Localization and Assembly of the *Vibrio cholerae* Type IV Pilus Secretin Channel

By

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Abstract

Type 4 pili (T4P) are filamentous structures found on the surfaces of many Gram-negative bacteria, including *Vibrio cholerae*. The *V. cholerae* T4P are the toxin-coregulated pili (TCP), which mediate bacterial aggregation and exoprotein secretion, critical functions in colonization of the human intestine to cause the diarrheal disease cholera. TCP assemble at the inner membrane (IM), grow through a multiprotein conduit in the periplasm and through a secretin channel in the outer membrane (OM). The multimeric secretin channel is formed by secretin subunits, which are translocated across the IM by the Sec apparatus, and in most T4P systems, are transported to the OM with the help of a lipoprotein co-chaperone. In the *V. cholerae* