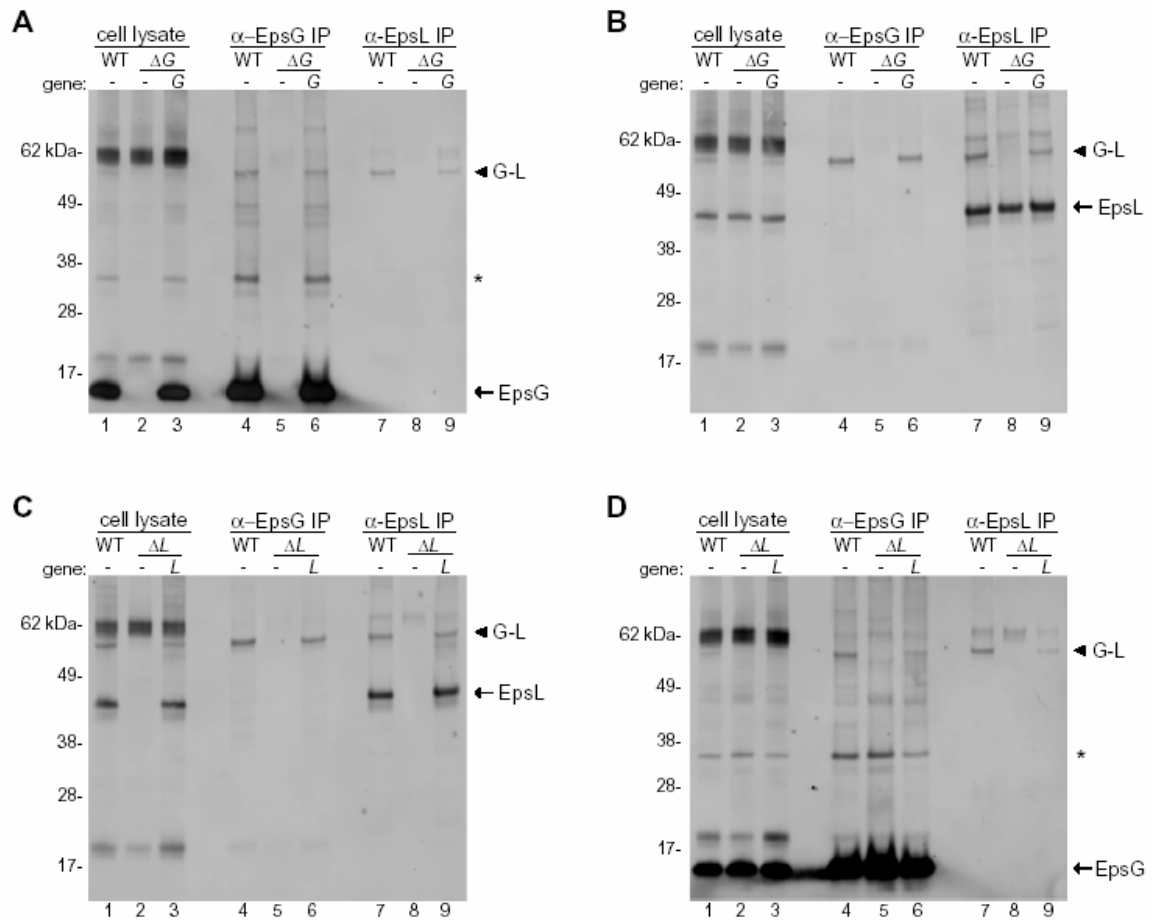

Figure 2.3. Co-immunoprecipitation of EpsG and EpsL. Triton X-100 cell extracts were prepared from P_{BAD}::*eps* wild-type, P_{BAD}::Δ*epsG* and complemented strain (Panels A and B), and P_{BAD}::Δ*epsL* and complemented strain (Panels C and D) after cross-linking with 0.25 mM DSP. Cleared cell extracts were immunoprecipitated with either anti-EpsG or anti-EpsL antibodies and subjected to SDS-PAGE and immunoblotting with biotinylated anti-EpsG (Panels A and D) or biotinylated anti-EpsL (Panels B and C) antibodies. In all panels the monomer for EpsG or EpsL is indicated with an arrow and the 60 kDa EpsG-EpsL complex is denoted by an arrow head. In panels immunoblotted for EpsG, the putative EpsG dimer is labeled with an asterisk. The molecular weight markers are shown in kilo Daltons. Lane numbers are indicated.

EpsG processing is required for the interaction with EpsL

We next wanted to determine if processing of EpsG by the prepilin peptidase, PilD, is required for the interaction with EpsL. The precursors of the T2S pseudopilins and type IV pilins are known to be processed by PilD, a unique peptidase that is responsible for both cleavage and methylation of the newly formed N-terminus (Nunn & Lory, 1993, Pugsley, 1993, Strom *et al*., 1993). In the absence of *pilD* secretion through the T2S apparatus is inhibited, indicating that processing of the pseudopilins is necessary for these proteins to be functional (Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). In order to address if PilD is required for EpsG and EpsL to associate we performed *in vivo* cross-linking using whole cells of wild-type and a *pilD* deficient strain of *V. cholerae*, followed by SDS-PAGE and immunoblotting for EpsG. The Δ*pilD* mutant strain did not exhibit the 60 kDa cross-linked complex observed in the wild-type strain although a very faint band slightly larger than the EpsG-EpsL complex was detected that may be indicative of unprocessed EpsG associating with EpsL (Figure 2.4A). However, the intensity of this band was greatly reduced in comparison to the band comprising EpsL and the mature, processed form of EpsG suggesting that the affiliation between unprocessed EpsG and EpsL was much less efficient. Furthermore, upon complementation of the Δ*pilD* strain the position and intensity of the EpsG-EpsL cross-linked species was restored to the levels observed in the wild-type cells.

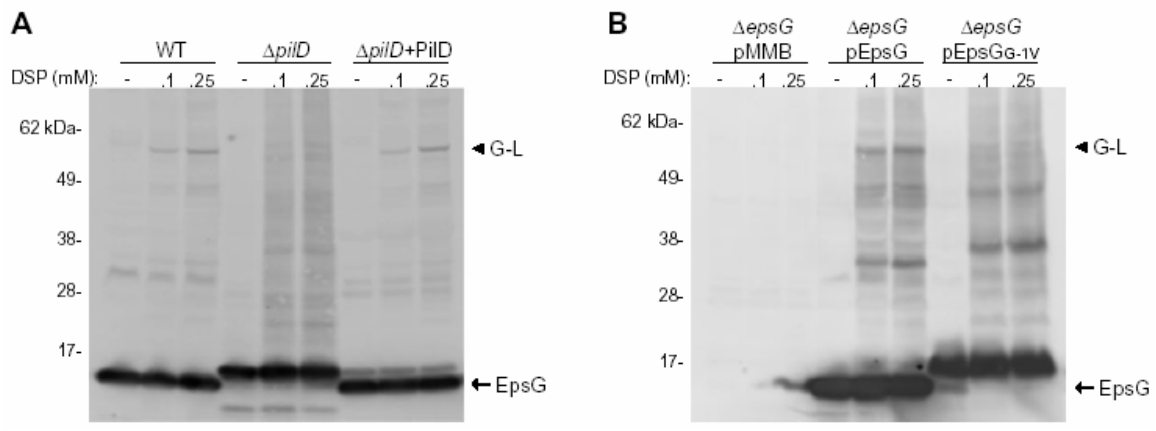

Figure 2.4. Processing of EpsG is necessary for EpsG-EpsL cross-linking. (A) Whole cells of *V. cholerae* C6706 wild-type, Δ*pilD*, and Δ*pilD* complemented with pACYC184 expressing *pilD* were cross-linked with the indicated concentrations of DSP, and immunoblotted with antibodies specific for EpsG. (B) Whole cells of P_{BAD}::Δ*epsG* expressing wild-type *epsG*, or *epsG* with mutation to residue G-1 were cross-linked and analyzed as described above. The processed monomer of EpsG is represented by an arrow, and the EpsG-EpsL complex is labeled with an arrow head. Position of molecular weight markers is shown.

To further verify that processing of EpsG is required for cross-linking with EpsL, we also examined an EpsG mutant that cannot be processed by PilD. Previous studies on type IV pilins and the EpsG homolog, PulG, from *K. oxytoca* demonstrated that substitution of the glycine residue (referred to as G-1) at the cleavage site G-F prevented cleavage by PilD, and inhibited pilus formation and secretion, respectively (Strom & Lory, 1991, Pugsley, 1993). We substituted EpsG residue G-1 for valine and expressed the mutant gene from a plasmid in the P_{BAD}::Δ*epsG* strain. Similar to the finding in *K. oxytoca*, $EpsG_{G-1V}$ was not processed (Figure 2.4B) and unable to support secretion of protease through the T2S apparatus (Figure 2.2). We next performed *in vivo* cross-linking with P_{BAD}::Δ*epsG* expressing wild type EpsG or $EpsG_{G-1V}$, and subjected samples to SDS-PAGE and immunoblotting for EpsG. As was observed for the *pilD* deficient strain, the $EpsG_{G-1V}$ mutant did not exhibit the 60 kDa cross-linked band indicative of an EpsG- EpsL interaction (Figure 2.4B). From these data we concluded that processing of EpsG by the prepilin peptidase is necessary for the association with EpsL.

EpsG and EpsL interact in the absence of all other T2S components

Since our data signified that processing by the prepilin peptidase is a prerequisite for EpsG to interact with EpsL, we wanted to examine whether any other components of the T2S machinery are essential for the interaction to occur. We first determined the *in vivo* cross-linking pattern of EpsG in strains deficient for each individual gene encoded by the *eps* operon and found that EpsG and EpsL still interacted, indicating that no specific component was required for their association (data not shown). To further explore the requirements necessary for EpsG and EpsL to assemble we expressed either

epsG alone (pEpsG), or in conjunction with *epsL* (pEpsGL), in a *V. cholerae* strain where the entire *eps* operon has been deleted (Δ*eps*) but still encodes the prepilin peptidase, PilD (Sikora *et a*l., 2007). Whole cells were incubated with DSP, subjected to SDS-PAGE, immunoblotted for EpsG, and the cross-linking profiles were compared to that of wild-type cells. As shown in Figure 2.5A, EpsG in the presence of only EpsL was sufficient for the 60 kDa complex to be restored and the band migrated in accordance to the EpsG-EpsL protein species observed in the wild-type strain. Expression of *epsG* alone did not result in the 60 kDa cross-linked band; however, when EpsG was produced alone or with EpsL we did observe other EpsG protein species, including the intense band around 35 kDa which is presumed to be an EpsG dimer. Despite the presence of these additional EpsG specific bands, the 60 kDa band was only observed in the presence of EpsL.

Although we were capable of establishing an EpsG-EpsL interaction in the absence of other Eps components in *V. cholerae*, we wanted to additionally determine if any other proteins specific to *V. cholerae* are required for EpsG and EpsL to form a complex by performing the cross-linking experiments in *E. coli*. Because processing of EpsG by PilD was required for the EpsG-EpsL association, we tested *E. coli* expressing *pilD* from a plasmid as well as an additional vector containing either *epsG* (pEpsG), *epsL* (pEpsL), or both *epsG* and *epsL* (pEpsGL). Whole cells were cross-linked, and analyzed by SDS-PAGE and immunoblotting with either EpsG or EpsL antisera. As was observed in *V. cholerae*, the 60 kDa protein species was only detected when both EpsG and EpsL were present, suggesting that no other protein unique to *V. cholerae* is required for their interaction (Figure 2.5B). In *E. coli* we also saw other EpsG specific protein species that

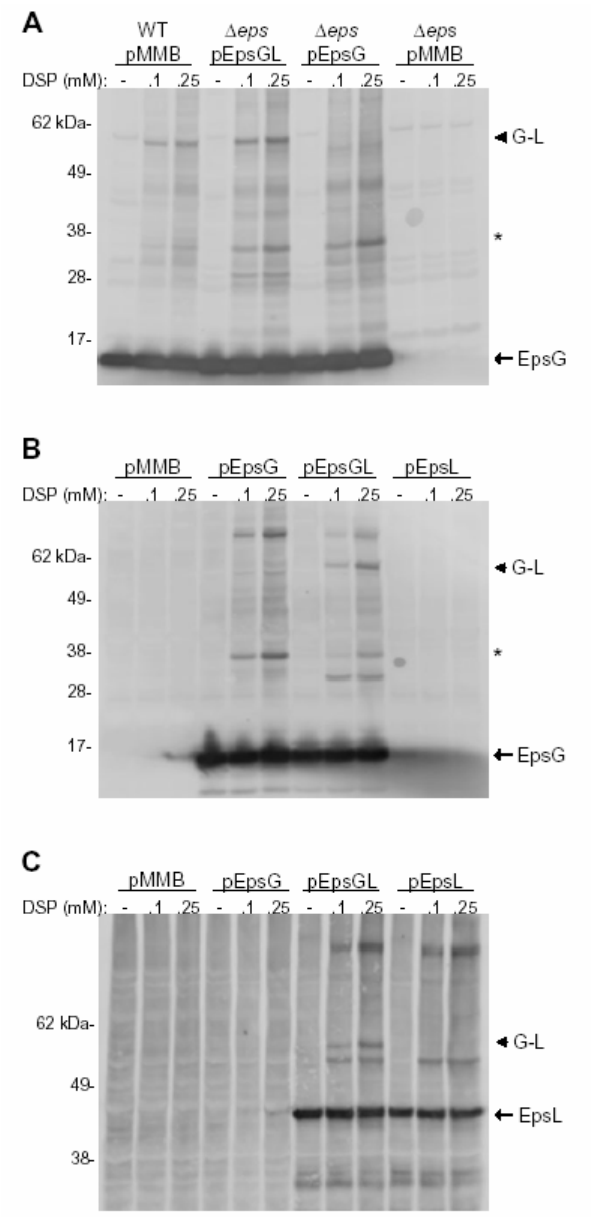

Figure 2.5. EpsG and EpsL interact in the absence of other T2S components. (A) Whole cells of *V. cholerae* TRH7000 wild-type, and TRH7000 with the entire *eps* operon removed (Δ*eps*) expressing plasmid-encoded *epsG*, or *epsG* and *epsL* (pEpsGL) were cross-linked with the indicated concentrations of DSP and immunoblotted for EpsG. (B and C) Whole cells of *E. coli* MC1061 with pACYC184-*pilD* and pMMB67 encoding *epsG*, *epsL*, or *epsG* and *epsL* concomitantly were cross-linked with the indicated concentrations of DSP and probed with antibodies specific for EpsG (B) or EpsL (C). The monomer for EpsG (Panels A and B) and EpsL (Panel C) is indicated by an arrow. An arrow head designates the EpsG-EpsL complex, and an asterisk represents the putative EpsG dimer in Panels A and B. Positions of molecular weight markers are indicated.

were present in the absence of EpsL, including the proposed dimer at 35 kDa. Interestingly, the 35 kDa band and another EpsG complex located at approximately 80kDa were reduced when *epsL* was expressed with *epsG*. This may imply that EpsG preferentially associates with EpsL and that the addition of EpsL titrated EpsG away from other protein complexes.

In *V. cholerae* we were unable to determine the cross-linking profile for EpsL due to cross-reactive bands recognized by our polyclonal EpsL antibodies, unless the cross-linking procedure was followed by immunoprecipitation and immunoblotting. However, in *E. coli* we were able to address whether an EpsG and EpsL interaction could be detected by immunoblotting with anti-EpsL antibodies. When comparing cells expressing EpsL either alone or in the presence of EpsG the only additional species that was detected when EpsG and EpsL were co-produced migrated to approximately 60 kDa, implying a specific EpsG-EpsL association (Figure 2.5C). Our findings that expression of *epsG* and *epsL* concomitantly in either *V. cholerae* Δ*eps* or *E. coli* results in an EpsG-EpsL cross-linked complex suggests that they do not require any additional members of the T2S machinery in order to associate. Furthermore, these data establish that the cross-linked EpsG species observed at 60 kDa in wild type *V. cholerae* is indicative of an EpsG-EpsL complex.

Mutation of EpsG residue T112 alters the interaction with EpsL

To further explain the original finding by Kagami *et al*. that showed that the threonine to leucine mutation isolated in XcpT, the *P. aeruginosa* homolog of EpsG, was suppressed by a second mutation in the EpsE homolog, XcpR, we created the equivalent

residue change, $EpsG_{T112L}$, as was reported for XcpT (Kagami *et al*., 1998). The recently solved structure for EpsG indicated that the side chain of residue T112 is within hydrogen bond distance to residue D91 and mutation of either residue should have a similar effect on EpsG (Figure 2.6A) (Korotkov *et al*., 2009); therefore, we also substituted D91 for glutamic acid. The mutant genes were expressed from a plasmid in the P_{BAD}::Δ*epsG* strain and we addressed whether the mutant proteins would interfere with secretion through the T2S apparatus by testing for the presence of a secreted protease in the culture supernatant. As shown in Figure 2.2, neither $EpsG_{T112L}$ nor $EpsG_{D91E}$ were able to support secretion of the protease, indicating that mutation of either residue resulted in a non-functional protein. The residue changes reduced the detection of EpsG protein seen by immunoblotting, implying that the mutant proteins may be less stable (data not shown).

In order to examine if replacement of residues T112 or D91 affected the ability of EpsG to cross-link with EpsL we needed to produce the mutant proteins at the same level as wild type EpsG in P_{BAD}::Δ*epsG*. We first determined the amount of IPTG required to produce the equivalent amount of all EpsG variants. Whole cells were then incubated with or without 0.1 mM DSP, and subjected to SDS-PAGE and immunoblotting with anti-EpsG antiserum. Neither mutant protein was able to cross-link with EpsL at the level observed in the complemented strain with both $EpsG_{T112L}$ and $EpsG_{D91E}$ exhibiting significantly reduced amounts of the EpsG-EpsL 60 kDa band (Figure 2.7). While wild type EpsG was found in the 60 kDa complex with EpsL the non-functional mutant proteins accumulated in a 45 kDa complex, similar to the cross-linking profile observed in an *epsL* deficient strain. Because the side chains of T112 and D91 are barely surface

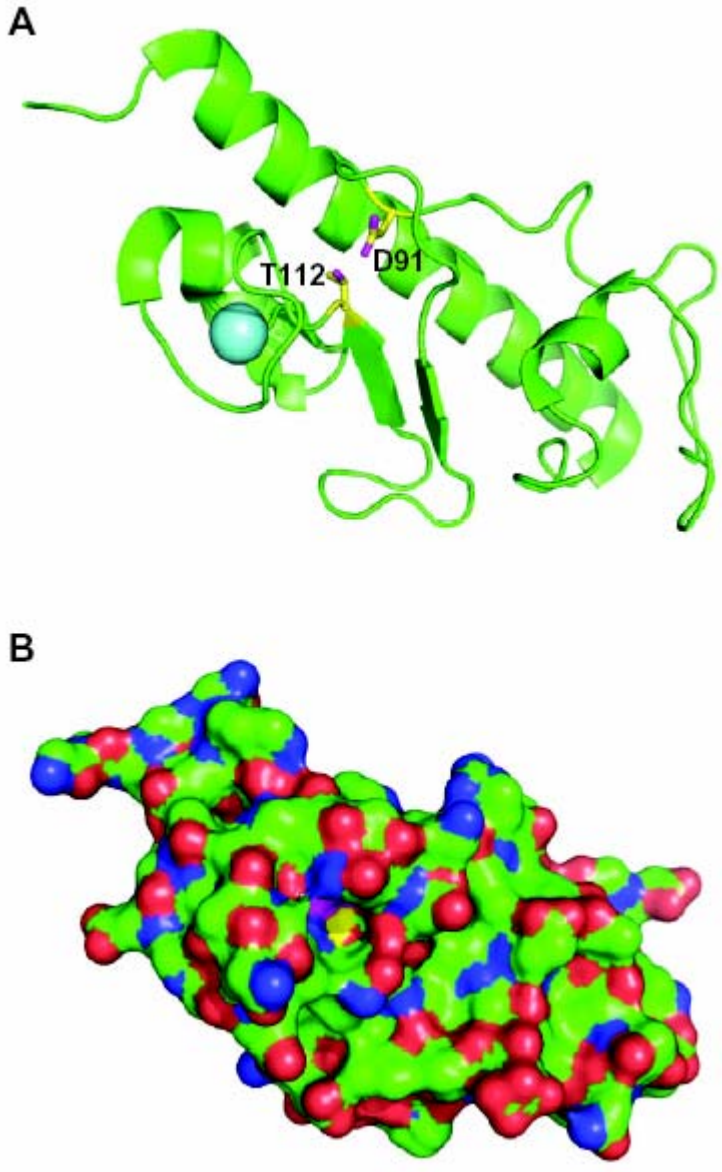

Figure 2.6. Location and interaction between residues Asp91 and Thr112 in EpsG. *V. cholerae* EpsG (Korotkov *et al*., 2009; PDB code 3FU1) depicted with the side chains of D91 and T112 colored with yellow carbons and magenta oxygens. The light blue sphere is a bound calcium ion. (A) View approximately perpendicular to the EpsG helix axis with the backbone cartoon in green and the side chains of D91 and T112 as sticks. (B) Surface representation of the same view with the side chains of D91 and T112 with the same color code as in Panel A, the other atoms are colored green for carbons, blue for nitrogens and red for oxygens. A deep cavity is apparent where only a few atoms of D91 and T112 are visible. Figure prepared with PyMOL (DeLano, 2002).

exposed and occur at the bottom of a deep crevice (Figure 2.6B) they may not mediate a direct contact with EpsL; therefore, replacement of these residues likely has an indirect effect on the EpsG-EpsL interaction. Additionally, we did not create the $EpsE_{S84F}$ suppressor originally isolated in the *P. aeruginosa* homolog XcpR to determine if it would restore the cross-linking band between $EpsG_{T112L}$ and EpsL. $XcpR_{S84F}$ only restored secretion ten percent (Kagami *et al*., 1998), and a slight increase would be difficult to measure using our cross-linking methodology. However, our data do clearly indicate that substitution of EpsG residues T112 or D91 prevent or alter the EpsG-EpsL interaction and suggest that the suppressor isolated in the *P. aeruginosa epsE* homolog may have occurred due to the interaction between the EpsE and EpsL homologs.

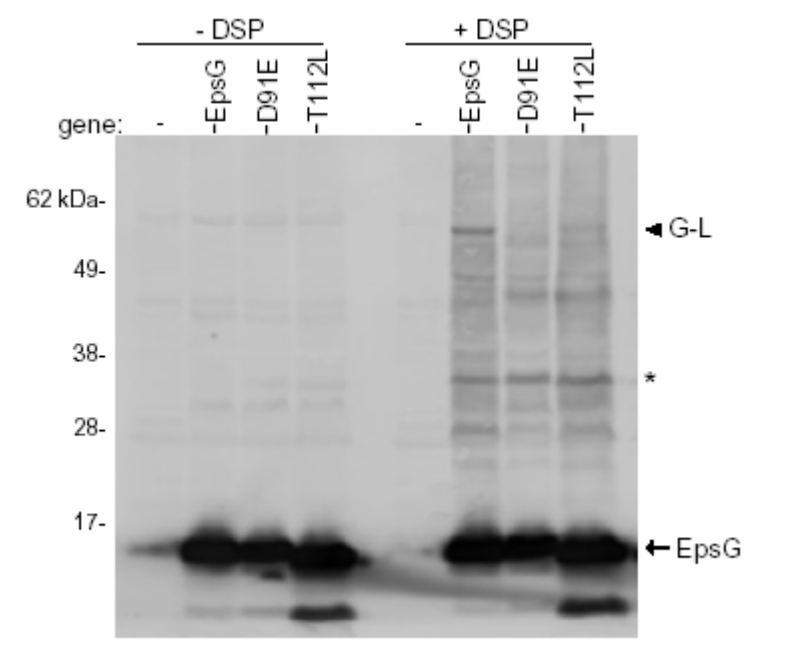

Figure 2.7. Replacement of residues D91 or T112 alters the cross-linking of EpsG with EpsL. Whole cells of P_{BAD}::Δ*epsG* expressing wild-type *epsG*, or *epsG* with mutations to either residue D91 or T112 were incubated in the absence or presence of 0.1 mM DSP and subjected to SDS-PAGE and immunoblotting with anti-EpsG antibodies. The EpsG monomer is designated by an arrow, the EpsG-EpsL complex is indicated by an arrow head, and a predicted EpsG dimer is labeled with an asterisk. Molecular weight markers are indicated.

Discussion

In order to better understand how the T2S apparatus assembles and promotes secretion through the gram-negative cell envelope, we have continued to examine the specific protein interactions that occur within this secretion complex. The T2S machinery is likely dynamic therefore in this study we elected to use *in vivo* cross-linking in whole cells in order to capture interactions within their native environment. Using this methodology, we have established a protein interaction between the major pseudopilin, EpsG, and the inner membrane component, EpsL. Additionally, we have determined the T2S components necessary for the EpsG-EpsL interaction, thus allowing us to begin to dissect the sequential order of events that leads to the incorporation of EpsG into the T2S complex, or pseudopilus. Previous studies have shown that homologs of EpsG are cotranslationally inserted into the inner membrane using the Signal Recognition Particle and SEC pathway, and do not require any components of the T2S inner membrane platform in order to be targeted to the inner membrane (Arts *et al.*, 2007, Francetic *et al.*, 2007). Following insertion into the inner membrane, our results indicate that EpsG must be processed by the prepilin peptidase before it can interact with EpsL. The processing results in the removal of 7 cytoplasmically exposed N-terminal residues of EpsG (Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). The processing and methylation events, both performed by PilD, have been speculated to cause a conformational change that alters the way that EpsG is presented within the inner membrane, thus enabling EpsG to interact with other components of the T2S apparatus (Nunn & Lory, 1993, Strom *et al.*, 1993). This hypothesis is supported by our finding that processing is a prerequisite for EpsG to cross-link with EpsL, suggesting that it is the mature form of EpsG that is

recognized by EpsL. Furthermore, we show that no other T2S component is required for the interaction between EpsG and EpsL. This suggests that EpsL may be the first protein that EpsG interacts with as it is incorporated into the T2S machinery, and that EpsL may be responsible for recruiting EpsG to this complex.

One implication as to the importance of the interaction between EpsG and EpsL is that EpsL may function to link the cytoplasmic ATPase, EpsE, with EpsG. The interaction between EpsE and the cytoplasmic domain of EpsL is necessary and sufficient to recruit EpsE to the inner membrane (Sandkvist *et al*., 1995, Abendroth *et al*., 2005). In addition, this interaction allows EpsE to adopt an active oligomeric state and *in vitro* studies have demonstrated that EpsL stimulates the ATPase activity of EpsE (Camberg *et al.*, 2007). Recent findings from our laboratory and others have shown that members of the type II/IV secretion ATPase family to which EpsE belongs undergo dynamic changes as they bind and hydrolyse ATP (Planet *et al*., 2001, Robien *et al*., 2003, Savvides *et al.*, 2003, Satyshur *et al.*, 2007, Yamagata & Tainer, 2007, Misic *et al*., 2010, Patrick *et al*., 2011). The entire N-terminal domain of EpsE undergoes a large movement relative to the nucleotide-binding C-terminal domain due to the presence of a flexible linker. The ensuing conformational changes do not only have substantial effects on the arrangement of subunits within the EpsE hexamer, but may also affect EpsL, which is interacting with a sub-domain of the N-terminal domain (Abendroth *et al.*, 2005). The conversion of chemical energy to mechanical work, translated through EpsL, may in turn promote assembly of the pseudopilus. In support of this suggestion, Py *et al*. have shown that the interaction between the cytoplasmic domain of the EpsL and EpsE homologs, OutL and

OutE, in *Dickeya dadantii* (previously classified as *Erwinia chrysanthemi*) results in a conformational change in the periplasmic domain of OutL (Py *et al.*, 2001).

We found that the T112L and D91E substitutions in EpsG severely reduced the amount of cross-linked EpsG-EpsL complex and negatively affected the secretion of protease via the T2S machinery. Sequence comparison of EpsG with other T2S homologs showed that T112 is highly conserved and is found as either a threonine or serine, while D91 is conserved as an aspartic acid in all homologs examined (Korotkov *et al.*, 2009). The structure of EpsG revealed that the side chain hydroxyl of T112 forms a solvent exposed hydrogen bond with one of the carboxylate oxygens from D91 at the bottom of a deep crevice (Figure 2.6) (Korotkov *et al.*, 2009). The second carboxylate atom of D91 is engaged in a hydrogen bond with the main chain NH group of Asn95. The mutation T112L disrupts the hydrogen bond between T112 and D91. As a result of the close packing of residues at this site the substitution D91E could also affect this hydrogen bond as well as the one between D91 and Asn95 and thus alter the conformation of loop 90-96. The resultant conformational change may explain why introducing an additional atom through the conservative replacement D91E is sufficient to have a negative effect on EpsG. The T112L and D91E mutations may cause a perturbation of the native fold and thus indirectly affect the EpsG-EpsL interaction. In preliminary attempts to identify the site(s) of interaction between EpsG and EpsL we have replaced every individual lysine residue in EpsG (data not shown). Only in one of the mutants ($EpsG_{K70A}$) was the EpsG-EpsL cross-linking reduced, but not abolished (data not shown). Therefore, future studies will be needed to determine the precise site of

interaction between EpsG and EpsL, which could occur either between the exposed periplasmic globular domains or within the membrane spanning portion of the proteins.

Because of the structural similarities between the pseudopilins, several intriguing questions remain regarding the minor pseudopilins, EpsH-K, which are proposed to form a cap on the tip of the pseudopilus (Korotkov & Hol, 2008, Douzi *et al*., 2009). It has yet to be determined if the minor pseudopilins also interact with EpsL; however, if the role of EpsL is to recruit EpsG to the T2S complex then it may also serve as a recruitment factor for one or more of the structurally related minor pseudopilins. Support for this suggestion comes from a yeast two hybrid study of T2S proteins from *D. dadantii* that detected an interaction between the EpsJ and EpsL homologs, OutJ and OutL (Douet *et al.*, 2004). It is possible that the minor pseudopilins also require an interaction with EpsL and energy provided by EpsE in order to become incorporated into the pseudopilus. Energy transduced by EpsL may be required to extract the N-terminal hydrophobic alpha-helix of both the major and minor pseudopilins from the inner membrane as the pseudopilus is formed in the periplasmic compartment.

A related mechanism to link the pilin subunits to the ATPase is likely to exist for the type IV pilus biogenesis machinery. Recently, the structure of *P. aeruginosa* PilN was found to be homologous to that of the periplasmic domain of EpsL (Sampaleanu *et al.*, 2009). PilN was suggested to interact with the cytoplasmic protein PilM, which has a predicted actin-like fold similar to the cytoplasmic domain of EpsL, in order to form a complex that is functionally equivalent to EpsL (Abendroth *et al.*, 2004, Ayers *et al.*, 2009). The findings that PilM and PilN, as well as homologs for EpsF and EpsM are required to form the type IV pilus suggests that the inner membrane complex of the type

IV pilus machinery may be analogous to the one formed by the T2S apparatus (Carbonnelle *et al.*, 2006, Ayers *et al*., 2009). Misic *et al*. have speculated that the type IV pilins may either directly interact with the ATPase or that their interaction may be mediated by the inner membrane complex composed of PilN, and the EpsF and EpsM homologs (Misic *et al*., 2010). Based on our findings, we propose that the PilM/PilN complex interacts with the pilin subunits and links them to the ATPase to support pilus assembly. At this point it is not understood why the EpsL equivalent is split into two proteins in the type IV pilus biogenesis system in *P. aeruginosa*, but it may be related to the requirement for several different ATPases to support pilus assembly and disassembly that lead to twitching motility (Pelicic, 2008). However, comparable to the T2S system, non-retractile type IV pili such as the toxin co-regulated pilus (TCP) in *V. cholerae* may only require the function of one ATPase. The cytoplasmic ATPase TcpT of the TCP system has been shown to interact with TcpR, a bitopic inner membrane protein that has suggested homology to EpsL (Tripathi & Taylor, 2007). Similar to the relationship between EpsE and EpsL, the interaction between TcpT and TcpR is responsible for bringing the ATPase to the inner membrane (Tripathi & Taylor, 2007). Intriguingly, the first 100 amino acids of TcpT are involved in the interaction with TcpR which is in agreement to the region of EpsE that interacts with EpsL, thus, it is likely that TcpR may interact directly with the pilin to support assembly.

In this study we have furthered our understanding of the T2S system by establishing a protein interaction between the major pseudopilin EpsG and the inner membrane component EpsL. Interestingly, every condition examined that disrupted or altered the interaction between EpsG and EpsL also prevented secretion, validating the

importance of their association within the T2S complex. Future work will be needed to completely understand the implications of this interaction; however, we propose that EpsL functions to link EpsG to the ATPase, providing a means by which energy is transduced between the two compartments in order for the pseudopilus to assemble.

Acknowledgements

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Experimental procedures

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani (LB) broth at 37°C. *V. cholerae* TRH7000 and its derivatives were cultured in the presence of 100 μg ml^{-1} thymine. *V. cholerae* P$_{BAD}$::*eps* strains were grown in the presence of 0.01% arabinose. Antibiotics (Sigma-Aldrich) were used at the following concentrations: ampicillin at 100 μg ml^{-1}, carbenicillin at 200 μg ml^{-1}, chlormanphenicol at 2 μg ml^{-1}, and kanamycin at 50 μg ml^{-1}. Expression of *eps* genes

was induced with isopropanyl-β-D-thiogalactopyranoside (IPTG) at the specified concentrations.

Construction of deletion strains

P_{BAD}::Δ*epsG* was constructed by replacing chromosomal *epsG* with the *cat* gene conferring chloramphenicol resistance in P_{BAD}::*eps* using the conditions previously described for creating TRH7000 Δ*epsG* (Lybarger *et al.*, 2009). Simlarly, P_{BAD}::Δ*epsL* was constructed in P_{BAD}::*eps* by replacing chromosomal *epsL* with *aph*-3 conferring kanamycin resistance using the conditions previously described for creating TRH7000 Δ*epsL* (Sikora *et al*., 2007).

Plasmid construction and conjugation

pEpsGL was constructed by excising *epsG* cloned in PCR-script (Stratagene) using the restriction sites PstI and SalI. The excised *epsG* fragment was cloned into similarly digested pMS44 using T4 DNA Ligase (New England BioLabs) in order to concomitantly express full length EpsG upstream of full length EpsL.

T112L mutation of *epsG* was introduced with QuickChange II site-directed mutagenesis kit (Stratagene) using previously constructed pEpsG as a template (Lybarger *et al*., 2009). Primers used for the site change were 5′-ggcacgattgatgtgttcctgttaggtgcggacggtcaag-3′ and 5′-cttgaccgtccgcacctaacaggaacacatcaatcgtgcc-3′. G-1V mutation of *epsG* was made similarly using the primers 5′-tgcgtaaacaaacggtctttaccctgctcgaagtaatg-3′ and 5′-cattacttcgagcagggtaaagaccgtttgtttacgca-3′.

pMMB872 was created for downstream subcloning by sequential removal of the BstEII sites from pMMB67 using the mutagenesis technique described previously (Allemandou *et al.*, 2003). The primer pairs used to remove the site from lacIQ were 5′-cagcgcgatttgctggtggcccaatgcgaccaga-3′ and 5′-gggctggacggtaactgagttcgcggcgggca-3′ (pMMBO109-1), and 5′-P-tgctccacgcccagtcgcgtaccgtcttcatgg-3′ and 5′-cgagacaggccctgcggggctgcacacgcgcc-3′ (pMMBO109-2). The primer pairs used to remove the site from repC were 5′-cttgcccgccgcgaactcagttaccgtccagcccag-3′ and pMMBO109-1 and 5′P-cgcgaccagctccggcaacgcctcgcgcac-3′ and pMMBO109-2. pMMB917 was generated by inserting *epsG* as a EcoRI-SalI fragment into pMMB872.

D91E was introduced with the primers 5′-aagcgtctgcctaaagaaccttggggtaacgac-3′ and 5′-gtcgttaccccaaggttctttaggcagacgctt-3′ by the same Stratagene technology described for T112L using truncated *epsG* encoding residues G25 through Q127 and the stop codon inserted in the vector pET28b (Novagen, Madison WI) as a template. A *Bst*EII – *Kpn*I fragment of the mutated gene was then excised from pET28b and used to replace the similar fragment in pMMB917. Sequences were verified at the University of Michigan DNA Sequencing Core.

All plasmid constructs were introduced into their bacterial strains by using the conjugative helper strain MM294/pRK2013 (Meselson & Yuan, 1968). Transconjugants were selected for antibiotic resistance encoded by the vector. All parental strains also received empty vectors except for C6706 Δ*pilD* which would not grow in the presence of vector only.

In vivo cross-linking

Overnight cultures were diluted in fresh media. The following concentrations of IPTG were used to induce the plasmid constructs equivalent to the P_{BAD}::*eps* chromosomal EpsG or EpsL levels: P_{BAD}::Δ*epsG* with pEpsG, $pEpsG_{G-1V}$, $pEpsG_{D91E}$, or $pEpsG_{T112L}$ at 15, 40, 40, and 20 μM, respectively, and P_{BAD}::Δ*epsL* with pEpsL at 10 μM. *E. coli* MC1061 containing pEpsG, pEpsL, or pEpsGL were induced with 10 μM IPTG. TRH7000 Δ*eps* containing pEpsG or pEpsGL were induced with 10 and 100 μM IPTG, respectively.

Diluted cultures were grown to an optical density at 600 nm between 1.5 and 2.0. Cells were harvested by centrifugation at 3,500 X g for 5 minutes and concentrated 8 fold by suspension in 500 μl of phosphate-buffered saline. Samples were cross-linked using dithiobis (succinimdyl propionate) (Pierce) for 1 hour at room temperature. The reactions were quenched for 10 minutes with 25 μl of 1 M Tris pH 8.0. Samples were centrifuged 8,000 X g for 5 minutes and suspended in 500 μl 50 mM Tris pH 8.0. To lyse, cells were incubated with 10 μl of 10 mg ml^{-1} lysozyme and 10 μl of 1 mg ml^{-1} DNase for 10 minutes followed by sonication at 30% amplitude for 10 seconds using one second pulses using the Vibra Cell Ultrasonic Processor (Sonics and Materials, Inc.). Samples were boiled for 5 minutes in SDS loading buffer and analyzed on NuPAGE 4 to 12% Bis-Tris polyacrylamide gels (Invitrogen) at the following concentrations: TRH7000 wild type and MC1061 were loaded at 10 μl of OD_{600}=2.0, all P_{BAD}::*eps* strains, Δ*eps*, and C6706 wild type and the Δ*pilD* complemented strain were loaded at 10 μl of OD_{600}=1.5, TRH7000 Δ*epsG* and Δ*epsL*, and C6706 Δ*pilD* were loaded at 10 μl of OD_{600}=1.0. Gels were transferred to nitrocellulose membranes in NuPAGE transfer

buffer and blocked in phosphate buffered-saline containing 3% BSA. Membranes were incubated with polyclonal antisera against EpsG (1:150,000 in Tris-buffered saline with 0.1% Tween-20) or EpsL (1:40,000 in Tris buffered saline with 0.1% Tween-20), followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (BioRad) diluted 1:15,000 in Tris-buffered saline containing 0.1% Tween-20. Blots were developed with ECL Plus Western blotting detection reagent (GE Healthcare) and protein was visualized using a Typhoon Trio variable mode imager system and Image Quant software.

Triton X-100 extraction and co-immunoprecipitation

Cultures were grown, cross-linked, and quenched as described above. Following quenching samples were subjected to Triton X-100 extraction as previously described (Johnson *et al.*, 2007). 100 µl of OD_{600}=2.0 of the Triton X-100 extracted material was immunoprecipitated with anti-EpsL or anti-EpsG antiserum bound to protein G-sepharose as previously detailed (Sandkvist *et al*., 1999). Antiserum labeled protein G sepharose beads bound to the cell extract were washed twice in high salt Triton X-100 buffer (Tris-buffered saline containing 500 mM NaCl, 0.01% Triton X-100, and 1M EDTA). Samples were then washed once in low salt buffer Triton X-100 buffer (Tris-buffered saline containing 150 mM NaCl, 0.01% Triton X-100, and 1M EDTA), followed by a final wash in Tris-buffered saline. 25 µl of Laemmli buffer was added to the washed beads and boiled for 10 minutes prior to centrifugation. Immunoprecipitated samples were divided equally and analyzed on NuPAGE 4-12% Bis-Tris polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in Tris-buffered

saline containing 5% milk, incubated with either biotinylated EpsG (1:5,000) or biotinylated EpsL antibodies (1:10,000) in Tris-buffered saline with 0.1% Tween-20, followed by incubation with horseradish peroxidase-conjugated streptavidin at 1:25,000 in Tris-buffered saline with 0.1% Tween-20. Blots were developed with ECL Plus Western blotting detection reagent (GE Healthcare) and protein was visualized using a Typhoon Trio variable mode imager system and Image Quant software.

Protease secretion assay

Secretion of extracellular protease was determined as previously described (Sikora *et al.*, 2007). The amount of fluorescence was compared to a standard curve of cleaved substrate in order to determine the nanograms of protease secreted.

Table 2.1. Strains and plasmids used in this study.

Strain or plasmid	Features	Reference or source
Strains		
V. cholerae		
TRH7000	El Tor strain, wild-type for type II secretion	Hirst *et al.*, 1984
Δ*epsG*	in frame replacement of *epsG* with Cm^R	Lybarger *et al.*, 2009
Δ*epsL*	in frame replacement of *epsL* with *aph-3* (Km^R)	Lybarger *et al.*, 2009
P_{BAD}::*eps*	TRH7000 P_{BAD}::*eps*	Sikora *et al.*, 2007
P_{BAD}::Δ*epsG*	TRH7000 P_{BAD}::*eps* with in frame replacement of *epsG* with Cm^R	This study
P_{BAD}::Δ*epsL*	TRH7000 P_{BAD}::*eps* with in frame replacement of *epsL* with *aph-3* (Km^R)	This study
Δ*eps*	TRH7000 in frame replacement of *epsC-N* with Cm^R	Sikora *et al.*, 2007
C6706str2	El Tor o1, Inaba, Sm^R	Thelin & Taylor, 1996
Δ*pilD* (JM313)	C6706str with in frame deletion of *pilD* (Cm^R)	Marsh & Taylor, 1998
E. coli		
MC1061	*F- lac-* K-12 laboratory strain	Casadaban & Cohen, 1980
MM294/pRK2013	helper strain for conjugations	Meselson & Yuan, 1968
Plasmids		
pMMB67	low copy number IPTG inducible vector (Ap^R)	Furste *et al.*, 1986
pMMB872	pMMB67 with *Bst*EII sites in lacIQ and repC removed	This study
pMMB917	*epsG* in pMMB872	This study
pEpsG	*epsG* in pMMB67	Lybarger *et al.*, 2009
$pEpsG_{D91E}$	substitution of residue D91 to glutamic acid in pMMB917	This study
$pEpsG_{T112L}$	substitution of residue T112 to leucine in pEpsG	This study
$pEpsG_{G-1V}$	substitution of residue G-1 to valine in pEpsG	This study
pEpsL (pMS44)	*epsL* in pMMB67	Sandkvist *et al.*, 1995
pEpsGL	*epsGepsL* in pMMB67	This study
pACYC184	low copy number vector (Cm^R, Tc^R)	Chang & Cohen, 1978
pPilD (pJM294)	*pilD* in pACYC184	Marsh & Taylor, 1998

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CHAPTER III

N-terminal methylation of the major pseudopilin EpsG is required for cross-linking with EpsL in the Type II Secretion complex in *Vibrio cholerae*

Summary

The type II secretion (T2S) system is proposed to form a periplasmic pilus-like structure, or pseudopilus, that aids in the extracellular secretion of proteins. The pseudopilus is comprised of several proteins referred to as pseudopilins that are structurally homologous to the type IV pilins. Additionally, both the T2S pseudopilins and the type IV pilins contain a conserved N-terminal sequence that is recognized and processed by the prepilin peptidase, PilD. PilD activity results in cleavage and methylation of the N-terminus, and is essential for secretion and pilus formation. Previously we used *in vivo* cross-linking to demonstrate an interaction between EpsG, the major component of the T2S pseudopilus, and the inner membrane protein EpsL in *Vibrio cholerae*. PilD was required for cross-linking to occur and mutations in EpsG that prevented cleavage also prevented cross-linking with EpsL, indicating that cleavage by PilD is a prerequisite for the interaction. In this study we have continued to examine the requirements for EpsG and EpsL to interact by addressing if methylation of the newly formed N-terminus following cleavage by PilD affects their association. Amino acid substitutions in EpsG that prevent methylation but not cleavage of the N-terminus inhibited secretion through the T2S apparatus and severely reduced *in*

vivo cross-linking with EpsL. These findings further validate the importance of PilD in the interaction between EpsG and EpsL, and suggest that N-terminal methylation of EpsG is necessary for it to associate with EpsL and perhaps other components of the T2S complex.

Introduction

Gram-negative bacteria utilize the T2S system for extracellular secretion of proteins, including many virulence factors (Sandkvist, 2001, Cianciotto, 2005). In *Vibrio cholerae* the T2S pathway is encoded by the extracellular protein secretion (*eps*) operon and is responsible for transporting the major virulence factor, cholera toxin, across the cell envelope (Sandkvist *et al.*, 1997). The *eps* products form a membrane spanning multi-protein complex that has structural homology to the type IV pilus biogenesis system (Filloux, 2004, Johnson *et al.*, 2006, Ayers *et al.*, 2010, Korotkov *et al.*, 2011). Five of these gene products, EpsG, H, I, J and K are structurally similar to the type IV pilins and have been termed the pseudopilins (Kohler *et al.*, 2004, Korotkov & Hol, 2008, Yanez *et al.*, 2008, Korotkov *et al.*, 2009). Whereas the type IV pilins form an extracellular appendage for diverse functions such as twitching motility, adhesion, and DNA uptake (Craig & Li, 2008), the T2S pseudopilins have been proposed to form a periplasmic pilus-like structure, or pseudopilus, primarily composed of the major pseudopilin EpsG in order to support secretion (Hobbs & Mattick, 1993, Pugsley, 1993a, Filloux, 2004, Johnson *et al*., 2006).

In addition to the structural homology shared by the T2S pseudopilins and type IV pilins, both contain a conserved N-terminal sequence that is processed by the prepilin

peptidase, PilD (Strom *et al.*, 1993, Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). PilD is required for type IV pilus biogenesis and T2S (Strom *et al.*, 1991, Nunn & Lory, 1993, Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). It is a bifunctional enzyme responsible for cleaving off the first seven residues of EpsG and then methylating the newly generated N-terminal residue, a phenylalanine (Marsh & Taylor, 1998, Fullner & Mekalanos, 1999). Studies of the *Pseudomonas aeruginosa* type IV pilin and the EpsG homolog PulG from *Klebsiella oxytoca* have indicated two specific residues within the N-terminus that are essential for cleavage and methylation by PilD. The glycine (referred to as G-1) at the cleavage site Gly-Phe is required for the cleavage event to occur (Strom & Lory, 1991, Pugsley, 1993b). A glutamic acid (referred to as E+5) located five residues post-cleavage is required for methylation by PilD (Pasloske & Paranchych, 1988, Strom & Lory, 1991, Macdonald *et al.*, 1993, Pugsley, 1993b, Aas *et al.*, 2007). Replacement of either the G-1 or E+5 residues prevents the formation of the type IV pilus and inhibits T2S, illustrating the importance of both cleavage and methylation in the formation of functional pilins or pseudopilins.

Previously we identified an interaction between EpsG and the inner membrane component EpsL by using *in vivo* cross-linking (Gray *et al.*, 2011). Deletion of *pilD* or mutagenesis of *epsG* to substitute the G-1 residue for valine prevented EpsG from cross-linking with EpsL, indicating that cleavage is required for EpsG to interact with EpsL (Gray *et al*., 2011). In this study we wanted to further dissect the processing requirement by determining if N-terminal methylation plays a role in allowing EpsG and EpsL to interact. We show that substitution of E+5 drastically inhibits EpsG from cross-linking with EpsL and abolishes secretion. Additionally, processing mutants prevented secretion

when produced in wild-type *V. cholerae* indicating that they interfere with the native T2S machinery.

Results

Substitution of EpsG residue E+5 prevents secretion

Studies on type IV pilins and the *K. oxytoca* EpsG homolog have shown that replacement of the conserved E+5 residue prevents the formation of the type IV pilus and secretion through the T2S apparatus; therefore, we first wanted to determine what effect substitution at this site in EpsG would have on secretion in *V. cholerae*. Based upon the residue changes that have previously been reported for the type IV pilin PilA from *P. aeruginosa* (Pasloske & Paranchych, 1988, Strom & Lory, 1991, Macdonald *et al*., 1993) and the EpsG homolog PulG from *K. oxytoca* (Pugsley, 1993b), we decided to substitute glutamic acid with several different amino acids: lysine, arginine, or valine. The mutant genes were generated by site-directed mutagenesis and expressed in P_{BAD}::Δ*epsG*, and then we monitored the secretion of an extracellular protease in overnight cultures. As shown in Figure 3.1 each of the substitutions prevented secretion through the T2S system, indicating that E+5 is required for EpsG to be functional.

N-terminal sequencing of $EpsG_{E+5K}$ confirms that the N-terminal phenylalanine is not methylated

Although it has been well documented that mutations to residue E+5 in EpsG homologs as well as type IV pilins prevents methylation of the phenylalanine post cleavage (Pasloske & Paranchych, 1988, Strom & Lory, 1991, Macdonald *et al*., 1993,

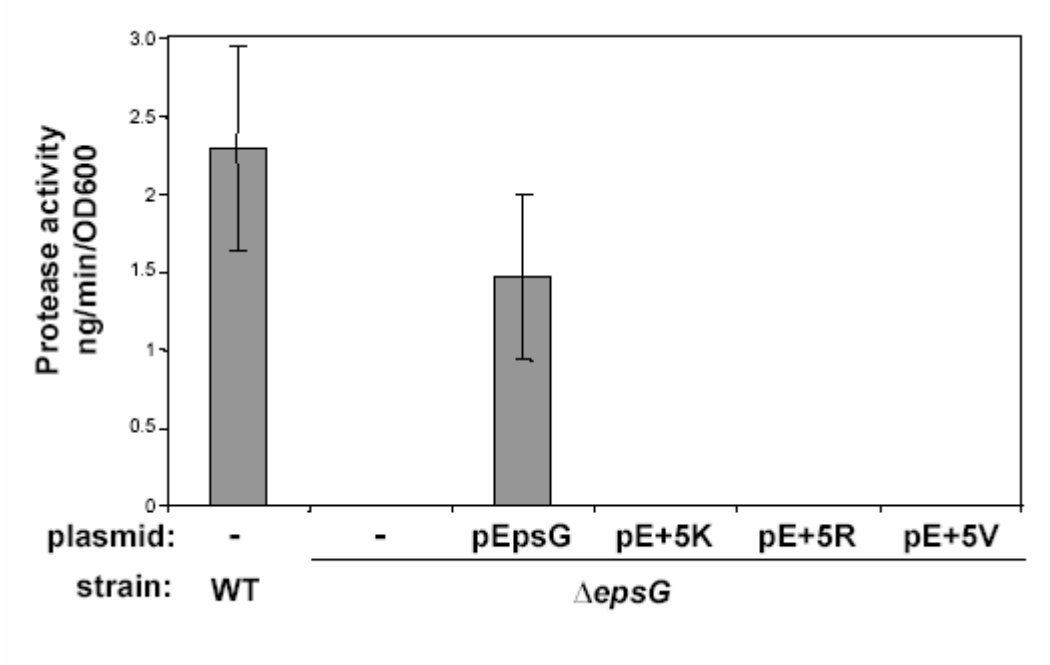

Figure 3.1. Replacement of EpsG residue E+5 prevents secretion. Liquid cultures of *V. cholerae* TRH7000 P_{BAD}::*eps* wild-type with empty vector, and P_{BAD}::Δ*epsG* with empty vector, vector encoded wild-type *epsG* or E+5 mutants were grown overnight at 37°C. Supernatants were separated from the cells and analyzed for the presence of a T2S specific extracellular protease using the cleavable fluorogenic substrate *N*-tert-butoxy-canbonyl-Gln-Ala-Arg-7-amide-4-methyl-coumarin. A minimum of three biological replicas for each sample was assayed. Protease activity for P_{BAD}::Δ*epsG* with empty vector or E+5 mutants was not detected above background. Standard error is indicated.

Nunn & Lory, 1993, Pugsley, 1993b, Aas *et al.*, 2006), we wanted to verify that our substitutions in EpsG also prevented methylation of the N-terminus. Because all three of our E+5 variants behaved similarly in the secretion assay above we elected to only sequence the E+5K substitution. EpsG and $EpsG_{E+5K}$ were partially purified by subjecting inner membrane proteins isolated from the P_{BAD}::Δ*epsG* strain over-expressing the wild-type and mutant genes to immunoprecipitation with anti-EpsG antiserum. The precipitated material was applied to SDS-PAGE analysis and transferred to PVDF membrane. Following staining with coomassie blue, the bands containing wild-type and mutant EpsG were excised and subjected to N-terminal sequencing by Edman degradation. A peak for the first residue of wild-type EpsG, predicted to be N-met-Phe, could not be detected; however, sequencing occurred past the first amino acid indicating that the N-terminus was not blocked (Table 3.1). The lack of a detectable Phe implies that the wild-type EpsG N-terminus is modified, and likely methylated, as has been shown for PulG (Pugsley, 1993b). In contrast, the N-terminal sequence for $EpsG_{E+5K}$ did detect Phe as residue one, suggesting that the N-terminus is no longer modified (Table 3.1). Additionally, residues 2-6 were identified as expected for both wild-type and mutant proteins, and confirmed the lysine substitution at position 5 for $EpsG_{E+5K}$.

Table 3.1. N-terminal sequencing reveals the first six residues for EpsG and $EpsG_{E+5K}$.

Residue #	**EpsG Sequence**	**$EpsG_{E+5K}$ Sequence**
1	ND[1]	F
2	T	T
3	L	L
4	L	L
5	E	K
6	V	V

[1]An amino acid peak was not detected above background.

Substitutions of EpsG residue E+5 alters the interaction with EpsL

We wanted to further our previous observation that PilD is necessary for *in vivo* cross-linking of EpsG to EpsL (Gray *et al*., 2011) by investigating whether both cleavage and methylation of EpsG are pre-requisites for cross-linking with EpsL. In order to observe similar protein levels as wild-type EpsG, the E+5 variants were induced with IPTG as described in the experimental procedures. The requirement for IPTG may indicate that the variants are less stable, or may be due to reduced recognition by our anti-EpsG antiserum. Following growth under the specified conditions, whole cells of P_{BAD}::Δ*epsG* expressing either wild-type *epsG* or the mutants were incubated in the absence or presence of DSP, subjected to SDS-PAGE, and then immunoblotted for EpsG. While cross-linking of wild-type EpsG produced a protein species around 60 kDa that corresponds to an EpsG-EpsL heterodimer, the E+5 mutants did not exhibit the EpsG-EpsL band (Figure 3.2). This finding suggests that interaction between EpsG and EpsL requires the N-terminus of EpsG to be methylated. Taken in conjunction with our previous finding that the G-1V substitution in EpsG that prevents cleavage by PilD also alters the ability of EpsG to cross-link with EpsL (Chapter II), our data indicate that both the cleavage and methylation activities of PilD are necessary for EpsG to associate with EpsL.

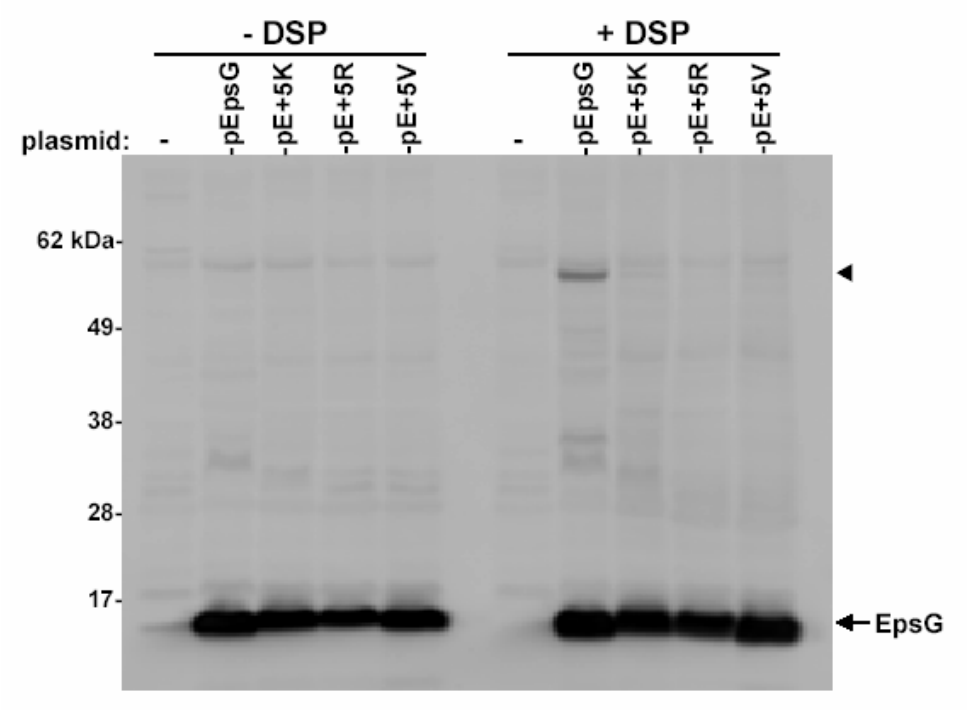

Figure 3.2. Substitutions of EpsG residue E+5 reduce the ability of EpsG to cross-link with EpsL. Whole cells of P_{BAD}::Δ*epsG* containing empty vector, or expressing wild-type *epsG* or variants of *epsG* were incubated in the absence or presence of 0.1 mM DSP. Samples were subjected to SDS-PAGE and immunoblotting with anti-EpsG antibodies. The EpsG monomer is labeled by an arrow and the EpsG-EpsL complex is indicated by an arrow head. Molecular weight markers are shown in kilo Daltons.

EpsG processing mutants are dominant-negative

Substitutions of E+5 in EpsG homologs have also been shown to be dominant-negative when produced in wild-type bacteria (Pasloske & Paranchych, 1988, Pugsley, 1993b, Aas *et al*., 2007). We next wanted to determine if our $EpsG_{E+5}$ variants exhibited dominance by over-expressing wild-type *epsG* or the $epsG_{E+5}$ mutants in *V. cholerae* TRH7000. Because we had previously observed that mutation to the G-1 residue that is required for cleavage also prevents secretion and cross-linking with EpsL (Gray *et al*., 2011), we also included the expression of $epsG_{G-1V}$ in our dominant-negative studies. The over-expression of plasmid-encoded wild-type *epsG* did not alter the secretion of an extracellular protease when grown in the absence or presence of IPTG (Figure 3.3). However, over-expression of the genes encoding processing mutants reduced the detection of secreted protease and completely abolished secretion when induced with 10 μM IPTG (Figure 3.3). Cells were also subjected to SDS-PAGE and immunoblotting with anti-sera against EpsG in order to verify induction of the genes with IPTG. Strains harboring plasmid-borne *epsG* or mutants showed a larger amount of protein in comparison to the strain with empty vector even in the absence of IPTG (Figure 3.3). Furthermore, the amount of protein detected was amplified upon treatment with IPTG, indicating that the loss of secretion observed with the variants was directly related to their abundance. These findings suggest that the EpsG processing mutants may interfere with the native T2S machinery.

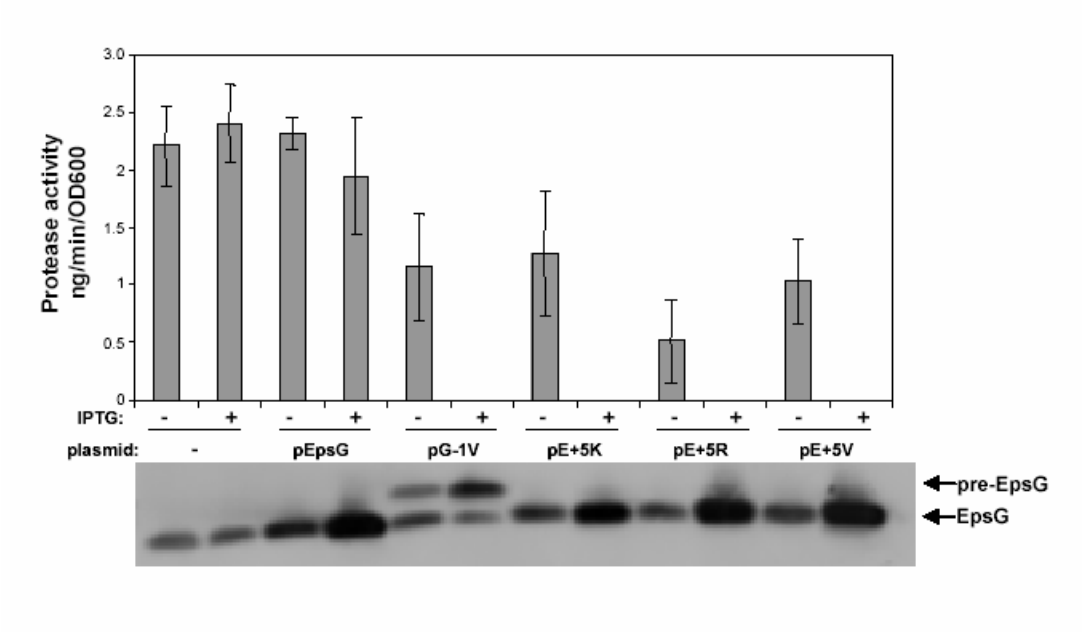

Figure 3.3. EpsG processing mutants exhibit dominance when expressed in wild-type *V. cholerae*. Overnight supernatants of TRH7000 expressing empty vector, wild-type *epsG* or indicated mutants were grown in the absence of presence of 10 μM IPTG and analyzed for the presence of an extracellular protease as described for Figure 3.1. Whole cells from overnight cultures were also immunoblotted for EpsG to verify IPTG induction of each vector. A representative blot from one of the secretion data sets is shown. The position of unprocessed (pre-EpsG) and processed EpsG is indicated.

Discussion

Here we have continued our investigation into the role that processing of EpsG by PilD plays in allowing for EpsG to be functional. Previously we determined that the cleavage activity of PilD was a pre-requisite for EpsG to support T2S as well as interact with the inner membrane component EpsL (Gray *et al*., 2011). In this study we wanted to elucidate the requirement for methylation of the newly formed EpsG N-terminus post cleavage. Although the cleavage and methylation events performed by PilD have been demonstrated for type IV pilins and the EpsG homolog from *K. oxytoca* (Strom & Lory, 1991, Macdonald *et al*., 1993, Nunn & Lory, 1993, Pugsley, 1993b, Aas *et al*., 2007), this report is the first time the methylation event has been analyzed in EpsG.

In agreement with the findings from *K. oxytoca* (Pugsley, 1993b), we determined that alteration to the E+5 residue in EpsG affects methylation and prevents secretion through the T2S complex. One explanation for the loss of secretion observed with our E+5 mutations is that they may alter the ability of EpsG to interact with other components of the T2S system, such as EpsL. Our data supports this hypothesis by demonstrating that substitution to E+5 greatly reduces the ability of EpsG to cross-link with EpsL. The loss of EpsG-EpsL cross-linking could be due to the N-terminus being involved in the interaction with EpsL. Alternatively, the loss in cross-linking may be due to the positioning of EpsG within the membrane as it has previously been proposed that modification of the N-terminus alters the way that EpsG is presented within the membrane (Nunn & Lory, 1993, Strom *et al*., 1993, Gray *et al*., 2011, Lemkul & Bevan, 2011); thus, loss of methylation could prevent EpsG from being accessible to EpsL. Recently, a structural homolog of EpsL, PilN, was identified in the *P.aeruginosa* type IV

pilus biogenesis system (Sampaleanu *et al.*, 2009); therefore, a similar mechanism may also explain the loss of type IV pili formation observed with E+5 substitutions in PilA (Strom & Lory, 1991, Macdonald *et al.*, 1993).

Another explanation for the loss in secretion is that substitution to E+5 prevents EpsG from multimerizing. Fiber assembly models based on the structures of type IV pilins and the EpsG homolog PulG from *K. oxytoca* suggest that residue E+5 may be involved in subunit interactions. It has been postulated that the N-met-Phe at position +1 in one subunit may form a hydrogen bond with the E+5 residue of the incoming subunit during pilus assembly (Craig *et al.*, 2003, Kohler *et al.*, 2004, Craig *et al.*, 2006). Alternatively, the E+5 in an incoming subunit may form a salt-bridge with a positively charged residue in the N-terminal alpha-helix of the neighboring subunit (Keizer *et al.*, 2001, Campos *et al.*, 2010).

We also observed a dominant-negative effect on T2S when the EpsG mutants with substitutions at either E+5 or G-1 were produced in a wild-type background. There are several interactions between the EpsG mutants and other T2S components that may have resulted in the dominance. First, it is possible that the variants interfered with PilD and prevented wild-type EpsG from being processed. However, our data suggested that EpsG was still cleaved in the presence of the $EpsG_{G\text{-}1V}$ variant, and similarly, there was not an accumulation of pre-EpsG in strains producing the $EpsG_{E+5}$ mutants. Another possibility is that the variants may have interfered with another member of the complex such as EpsL in order to prevent the native interaction that occurs with wild-type EpsG. Although our cross-linking data indicated a drastic decrease between the E+5 mutants and EpsL, we can not rule out the possibility that the mutants associate with EpsL in a

non-productive way that is no longer detectable through our methodology. A more plausible explanation is that the variants titrated wild-type EpsG away from the T2S apparatus due to the strong interaction between the hydrophobic alpha-helices of EpsG (Korotkov *et al*., 2009). In support of this hypothesis, E+5 variants in the *P. aeruginosa* type IV pilin PilA were capable of forming heterodimers with the wild-type subunit (Pasloske *et al.*, 1989). Additionally, crystallization of EpsG and the *K. oxytoca* homolog, PulG, revealed the formation of non-functional dimers (Kohler *et al*., 2004, Korotkov *et al*., 2009).

Interestingly, substitutions to the E+5 residue in the type IV pilin PilE from *Neisseria gonorrhoeae* were also dominant and resulted in loss of pili; however, the dominance was recessive in a background lacking PilT, the ATPase required for type IV pilus retraction (Aas *et al*., 2007). Aas *et al*. proposed that PilT may act as a quality control mechanism of the type IV pilus and favor retraction in the presence of mixed multimers, either through a direct interaction with the pilus or through an indirect association with an inner membrane component. This hypothesis is supported by our finding that $EpsG_{E+5}$ mutants alter the interaction with EpsL. Alternatively, mixed multimers may have reduced the efficiency of polymerization; thus, the defect in assembly was overcome in the absence of the retraction motor.

Our findings have established that both cleavage and methylation of EpsG is required for the association with EpsL and further identifies the steps that EpsG undergoes as it is recruited and incorporated into the T2S complex**.** Previously we had shown that PilD was the only component required for EpsG and EpsL to interact (Chapter II), and this finding may imply that the interaction occurs before EpsG assembles into the

pseudopilus. Although it is difficult to discern the exact role of the E+5 residue and how it affects N-terminal methylation, E+5 could be important for both associating with EpsL within the inner membrane prior to pseudopilus assembly and in forming the interface for EpsG subunits as they are assembled. Future studies will be needed to fully understand the importance of N-terminal methylation for both the T2S pseudopilins and type IV pilins.

Acknowledgements

We thank Michael Bagdasarian for plasmid pEpsG$_{E+5V}$.

Experimental procedures

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 3.2. For all experiments, cultures were grown at 37°C in Luria-Bertani supplemented with 100 μg ml^{-1} thymine. *V. cholerae* P$_{BAD}$::*eps* strains were also grown in the presence of 0.01% arabinose. When necessary, antibiotics (Sigma-Aldrich) were included at the following concentrations: carbenicillin at 200 μg ml^{-1}, ampicillin at 100 μg ml^{-1}, and kanamycin at 50 μg ml^{-1}. IPTG was added at the specified concentration for each experiment.

Construction of EpsG E+5 plasmids

All *epsG$_{E+5}$* mutations were introduced with the QuickChange II site-directed mutagenesis kit (Stratagene) using pEpsG as a template (Lybarger *et al.*, 2009). Primers used for the site change are as follows: E+5K was constructed using 5′-

cgggctttaccctgctcaaagtaatggtggttgtgg-3′ and 5′-ccacaaccaccattactttgagcagggtaaagcccg-3′, E+5R with 5′-gggctttaccctgctcagagtaatggtggttgtggtg-3′ and 5′-caccacaaccaccattactctgagcagggtaaagccc-3′, and E+5V with 5′-gggctttaccctgctcgttgtaatggtggttgtggtg-3′ and 5′-caccacaaccaccattacaacgagcagggtaaagccc-3′. Sequences were verified at the University of Michigan DNA sequencing core. Plasmid constructs were first obtained using *E.coli* MC1061 and subsequently conjugated into *V. cholerae* P_{BAD}::Δ*epsG* using the helper strain MM294/pRK2013 (Meselson & Yuan, 1968). Transconjugants were selected for plasmid retention by plating on carbenicillin.

Protease secretion assay

Secretion of extracellular protease was determined as previously described (Sikora *et al.*, 2007).

In vivo cross-linking

Overnight cultures were diluted in fresh media. Genes encoding the E+5 mutants were induced with IPTG in order to express an amount equivalent to that of the wild-type *epsG* gene. Specifically, P_{BAD}::Δ*epsG* with empty vector or wild-type *epsG* were grown in the absence of IPTG. P_{BAD}::Δ*epsG* with pEpsG$_{E+5K}$, pEpsG$_{E+5R}$, and pEpsG$_{E+5V}$ were induced with 5, 10, and 10 μM IPTG, respectively. Cross-linking and immunoblotting for EpsG was performed as previously detailed (Gray *et al*., 2011).

Isolation of inner membrane fractions

Overnight cultures of P_{BAD}::Δ*epsG* containing pEpsG were diluted into 100 mL of LB and induced with 1 mM IPTG. P_{BAD}::Δ*epsG* containing $pEpsG_{E+5K}$ were diluted into 1 L of LB and induced with 100 μM IPTG. Both strains were grown at 37°C to an optical density of OD_{600}=1.5. Cultures were centrifuged at 4°C to pellet the cells and then suspended in 10 mL of 50 mM Tris pH 8.0 containing protease inhibitors and placed on ice. Samples were put through a French press three times each in order to disrupt the membranes. Samples were then centrifuged at 2650 x *g* to remove any cellular debris and the supernatant was subjected to centrifugation at 150,000 x *g* for 30 min at 4°C to collect the membrane fractions. Membranes were suspended in 1 mL of 50 mM Tris pH 8.0, 10 mM MgCl2, 1% Triton X-100 and incubated on ice for 30 min. Samples were centrifuged at 242,000 x *g* for 30 min at 4°C and the supernatant containing the inner membrane fraction was removed and stored at -80°C.

N-terminal sequencing

Inner membrane fractions were immunoprecipitated with anti-EpsG antiserum as previously described (Gray *et al*., 2011). Precipitated samples were subjected to SDS-PAGE and transferred to PVDF membranes in CAPS buffer (10 mM CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid), 10% methanol, pH 11.0). The membranes were coomassie stained using Brilliant Blue R-250 (Fisher Scientific) and the bands corresponding to wild-type and mutant EpsG were cut out and sequenced at the Protein Chemistry Laboratory at Texas A&M using an Applied Biosystems model 492 analyzer.

Table 3.2. Strains and plasmids used in this study.

Strain or plasmid	Features	Reference or source
Strains		
V. cholerae		
TRH7000	El Tor strain, wild-type for type II secretion	Hirst *et al.*, 1984
P_{BAD}::*eps*	TRH7000 P_{BAD}::*eps*	Sikora *et al*., 2007
P_{BAD}::Δ*epsG*	TRH7000 P_{BAD}::*eps* with in frame replacement of *epsG* with Cm^R	Gray *et al*., 2011
E. coli		
MC1061	*F- lac-* K-12 laboratory strain	Casadaban & Cohen, 1980
MM294/pRK2013	helper strain for conjugations	Meselson & Yuan, 1968
Plasmids		
pMMB67	low copy number IPTG inducible vector (Ap^R)	Furste *et al.*, 1986
pEpsG	*epsG* in pMMB67	Lybarger *et al*., 2009
$pEpsG_{G-1V}$	substitution of residue G-1 to valine in pEpsG	Gray *et al*., 2011
$pEpsG_{E+5K}$	substitution of residue E+5 to lysine in pEpsG	This study
$pEpsG_{E+5R}$	substitution of residue E+5 to arginine in pEpsG	This study
$pEpsG_{E+5V}$	substitution of residue E+5 to valine in pEpsG	This study

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CHAPTER IV

Identification of EpsG residues required for cross-linking with EpsL in the Type II Secretion system of *Vibrio cholerae*

Summary

EpsG is the major component of the type II secretion (T2S) pseudopilus in *Vibrio cholerae*. The pseudopilus is proposed to be a fiber-like structure that spans the periplasm and assists in the secretion of proteins. Similar to the type IV pilus biogenesis system, assembly of the pseudopilus is thought to require the energy provided by a cytoplasmic ATPase. Previously we identified an interaction between EpsG and the inner membrane component EpsL and hypothesized that their interaction serves to link EpsG to the ATPase. Here we have continued to investigate the interaction between EpsG and EpsL by determining residues in EpsG that are required for their association. Using site-directed mutagenesis and *in vivo* cross-linking we have determined that disruption to the calcium binding site in the EpsG C-terminus increases the cross-linking with EpsL. Additionally, we have identified several residues located in the αβ loop of EpsG that when substituted reduce the amount of EpsG-EpsL complex. As alteration to both sites also interferes with secretion, we propose that substitutions to the calcium binding site may result in localized misfolding that stabilizes the interaction with EpsL, whereas, the substitutions within the αβ loop reside in a region that is surface exposed and may represent a site of direct interaction with EpsL. The type IV pilus biogenesis

system contains a component that has structural homology to EpsL and the αβ loop of the type IV pilins is also surface exposed. This region may represent an important site in linking both the pseudopilus and type IV pilus to the inner membrane components to support secretion and formation of the pilus, respectively.

Introduction

The T2S system is a multi-protein complex employed by gram-negative bacteria to transport proteins, including virulence factors, across the cell envelope (Sandkvist, 2001a, Filloux, 2004, Cianciotto, 2005). *Vibrio cholerae* utilizes the T2S apparatus to secrete a wide array of proteins including cholera toxin which is responsible for the major diarrheal symptoms of the disease cholera (Overbye *et al.*, 1993, Sack *et al.*, 2004, Sikora *et al.*, 2011). Understanding how the T2S system forms and the specific interactions that occur between the components is paramount in order to identify novel therapeutics that can specifically inhibit the T2S complex and prevent the secretion of proteins involved in pathogenesis.

In *V. cholerae* the T2S system requires at least 12 proteins, EpsC-M and PilD, to support secretion. The assembled apparatus consists of an outer membrane secretin pore formed by EpsD, an inner membrane platform composed of EpsC, F, L and M, a cytoplasmic ATPase EpsE, and a pilus-like structure (or pseudopilus) made up of EpsG, H, I, J and K (Filloux, 2004, Johnson *et al.*, 2006). EpsG-K are referred to as pseudopilins due to their structural homology to the type IV pilins and require processing of their N-terminus by the prepilin peptidase PilD in order to be functional (Marsh & Taylor, 1998, Fullner & Mekalanos, 1999, Korotkov & Hol, 2008, Yanez *et al.*, 2008,

Korotkov *et al.*, 2009). The pseudopilins are hypothesized to form a periplasmic pilus, primarily composed of the major pseudopilin EpsG, which may act as a piston to push secreted proteins through the secretin pore (Hobbs & Mattick, 1993, Sandkvist, 2001b, Filloux, 2004).

Assembly of the EpsG is proposed to occur due to the energy generated by the cytoplasmic ATPase EpsE; however, it is unclear how these two components may directly interact since they reside in different cellular compartments. EpsE has been shown to interact with the bitopic inner membrane protein EpsL, and their association is necessary for recruiting EpsE to the cytoplasmic membrane and the T2S complex (Sandkvist *et al.*, 1995, Abendroth *et al.*, 2005). Recently we established an interaction between EpsG and EpsL by utilizing the cross-linker dithiobis (succinimidyl propionate), DSP (Gray *et al.*, 2011). We hypothesized that EpsL links EpsE to EpsG, perhaps by functioning as a mechanical lever to transfer the energy needed to support assembly of the pseudopilus.

In this study we sought to better understand the interaction between EpsG and EpsL by identifying EpsG residues that are involved in cross-linking EpsG to EpsL. EpsG adopts a typical pilin fold consisting of an N-terminal alpha-helix, a variable or αβ loop region, and a C-terminal domain that binds a calcium ion (Korotkov *et al.*, 2009). Previously we found that substitution of EpsG residues within the N-terminus drastically reduced the amount of cross-linking with EpsL, affected processing by PilD, and inhibited T2S (Chapters II and III). In Chapter II we also investigated EpsG residues towards the C-terminus that reduced cross-linking with EpsL and inhibited secretion when substituted. Here we further investigated the C-terminus of EpsG by demonstrating

that replacement of residues involved in the calcium coordination increases cross-linking with EpsL. Additionally, we have identified amino acids located in the αβ loop of EpsG between residues 70 and 80 that when substituted reduce the amount of cross-linking with EpsL. The loss of EpsG-EpsL cross-linking observed with the αβ loop mutations correlated with a similar loss in secretion. These findings further validate the importance of the interaction between EpsG and EpsL, and provides insight as to how the structural domains of EpsG may affect it's function.

Results

Replacement of residues in the EpsG calcium binding site increases cross-linking with EpsL

The structure of EpsG revealed a unique calcium binding site in the C-terminus that is coordinated by six amino acids (Leu-113, Asp-116, Gln-118, Gly-120, Gly-125, and Asp-127) (Korotkov *et al*., 2009). Substitution of the two aspartic acids highlighted in Figure 4.1 for alanine was previously shown to disrupt secretion, indicating that calcium coordination is essential for EpsG to be functional (Korotkov *et al*., 2009). Thus, we wanted to determine if $EpsG_{D116AD127A}$ affected the ability of EpsG to cross-link with EpsL *in vivo*. Log cultures of P_{BAD}::Δ*epsG* containing plasmid-encoded wild-type or mutant *epsG* were grown in the absence of IPTG and cross-linked with 0.1 mM DSP; samples were then subjected to SDS-PAGE and immunoblotted with anti-EpsG antibodies as previously described (Gray *et al*., 2011). Although $EpsG_{D116AD127A}$ appeared to be produced at a lower level than EpsG, a similar amount of EpsG-EpsL cross-linked complex was detected for both mutant and wild-type EpsG (Figure 4.2A).

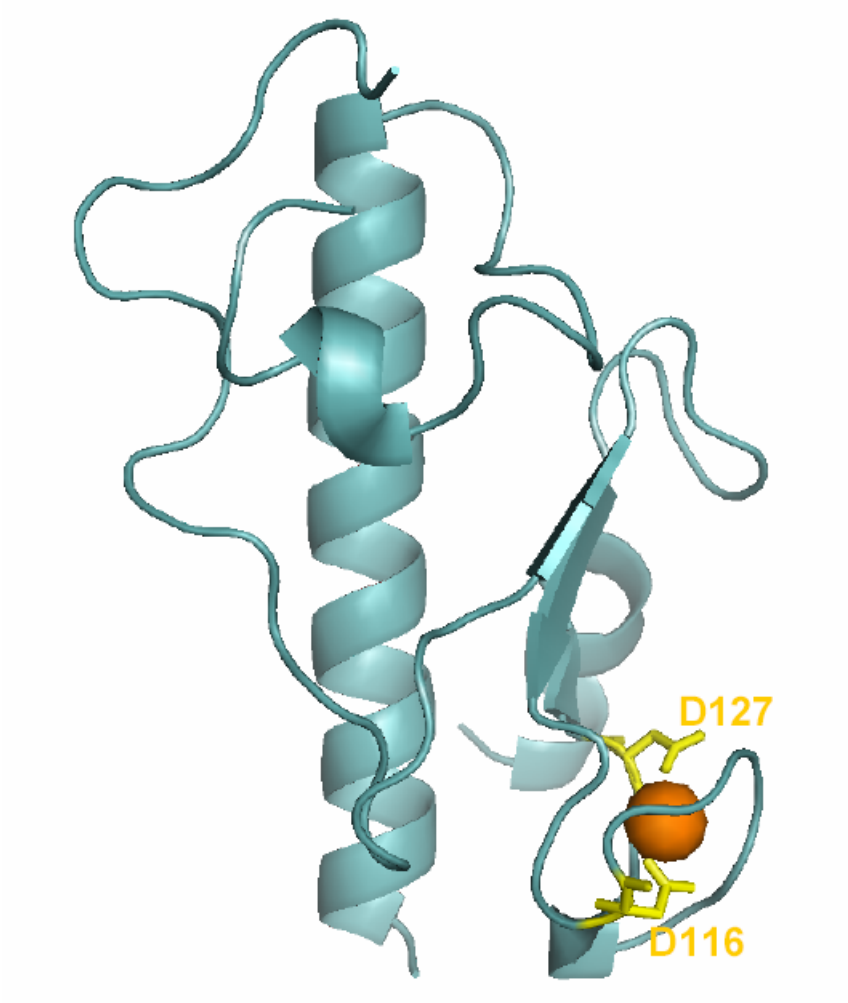


Figure 4.1. The calcium is coordinated by residues Asp-116 and Asp-127 in EpsG. *V. cholerae* EpsG (Korotkov *et al*., 2009; PDB code 3FU1) is shown with the calcium ion represented by an orange sphere and the side chains of D116 and D127 in yellow. Figure prepared with PyMOL (DeLano, 2002).

The reduction in protein level for the calcium binding mutant was apparent when the cross-linked samples were diluted 1:50 and then immunoblotted for EpsG (Figure 4.2B).

To further investigate if substitution of residues in the calcium binding site in EpsG increases the amount of EpsG-EpsL cross-linked complex, we determined the amount of IPTG necessary to produce the wild-type and mutant proteins at a similar level (data not shown). Equivalent levels for EpsG and $EpsG_{D116AD127A}$ were obtained with 15 and 40 μM IPTG, respectively. Using these conditions, samples were then subjected to *in vivo* cross-linking and immunoblotting for EpsG as above. As shown in Figure 4.2A, the EpsG-EpsL cross-linked band was substantially increased for $EpsG_{D116AD127A}$ in comparison to the complemented strain when similar levels of monomers were produced. Diluting the IPTG induced cross-linked samples 1:500 further demonstrated that both wild-type and mutant EpsG were being produced at comparable levels (Figure 4.2B).

Finally, we quantitated the amount of EpsG-EpsL complex that was cross-linked for both wild-type and mutant proteins. Using a Typhoon Trio variable mode imager system and Image Quant software we determined the amount of EpsG-EpsL cross-linked complex shown in Figure 4.2A. The EpsG-EpsL protein species for wild-type or mutant EpsG was then normalized to the amount of its respective diluted monomer (Figure 4.2B). For the samples grown in the absence of IPTG, $EpsG_{D116AD127A}$ exhibited a 3 fold increase in EpsG-EpsL cross-linked complex in comparison to wild-type EpsG. Similarly, when grown in the presence of IPTG the calcium binding mutant displayed a 3.4 fold increase in EpsG-EpsL cross-linking compared to wild-type protein.

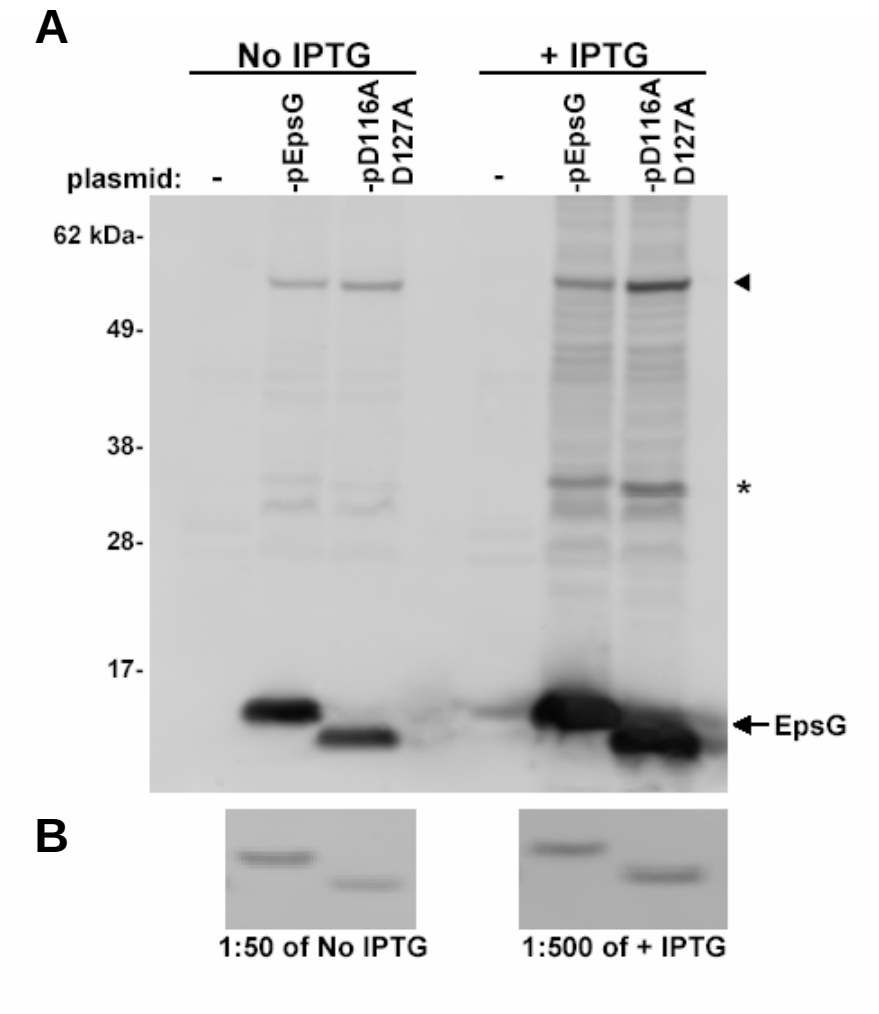


Figure 4.2. Substitution of EpsG residues involved in calcium coordination increases the amount of EpsG-EpsL cross-linked complex.
A. Whole cells of P_{BAD}::Δ*epsG* containing pEpsG or pEpsG$_{D116AD127A}$ were grown in the absence or presence of IPTG (15 and 40 μM, respectively). Samples were incubated with 0.1 mM DSP, followed by SDS-PAGE analysis and immunoblotting with antibodies specific for EpsG. The position of the EpsG monomer (EpsG$_{D116AD127A}$ migrates at a slightly faster rate) is indicated by an arrow, the EpsG-EpsL cross-linked species with an arrow head, and the putative EpsG dimer is labeled with an asterick. The molecular weight markers are shown in kilo Daltons.

Identification of lysine residues in EpsG that are involved in cross-linking with EpsL

In order to identify other residues of EpsG that are involved in cross-linking with EpsL we took advantage of the properties of our cross-linker. DSP is a homobifunctional membrane permeable cross-linker that recognizes primary amines. Because the N-terminus of EpsG is modified by the prepilin peptidase (Marsh & Taylor, 1998, Fullner & Mekalanos, 1999, Chapter III), the only amino acids available for cross-linking are lysines. Using site-directed mutagenesis, we replaced each individual lysine residue in the mature form of EpsG (K28, K30, K35, K51, K70, K86, K90, and K105). P_{BAD}::Δ*epsG* producing wild-type EpsG or the lysine mutants were cross-linked with 0.1 mM DSP, and then analyzed by SDS-PAGE and immunoblotting with anti-EpsG antiserum (Figure 4.3). Substituting the lysine at position 70 for an alanine reduced the ability of EpsG to cross-link with EpsL, implying that this lysine may be involved in the cross-linking. Replacement of K51 with glutamic acid also reduced the amount of EpsG-EpsL complex; however, substitution to K51 with alanine did not exhibit a decrease in G-L cross-linking. This suggests that the lysine at position 51 is not involved in cross-linking with EpsL, rather, the charge reversion introduced with $EpsG_{K51E}$ may have interfered with other interactions that indirectly altered the cross-linking with EpsL. Thus, our data indicate that the lysine at position 70 may be the primary residue involved in cross-linking with EpsL. However, since the EpsG-EpsL complex was not completely lost another lysine must also be contributing to the cross-linking, although not as significantly.

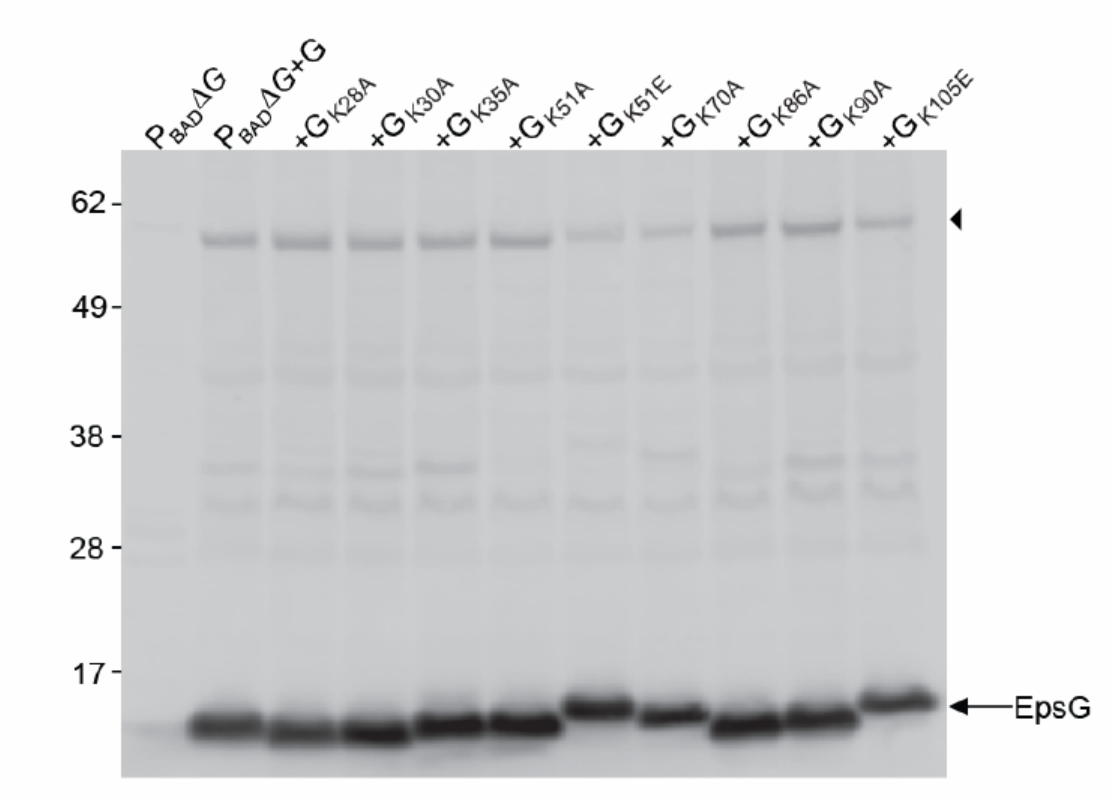
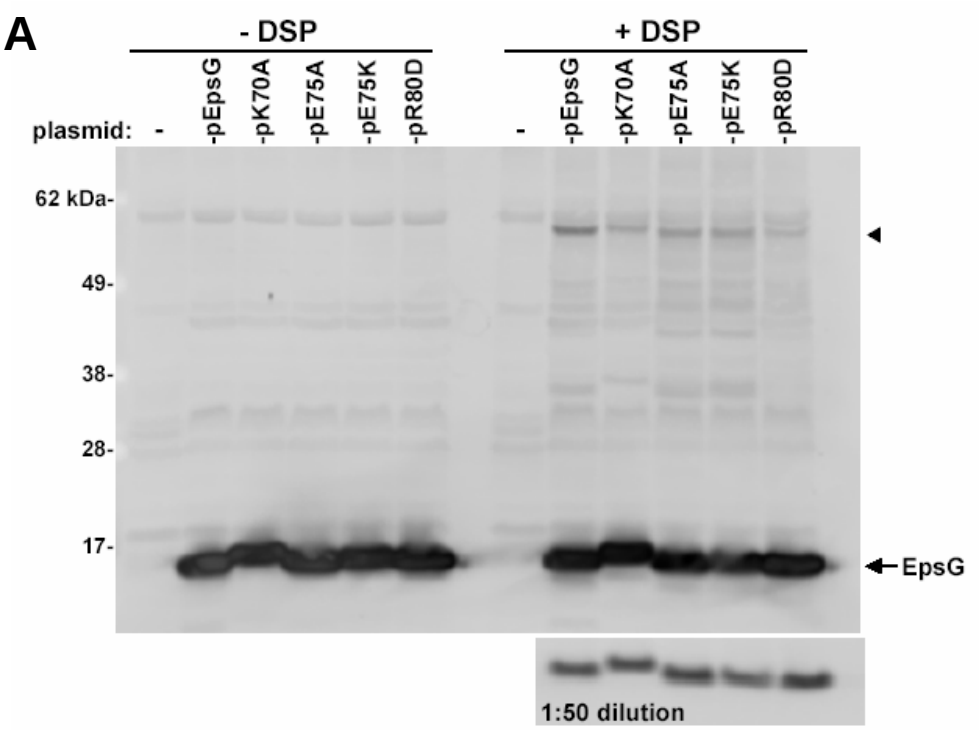


Figure 4.3. Substitution of the lysine residues in EpsG suggests that position K70 is involved in the cross-linking event.
Whole cells of P_{BAD}::Δ*epsG* containing pEpsG or the indicated mutants were incubated in the presence of 0.1 mM DSP, subjected to SDS-PAGE, and then immunoblotted for EpsG. The position of the EpsG monomer is indicated by an arrow and the EpsG-EpsL cross-linked band is designated with an arrow head. The molecular weight markers are shown in kilo Daltons.

Substitutions in EpsG between residues 70 and 80 reduces cross-linking with EpsL

We wanted to further examine the region of EpsG where K70 is located in order to determine the role this structural domain plays in the interaction with EpsL. We chose to replace surface exposed residues around K70 that would have a higher potential to be involved in protein-protein interactions. Specifically we substituted the glutamic acid at position 75 for either an alanine or lysine, and the arginine at residue 80 for an aspartic acid. P_{BAD}::Δ*epsG* containing pEpsG, pEpsG$_{K70A}$, pEpsG$_{E75A}$, pEpsG$_{E75K}$, or pEpsG$_{R80D}$ were incubated in the absence or presence of 0.1 mM DSP. Samples were subjected to SDS-PAGE and then immunoblotted with anti-sera against EpsG. Wild-type EpsG produced a strong band around 60 kDa corresponding to an EpsG-EpsL complex; however, each of the EpsG substitutions greatly reduced the amount of EpsG-EpsL cross-linking (Figure 4.4A). In order to verify that the reduced complex was not due to expression or loading discrepancies between the different samples we quantitated the amount of EpsG-EpsL complex and monomer for each sample. To measure the amount of EpsG monomer, cross-linked samples were diluted 1:50 and ran on a separate SDS-PAGE gel and immunoblotted for EpsG (Figure 4.4A). Bands were quantitated using a Typhoon Trio imager as described for the calcium binding mutant above. The amount of diluted EpsG monomer was used to normalize the amount of EpsG-EpsL cross-linking; wild-type EpsG cross-linked with EpsL was then set to 100% and the EpsG mutants were compared to the percent of wild-type cross-linking. As shown in Figure 4.4B, the mutants exhibited a 60-80% decrease in the amount of EpsG-EpsL cross-linked complex, demonstrating that substitutions of K70, E75, and R80 reduced EpsG's ability to cross-link with EpsL.



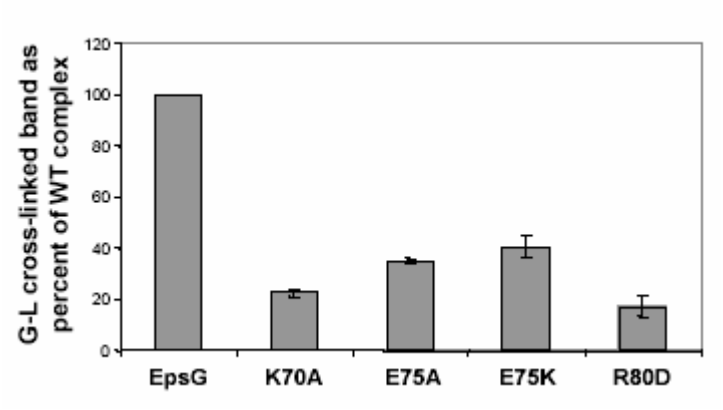


Figure 4.4. Replacement of EpsG residues K70, E75, and R80 reduce the amount of cross-linked EpsG-EpsL complex.

A. Whole cells of P_{BAD}::Δ*epsG* containing pEpsG or variants were incubated in the absence or presence of 0.1 mM DSP, followed by SDS-PAGE analysis and immunoblotting with antibodies specific for EpsG. The position of the EpsG monomer is indicated by an arrow, and the EpsG-EpsL cross-linked species with an arrow head. Molecular weight markers are shown in kilo Daltons. Below is the amount of monomer from the DSP treated samples when diluted 1:50.

B. Quantitation of EpsG-EpsL cross-linked protein species. Bands were analyzed with Image Quant software as described in the text. The percentage of EpsG-EpsL complex was determined from three separate experiments. Standard Error is indicated.

EpsG substitutions between residues 70 and 80 reduces protease secretion

We next wanted to determine if the reduction in cross-linking to EpsL observed with the replacement of EpsG residues K70, E75, and R80 also altered their ability to support T2S. Supernatants from overnight cultures of P_{BAD}::Δ*epsG* producing wild-type EpsG or the mutants were analyzed for the presence of an extracellular protease secreted by the T2S system. Substitution to K70 and R80 displayed a statistically significant reduction in secretion, with $EpsG_{R80D}$ being completely unable to complement the secretion defect in the P_{BAD}::Δ*epsG* strain (Figure 4.5). Although substitution of residue E75 appeared to have a slight negative effect on secretion, it was not statistically significant. Interestingly, the amount of secretion detected corresponded to the trend observed for the percent loss of EpsG-EpsL cross-linked complex. That is, replacement of R80 showed the greatest loss of cross-linking whereas substitution to E75 exhibited the highest amount of cross-linked complex.

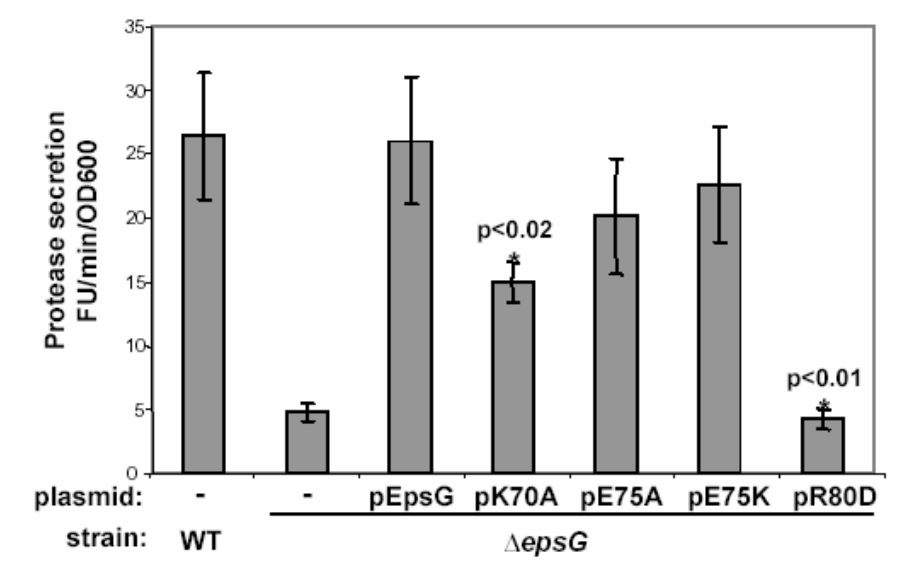


Figure 4.5. EpsG substitutions within the K70-R80 loop reduce protease secretion. Supernatants of overnight cultures of P_{BAD}::*eps* wild-type, and P_{BAD}::Δ*epsG* with vector encoded wild-type or mutant *epsG* were separated from cells and analyzed for the presence of an extracellular protease as described in the experimental procedures. A minimum of three biological replicas of each sample was assayed and p values were determined compared to the complemented strain. Standard error is indicated.

Discussion

In this study we have continued our efforts to better understand the interaction between EpsG and EpsL within the T2S complex by determining residues in EpsG that are required for *in vivo* cross-linking with EpsL. Previously we found that replacement of EpsG residues D91 and T112 severely reduced the amount of EpsG-EpsL cross-linked complex and prevented secretion; however, these amino acids are found in a crevice that is barely surface exposed and the substitutions likely altered the localized folding of EpsG and may have had an indirect effect on the EpsG-EpsL interaction (Gray *et al*., 2011). Here we used site-directed mutagenesis in order to determine other regions of EpsG that are involved in cross-linking with EpsL, with the aim of identifying the site in EpsG that may be directly interacting with EpsL.

Our findings have illustrated that K70 is one of the residues involved in the cross-linking event as replacement of K70 for alanine resulted in an 80% decrease in cross-linking efficiency. Furthermore, substitution of nearby residues, E75 or R80, also reduced the amount of EpsG-EpsL cross-linked complex. EpsG residues K70, E75, and R80 are found within the variable segment (also referred to as the αβ loop) and located in a loop near the tip of the N-terminal alpha-helix (Figure 4.6A). The K70-R80 loop is surface exposed in the monomer, therefore, these residues would be available to interact with EpsL. Furthermore, modeling of the pseudopilus formed by *V. cholerae* EpsG or PulG from *Klebsiella oxytoca* suggested that the K70-R80 loop would remain partially surface exposed following assembly, although it may also be important for interactions between EpsG subunits (Kohler *et al*., 2004, Korotkov *et al*., 2009, Campos *et al*., 2010). Thus, it is possible that this region may represent a site of direct interaction with EpsL.

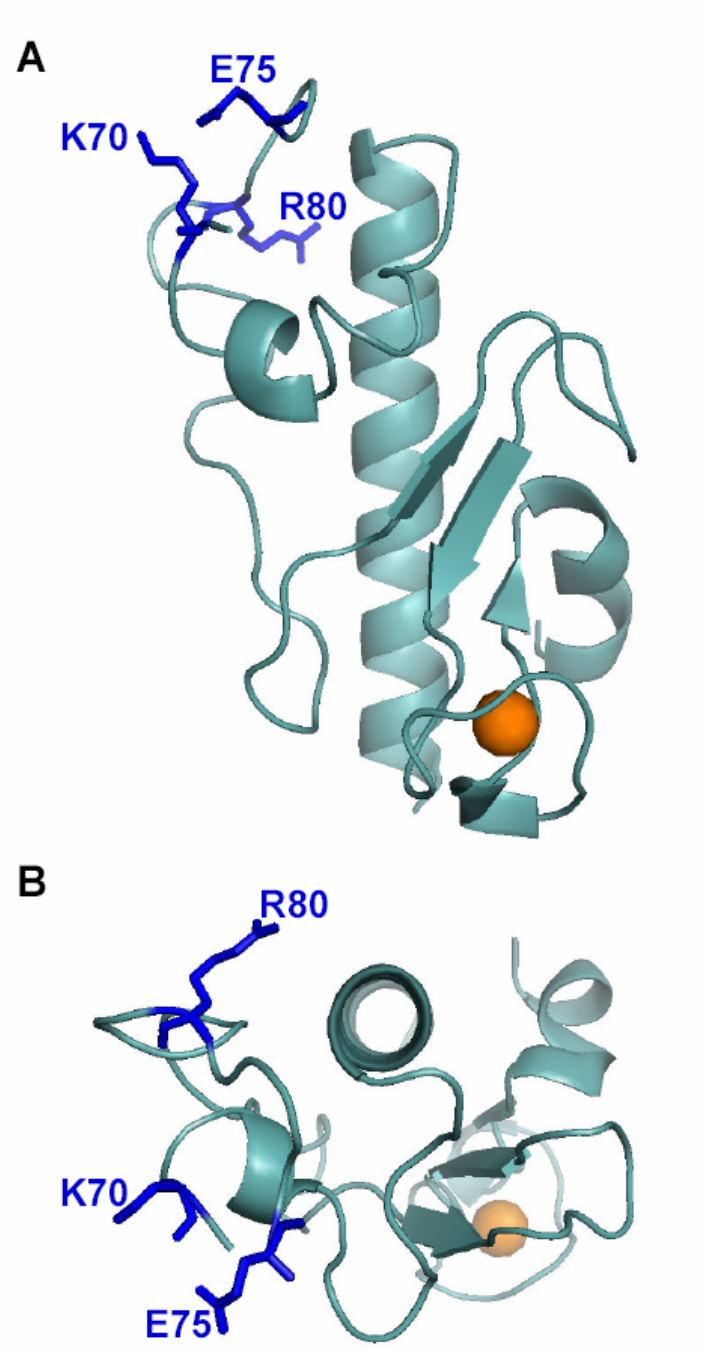


Figure 4.6. *V. cholerae* EpsG residues Lys-70, Glu-75, and Arg-80 are located in the αβ loop.
A. The structure of *V. cholerae* EpsG has a typical pilin fold (Korotkov *et al*., 2009; PDB code 3FU1). The side chains of K70, E75, and R80 are depicted as sticks in blue. The orange sphere represents a calcium ion.
B. Bird's eye view of EpsG structure along the alpha-helix demonstrates the location of the side chains for K70, E75, and R80 in relation to each other. Figure prepared with PyMOL (DeLano, 2002).

The ability of substitutions within the αβ loop to interfere with secretion varied. Mutation to K70 caused a significant 40% reduction in secretion while substitution to E75 did not significantly reduce protease secretion. It is likely that the interface between EpsG and EpsL is composed of several residues; thus, single substitutions may not be sufficient to completely disrupt secretion. One explanation for the difference in the ability to secrete and the ability to cross-link with EpsL is based on our finding that K70 is one of the lysines being cross-linked by DSP. Substitution to E75 may have reduced the cross-linking with EpsL by slightly altering the αβ loop in such a way that K70 is no longer available for cross-linking. That is, while changes within the αβ loop can have substantial effects on cross-linking, the interaction with EpsL is not sufficiently altered as to prevent secretion. Furthermore, the substitutions within the αβ loop did not fully prevent cross-linking with EpsL, therefore, the amount of cross-linking observed may be adequate to promote the levels of secretion. Interestingly, the structure of EpsG indicated that K70 and E75 form a salt bridge; however, there are several residues within the αβ loop that were undefined in the structure including three amino acids between K70 and E75. Because of this the orientation of K70 and E75 may be slightly different than what is seen in the structure and this possibility may also explain the difference in secretion for the substitutions. It is intriguing that the αβ loop was the only part of the structure that was undefined, implying that this region may be flexible and become stabilized through an interaction with another component such as EpsL.

Conversely, substitution of R80 completely abolished secretion and showed the most dramatic loss in EpsG-EpsL cross-linking. The severe defects observed with $EpsG_{R80D}$ may be a result of the charge reversion introduced and the orientation of its side

chain in relation to the K70 and E75 residues (Figure 4.6B). Multimerization of EpsG is proposed to involve interactions between the N-terminal alpha-helix of each subunit (Kohler *et al.*, 2004, Korotkov *et al*., 2009, Campos *et al.*, 2010); therefore, the R80D mutation may cause subunit-subunit hindrance as well as interfere with the ability of EpsG to associate with EpsL. Additionally, the structure of EpsG revealed that R80 is forming an intramolecular salt bridge with D53, a residue found along the alpha-helix. Thus, disruption of this bond may have also resulted in the more severe phenotype observed with $EpsG_{R80D}$.

Modeling of the type IV pilus formed by either *Neisseria gonorrhoeae* PilE or *Pseudomonas aeruginosa* PilA also suggest that the αβ loop is surface exposed in the assembled type IV fiber (Forest *et al.*, 1999, Keizer *et al.*, 2001, Craig *et al.*, 2003, Craig *et al.*, 2006). Moreover, antisera raised against regions of the αβ loop in PilE were able to bind to the surface of purified pili (Forest *et al.*, 1996). Interestingly, PilE is post-translationally modified at several residues within the αβ loop (Parge *et al.*, 1995, Marceau *et al.*, 1998, Hegge *et al.*, 2004, Craig *et al*., 2006, Hansen & Forest, 2006). Modification of PilE is unique to *Neisseri*a species and has been hypothesized to aid in antigenic variation and provides a means for escape from the host immune system (Hegge *et al.*, 2004). Alternatively, it has also been proposed that modification may affect assembly or disassembly of the pilus (Forest *et al*., 1999). Recently, Chamot-Rooke *et al*. showed that modification to PilE in *N. meningitidis* allowed for dissemination of the bacteria during infection (Chamot-Rooke *et al.*, 2011). They proposed that modification altered the aggregative properties of the pilus by disrupting subunit interactions within the fiber. However, it is also possible that modification

signaled disassembly by affecting the interaction between pilin and a component of the inner membrane platform, such as the EpsL homolog PilM/N (Carbonnelle *et al.*, 2006, Sampaleanu *et al.*, 2009).

In addition to our investigation into the αβ loop, we also determined how substitution to residues involved in calcium coordination by EpsG altered cross-linking with EpsL. Substitution to residues D116 and D127 were previously shown to prevent secretion through the T2S complex (Korotkov *et al*., 2009), and here we show that their replacement increases cross-linking with EpsL. The interaction between EpsG and EpsL is presumed to be transient due to the dynamic nature of the T2S complex (Johnson *et al.*, 2006, Gray *et al*., 2011); therefore, one explanation for this finding is that alteration to the calcium binding site may stabilize the interaction between EpsG and EpsL. If a transient interaction between EpsG and EpsL is a prerequisite for function of the T2S complex, then substitutions that either strengthen or weaken the EpsG-EpsL interaction would have a negative outcome on secretion. Consistent with this, we have now identified mutations that either stabilize (this Chapter) or destabilize (Chapters II-IV) the EpsG-EpsL complex, and most of these mutations reduce or completely abolish secretion.

It is of note that the calcium binding site is located near residue T112 that was proposed in Chapter II to result in localized misfolding of EpsG. It is possible that replacement of the calcium coordinating residues also resulted in localized misfolding. In support of this hypothesis, our previous study on the calcium binding site indicated that substitution of the aspartic acids resulted in a protein that was slightly less thermodynamically stable than wild-type EpsG (Korotkov *et al*., 2009); this finding

suggested that calcium coordination allowed for stabilization of the C-terminus, similar to how type IV pili utilize a disulfide bridge to stabilize their C-terminus (Hansen & Forest, 2006). Although replacement of T112 exhibited a reduction in cross-linking with EpsL compared to the increase observed with $EpsG_{D116AD127A}$, it is likely that localized misfolding could result in either an increase or decrease in cross-linking. That is, $EpsG_{D116AD127A}$ may alter the folding such that the cross-linked lysines in EpsG are brought into a position that allows for improved cross-linking whereas substitution to T112 relocated these lysines such that the EpsG-EpsL complex was reduced. Both explanations for the increase in EpsL cross-linking, either stabilization or misfolding, are not mutually exclusive; thus, if disruption of the calcium ion slightly alters the structure of EpsG it is still plausible that such variation could stabilize the interaction with EpsL.

Overall, these findings further validate the importance of the interaction between EpsG and EpsL and allow us to better understand the structural domains that are required for the interaction to occur. Our data indicate that substitutions to either the calcium binding site in the C-terminus or to the αβ loop of EpsG can affect the ability of EpsG to cross-link with EpsL. Future studies will be needed to fully understand how these substitutions alter the interaction with EpsL; however, we propose that disruption to the calcium coordinating residues in EpsG resulted in localized misfolding that altered the cross-linking with EpsL. Additionally, due to the αβ loop in EpsG being surface exposed we propose that this region may represent a site of direct interaction with EpsL. Based on the similarity to the type IV pilus biogenesis apparatus, it is likely that the type IV pilin αβ loop may also be involved in an interaction with the EpsL homolog, PilM/N, to aid in assembly or disassembly of the pilus.

Acknowledgements

We thank Michael Bagdasarian for plasmids pMMB917, pEpsG$_{K51E}$, pEpsG$_{E75A}$, pEpsG$_{E75K}$, pEpsG$_{K86A}$, pEpsG$_{K90A}$, and pEpsG$_{K105E}$.

Experimental procedures

Bacterial strains and growth conditions

All strains and plasmids used in this study are listed in Table 4.1. Bacteria were grown at 37°C in Luria-Bertani supplemented with 100 μg ml^{-1} thymine for *V. cholerae* strains and 0.01% arabinose for P$_{BAD}$::*eps* strains. Antibiotics (Sigma-Aldrich) were included at the following concentrations: carbenicillin at 200 μg ml^{-1}, ampicillin at 100 μg ml^{-1}, and kanamycin at 50 μg ml^{-1}.

Construction of EpsG point mutations

Mutations to substitute K28A, K30A, K35A, K51A, K70A and R80D were introduced with the QuickChange II site-directed mutagenesis kit (Stratagene) using pEpsG as a template and the primers in Table 4.2. Substitution to K51 for glutamic acid, and mutation to E75, K86, K90, and K105 were constructed as previously described for creating pEpsG$_{D91E}$ (Gray *et al*., 2011). Briefly, the mutations were introduced into pET28b encoding a truncated form of *epsG* with the primers listed in Table 4.2. A BstEII-KpnI fragment containing the mutated gene was subsequently used to replace a similar fragment in pMMB917 containing full length *epsG*. Sequences were verified at the University of Michigan DNA sequencing core. Plasmid constructs were introduced into *V. cholerae* as previously described (Gray *et al*., 2011).

Protease secretion assay

Secretion of extracellular protease was determined as previously described (Sikora *et al.*, 2007).

In vivo cross-linking

Cross-linking with DSP and immunoblotting for EpsG was performed as previously described in Chapter II (Gray *et al*., 2011) using the following amounts of IPTG to induce plasmid expression to equal amounts for: P_{BAD}::Δ*epsG* with empty vector or pEpsG were grown in the absence of IPTG. P_{BAD}::Δ*epsG* containing $pEpsG_{K70A}$, $pEpsG_{E75A}$, $pEpsG_{E75K}$, and $pEpsG_{R80D}$ were induced with 5 μM IPTG.

Table 4.1. Strains and plasmids used in this study.

Strain or plasmid	Features	Reference or source
Strains		
V. cholerae		
P_{BAD}::*eps*	TRH7000 P_{BAD}::*eps*	Sikora *et al*., 2007
P_{BAD}::Δ*epsG*	TRH7000 P_{BAD}::*eps* with in frame replacement of *epsG* with Cm^R	Gray *et al*., 2011
Plasmids		
pMMB67	low copy number IPTG inducible vector (Ap^R)	Furste *et al.*, 1986
pMMB917	*epsG* in pMMB67 that has *Bst*EII sites in lacIQ and repC removed	Gray *et al*., 2011
pEpsG	*epsG* in pMMB67	Lybarger *et al.*, 2009
$pEpsG_{K28A}$	substitution of residue K28 to alanine in pEpsG	This study
$pEpsG_{K30A}$	substitution of residue K30 to alanine in pEpsG	This study
$pEpsG_{K35A}$	substitution of residue K35 to alanine in pEpsG	This study
$pEpsG_{K51A}$	substitution of residue K51 to alanine in pEpsG	This study
$pEpsG_{K51E}$	substitution of residue K51 to glutamic acid in pMMB917	This study
$pEpsG_{K70A}$	substitution of residue K70 to alanine in pEpsG	This study
$pEpsG_{E75A}$	substitution of residue E75 to alanine in pMMB917	This study
$pEpsG_{E75K}$	substitution of residue E75 to lysine in pMMB917	This study
$pEpsG_{R80D}$	substitution of residue R80 to aspartic acid in pEpsG	This study
$pEpsG_{K86A}$	substitution of residue K86 to alanine in pMMB917	This study
$pEpsG_{K90A}$	substitution of residue K90 to alanine in pMMB917	This study
$pEpsG_{K105E}$	substitution of residue K105 to glutamic acid in pMMB917	This study
$pEpsG_{D116AD127A}$	substitution of residues D116 and D127 to alanine	Korotkov *et al*., 2009

Table 4.2 Primers used for generation of *epsG* mutations.

Substitution to EpsG	Primers
K28A	5′-cccaacctcttaggtaacgcagagaaagcggatcaacag-3′
	5′-ctgttgatccgctttctctgcgttacctaagaggttggg-3′
K30A	5′-cctcttaggtaacaaagaggcagcggatcaacagaaagcgg-3′
	5′-ccgctttctgttgatccgctgcctctttgttacctaagagg-3′
K35A	5′-gagaaagcggatcaacaggcagcggtgaccgatatcgtc-3′
	5′-gacgatatcggtcaccgctgcctgttgatccgctttctc-3′
K51A	5′-atgcgttggatatgtacgcgcttgacaacagcg-3′
	5′-cgctgttgtcaagcgcgtacatatccaacgcat-3′
K51E	5′-gcgttggatatgtacgagcttgacaacagcgtttacccg-3′
	5′-cgggtaaacgctgttgtcaagctcgtacatatccaacgc-3′
K70A	5′-ggaagcgttagtgactgcgccaaccaatccagag-3′
	5′-ctctggattggttggcgcagtcactaacgcttcc-3′
E75A	5′-ccaaccaatccagcgccgcgtaactatcg-3′
	5′-cgatagttacgcggcgctggattggttgg-3′
E75K	5′-ccaaccaatccaaagccgcgtaactatcgcg-3′
	5′-cgcgatagttacgcggctttggattggttgg-3′
R80D	5′-ccagagccgcgtaactatgacgaaggcggttacatc-3′
	5′-gatgtaaccgccttcgtcatagttacgcggctctgg-3′
K86A	5′-gcgaaggcggttacatcgcacgtctgcctaaagatcc-3′
	5′-ggatctttaggcagacgtgcgatgtaaccgccttcgc-3′
K90A	5′-gcgtctgcctgcagatccttggggtaac-3′
	5′-gttaccccaaggatctgcaggcagacg-3′
K105E	5′-gagcccaggcgatgaaggcacgattg-3′
	5′-caatcgtgccttcatcgcctgggctc-3′

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CHAPTER V

Discussion

The goal of this study was to determine if the major pseudopilin, EpsG, interacts with the bitopic inner membrane protein, EpsL, in the Type II Secretion (T2S) system of *Vibrio cholerae* and to identify the additional T2S components and structural domains of EpsG that are required for their association. The importance and implication of these findings will be discussed in the sections below.

EpsG and EpsL interact

I have identified an interaction between EpsG and EpsL by *in vivo* cross-linking using the chemical cross-linker dithiobis (succinimidyl propionate), or DSP (Chapter II). The interaction was further established through co-immunoprecipitation and allowed me to verify that the cross-linked complex observed was indeed specific for EpsG and EpsL. Because the interaction between EpsG and EpsL could not be detected without DSP this may suggest that their association is weak or transient. In Chapter II I also created a point mutation originally isolated during a genetic screen of *Pseudomonas aeruginosa* that proposed that the EpsG and EpsE homologs may directly interact (Kagami *et al*., 1998). Substitution of EpsG residue T112 drastically reduced the amount of EpsG-EpsL cross-linked complex. Taken together, my findings suggest that EpsL may function as a scaffold between EpsG and EpsE.

An important implication to my findings is that EpsL may act to transduce the energy generated through EpsE mediated ATP hydrolysis in order to support secretion, perhaps by aiding in the assembly or disassembly of the pseudopilus. EpsE is associated with the inner membrane due to the interaction between EpsE's N-terminus and the cytoplasmic domain of EpsL (Sandkvist *et al.*, 1995, Abendroth *et al.*, 2005). Based upon modeling of the type IV pilus ATPase PilT, EpsE is predicted to be a hexamer that can adopt both an open and closed conformation dependent upon whether ATP is bound (Camberg *et al.*, 2007, Misic *et al.*, 2010, Patrick *et al*., 2011). ATP hydrolysis results in movement to the flexible N-terminus that is interacting with EpsL; therefore, EpsL may undergo a similar movement that could affect EpsG due to the EpsG-EpsL interaction. In support of this hypothesis, a study from *Dickeya dadantii* indicated that the interaction between the EpsE homolog and the cytoplasmic domain of the EpsL homolog resulted in a conformational change to the periplasmic domain of the EpsL homolog (Py *et al.*, 2001).

Models for how mechanical energy may be transduced to EpsG to support either assembly or disassembly are illustrated in Figure 5.1. In the EpsG assembly model, ATP hydrolysis would result in movement of the periplasmic domain of EpsL that would then allow for extraction of the hydrophobic N-terminal alpha-helix of EpsG from the inner membrane and insertion into the growing pseudopilus. Modeling of the pseudopilus formed by EpsG or the homolog from *Klebsiella oxytoca* has indicated that multimerization occurs primarily through interactions between the alpha-helices of the

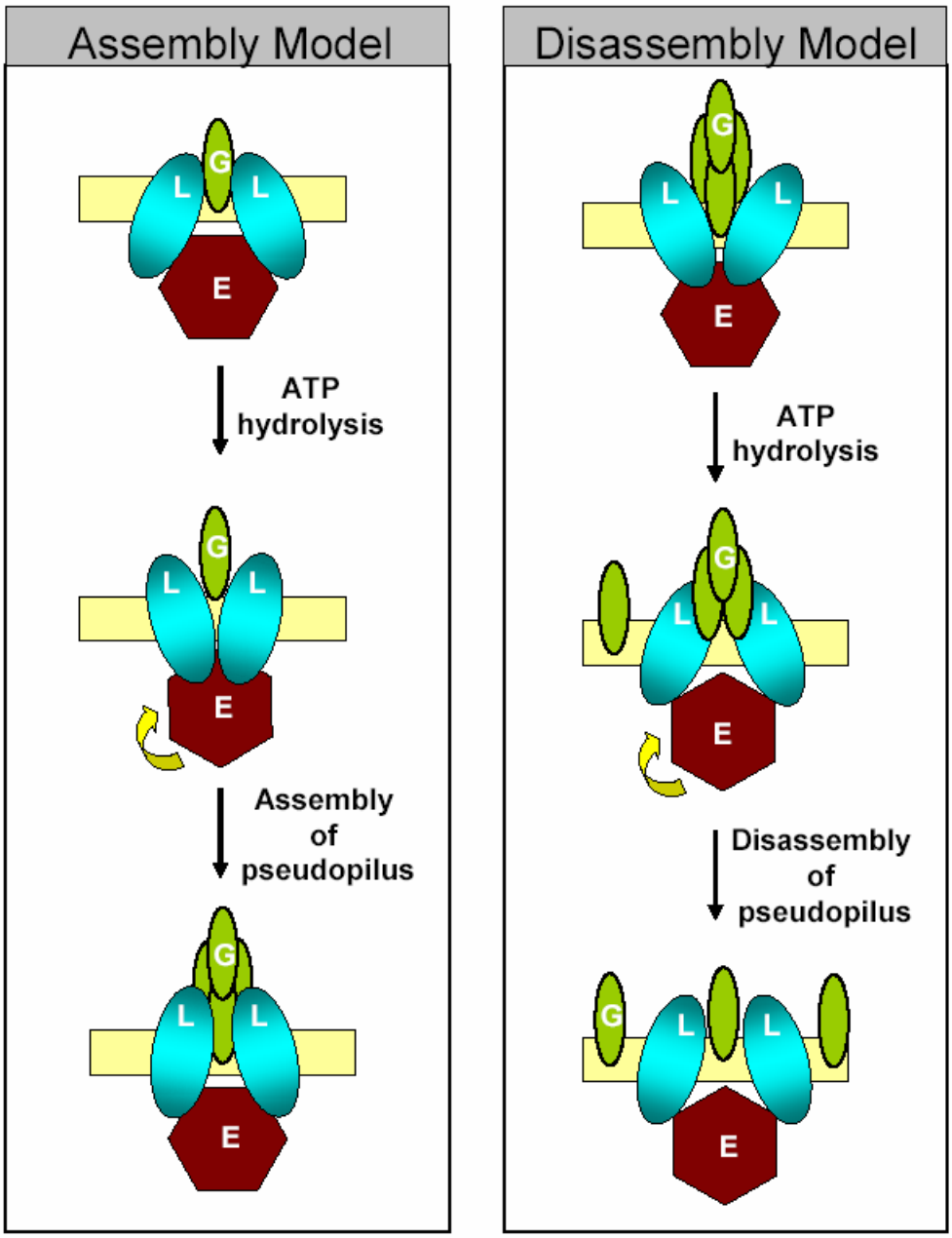

Figure 5.1. Assembly and disassembly models for EpsG multimerization.
The energy generated by ATP hydrolysis from EpsE may be transduced through EpsL to allow for either assembly or disassembly of EpsG into the pseudopilus. Conformational changes within the N-terminus of EpsE, which is interacting with the cytoplasmic domain of EpsL, may cause a conformational change in the periplasmic domain of EpsL that would allow for extraction of the hydrophobic N-terminus of EpsG from the membrane to aid in assembly. Conversely, a conformational change in the periplasmic domain of EpsL may pull on EpsG after assembly to retract the polymerized subunits and result in disassembly.

subunits (Kohler *et al.*, 2004, Korotkov *et al.*, 2009, Campos *et al.*, 2010). Upon multiple rounds of ATP hydrolysis and membrane extraction of EpsG, the N-terminal alpha-helix from each subunit would tightly pack together in order to form the pilus-like structure. As the pseudopilus assembles it would become proximal to EpsD (Figure 1.1, page 4) and the interaction between the minor pseudopilins and the periplasmic domain of EpsD may trigger a conformational change within the periplasmic gate of EpsD to allow for secretion of substrates (Reichow *et al*., 2010, Korotkov *et al*., 2011). Conversely, in the disassembly model EpsG may self-polymerize without a requirement for ATP hydrolysis due to the hydrophobic nature of the N-terminal alpha-helix. The energy generated during ATP hydrolysis by EpsE would then result in a conformational change in the periplasmic domain of EpsL that would tug on EpsG and disassociate the subunits from the assembled pseudopilus. In this model the interaction between the minor pseudopilin cap and the periplasmic domain of EpsD may act as a plug to prevent the secretion of proteins. As the pseudopilus disassembles the disengagement of the minor pseudopilins from EpsD may allow for the periplasmic gate of EpsD to open (Reichow *et al*., 2010, Korotkov *et al*., 2011), resulting in an efflux of secreted proteins.

Several findings have led to more support for the assembly model. Studies on *P. aeruginosa* and *K. oxytoca* demonstrated that the production of surface pseudopili when the *epsG* homolog was over-expressed was dependent upon the presence of the EpsE homolog (Sauvonnet *et al.*, 2000, Durand *et al.*, 2005). The EpsL homolog was also required for the over-production of the EpsG homolog to form surface pseudopili, supporting my hypothesis that EpsL may link EpsG to EpsE. Furthermore, sequence alignment of EpsE with related ATPases has shown that it is more homologous to the

type IV pilus assembly ATPase, PilB, than the retraction ATPase, PilT (Robien *et al.*, 2003). Additionally, the EM structure of the secretin formed by EpsD showed that the pore was closed (Reichow *et al*., 2010), indicating that the pseudopilus may not act as a plug to prevent secretion. Assembly of the type IV pilus also requires several components of the inner membrane platform including the homologs for EpsL, PilM and PilN (Carbonnelle *et al.*, 2006, Ayers *et al.*, 2009). Based upon my findings, this may suggest that a similar interaction occurs between the type IV pilin and PilM/N to support assembly of the pilus.

My studies may also lead to support for the assembly model. In wild-type *V. cholerae* other EpsG specific cross-linked complexes were detected and these bands were absent when any single gene from the *eps* operon was removed. These bands may be indicative of EpsG multimers; therefore, determining the other EpsG containing species will be important to further understand the EpsG-EpsL interaction. Other future studies investigating if the minor pseudopilins also interact with EpsL, as well as exploring the interaction between EpsG and the minor pseudopilins and addressing if substitutions of EpsG that prevent cross-linking with EpsL effects the interaction with the minor pseudopilins, will provide more insight into how the pseudopilus assembles.

Prerequisites for EpsG and EpsL to interact

In Chapter II I determined that PilD was the only component of the T2S system required for EpsG to cross-link with EpsL. PilD is a bifunctional enzyme that cleaves off the first seven amino acids from EpsG and subsequently methylates the newly formed N-terminus, resulting in the mature subunit (Marsh & Taylor, 1998, Fullner & Mekalanos,

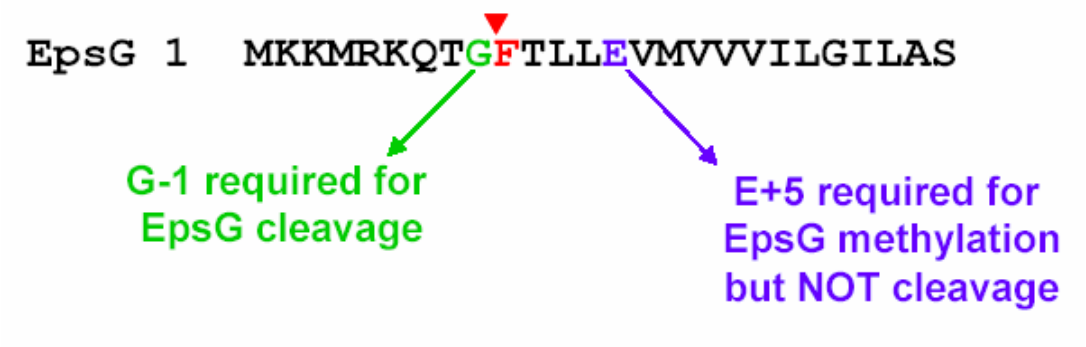

Figure 5.2. EpsG residues in the N-terminus involved in processing by PilD.
The N-terminal amino acid sequence of EpsG indicates the Phe (red) where cleavage occurs and is subsequently methylated post-cleavage. Substitution of the Gly residue (shown in green) results in a protein that is no longer cleaved. Substitution to the Glu residue (shown in blue) results in a protein that is cleaved but the Phe is no longer methylated.

1999). Studies on the EpsG homolog from *K. oxytoca* and type IV pilins have demonstrated that cleavage occurs between the glycine (referred to as G-1) and phenylalanine (position +1) indicated in Figure 5.2 (Strom & Lory, 1991, Pugsley, 1993). Replacement of G-1 prevents cleavage of the N-terminus, whereas substitution of the glutamic acid (referred to as E+5) prevents methylation of the phenylalanine post-cleavage (Figure 5.2) (Strom & Lory, 1991, Macdonald *et al.*, 1993, Pugsley, 1993). In my studies I demonstrated that replacement of either residue (Chapters II and III) drastically reduced the amount of EpsG-EpsL cross-linked complex, indicating that a fully mature EpsG is required for the interaction to occur.

The exact purpose for the N-terminal modification of pseudopilins and type IV pilins is unknown; however, several possibilities have been speculated upon. It has been proposed for the monomer that methylation to Phe+1 may neutralize the Glu+5 charge within the membrane environment prior to assembly (Kohler *et al*., 2004, Johnson *et al*., 2006). Following assembly, models for the *K. oxytoca* pseudopilus and type IV pilus have suggested that residues Phe+1 and Glu+5 are then involved in subunit-subunit interactions (Craig *et al.*, 2003, Craig *et al.*, 2006, Campos *et al.*, 2010). Alternatively, Strom *et al*. postulated that maturation may alter the way that the subunit is presented in the membrane (Strom *et al.*, 1993). Recently, molecular dynamics simulations were applied to the *P. aeruginosa* type IV pilin PilA to investigate its position and orientation within a model membrane and indicated that these hypotheses may not be mutually exclusive (Lemkul & Bevan, 2011). Modeling suggested that residues Phe+1 and Glu+5 initially formed an intramolecular salt bridge to shield the charges from the hydrophobic membrane; however, as PilA stabilized within the membrane the position of the pilin

tilted and broke the interaction. Lemkul and Bevan hypothesized that the separation of Phe+1 and Glu+5 in the membrane stabilized monomer may then aid in allowing these residues to take part in subunit-subunit interactions during assembly. As it is still unclear at what point during secretion EpsG and EpsL interact, membrane positioning and/or a deficiency in multimerization could explain the loss in cross-linking observed with my EpsG processing mutants.

Interestingly, no product of the *eps* operon (*epsC-N*) was required for EpsG and EpsL to interact (Chapter II). From this finding it is tempting to speculate that the EpsG-EpsL interaction may occur prior to EpsG polymerization. If the EpsG-EpsL interaction does precede EpsG polymerization then this may support the assembly model in which the energy generated from ATP hydrolysis by EpsE drives multimerization of EpsG. Furthermore, some of the additional EpsG specific cross-linked species that were present in wild-type *V. cholerae* may represent EpsG cross-linking to other T2S components. Future studies to determine if EpsG interacts with other T2S components may allow for a better understanding of when during assembly of the pseudopilus that EpsG and EpsL interact.

Structural domains of EpsG involved in the interaction with EpsL

Throughout the course of this study I have identified residues in all three of the structural domains of EpsG that affect the ability of EpsG to cross-link with EpsL: the N-terminal alpha-helix, the αβ loop, and the C-terminus (Figure 5.3). Analysis of substitutions within these regions have provided insight into what function each domain may provide for EpsG, and moreover, how disruption of each region may affect the

ability of EpsG to interact with EpsL. Additionally, substitution of residues within each domain affected the ability of EpsG to support secretion further highlighting the importance of the EpsG-EpsL interaction within the T2S complex. The N-terminal residues that were examined prevented maturation of EpsG and were discussed above; therefore, this section will focus on my findings that relate to the C-terminus and αβ loop.

The residues within the C-terminus that were examined are D91, T112, D116, and D127. D91 and T112 are forming a hydrogen bond and substitution of either residue reduced the amount of EpsG-EpsL complex (Chapter II). These residues are found in a deep crevice of the EpsG structure and are barely surface exposed, therefore, it is likely that replacement of these amino acids resulted in localized misfolding of EpsG. Residues D116 and D127 are involved in the coordination of a calcium ion located in the C-terminus (Korotkov *et al*., 2009). A mutant protein in which both Asp's were exchanged for an alanine increased the amount of EpsG-EpsL cross-linked complex approximately 3 fold (Chapter IV); however, $EpsG_{D116AD127A}$ has been shown to be incapable of supporting secretion and is less thermodynamically stable than wild type EpsG, suggesting that this variant may also have a folding deficiency (Korotkov *et al*., 2009). Based on these findings, I propose that the C-terminus is necessary for stabilizing EpsG and that the resultant misfolding of the protein due to substitutions in this region altered the way that EpsG was able to cross-link with EpsL.

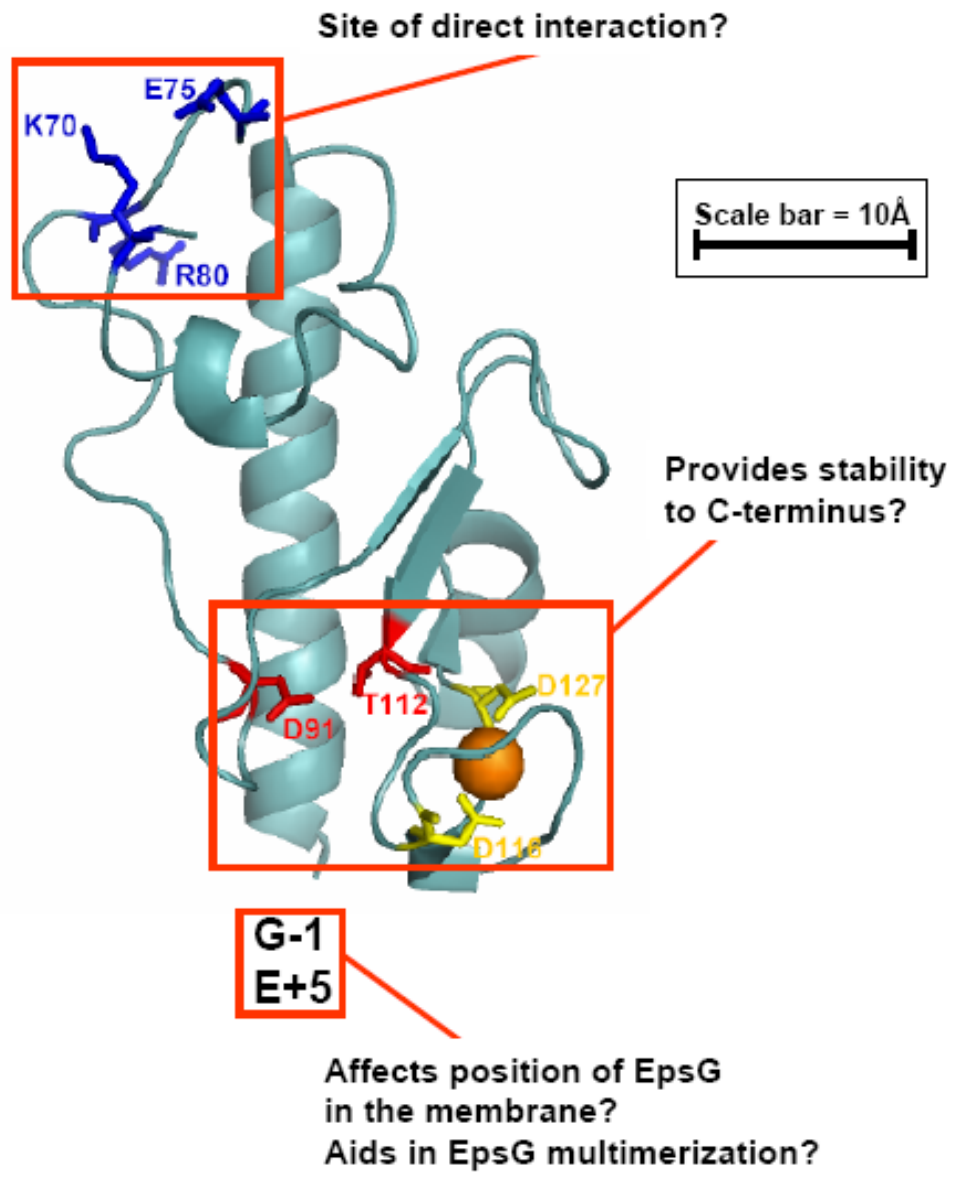

Figure 5.3. Location of EpsG residues that alter the interaction with EpsL. EpsG has an N-terminal alpha-helix, a variable or αβ loop region, and a C-terminal region that contains a calcium ion indicated by the orange sphere (Korotkov *et al*., 2009; PDB code 3FU1). Substitutions within each of the domains affected secretion and altered the interaction with EpsL. The residues that were investigated in this study are highlighted and possible explanations as to why these regions affected the EpsG-EpsL interaction are listed. See text for details. Figure prepared with PyMol (DeLano, 2002).

The residues within the αβ loop that were examined are K70, E75, and R80. Substitution to these residues decreased the amount of EpsG-EpsL cross-linked complex to varying degrees which correlated to a similar loss in secretion, implying that there is a parallel between the ability of EpsG to associate with EpsL and the ability to support secretion (Chapter IV). K70, E75, and R80 are located in a surface exposed region of the αβ loop near the tip of the alpha-helix and I propose that this site is involved in a direct interaction with EpsL. It is likely that the interaction interface between EpsG and EpsL is composed of several residues, therefore, this may explain why single substitutions to this region was not sufficient to completely abolish cross-linking with EpsL. However, since I was still able to detect a low level of cross-linking to EpsL with the αβ loop variants I cannot rule out the possibility that another region of the protein is also involved in the interaction.

Substitution to K70 or E75 showed a moderate loss in secretion whereas substitution of R80 for aspartic acid completely abolished the ability of EpsG to support secretion and exhibited the most severe loss in cross-linking with EpsL. The side chain of R80 is facing towards the N-terminal alpha-helix rather than the surface of the protein (Figure 5.3), thus, the charge reversion introduced may have affected the ability of EpsG to form intermolecular interactions with other subunits during multimerization, as well as altered the αβ loop. Interestingly, my data also indicated that substitution of K51 for a glutamic acid reduced the EpsG-EpsL complex whereas a K51A mutation did not (Chapter IV). K51 is located in the alpha-helix near the tip of the protein; therefore, the charge reversion introduced by K51E may have also affected the ability of EpsG to multimerize. This may imply that there is a correlation between the ability of EpsG to

form multimers and the ability to interact with EpsL. Conversely, as K51 is located near the tip of the alpha-helix $EpsG_{K51E}$ may have also altered the αβ loop, supporting my hypothesis that this is a site of direct interaction with EpsL. Future experimentation analyzing other charged residues along the alpha-helix may aid in distinguishing between these possibilities and provide a better understanding of the requirements that are needed in order for EpsG and EpsL to interact.

Conclusions

In this study I have identified an interaction between the T2S components EpsG and EpsL, and analysis of the structural domains of EpsG suggests a distinct role for each region that allow for EpsG to interact with EpsL and support secretion. From my findings I propose that EpsL functions to link EpsG to the ATPase, EpsE, and thereby act as a mechanical lever to transduce the energy from ATP hydrolysis to allow for polymerization and/or depolymerization of EpsG. Future studies will be needed to fully understand the importance of the EpsG-EpsL interaction; however, these findings have begun to address a fundamental question within the T2S field by providing insight as to how the ATPase may be connected to the pseudopilus. Furthermore, because of the similarities between the type IV pilus biogenesis machinery and the T2S complex, these findings also have implications for how the type IV pilus may be linked to its respective ATPase(s).

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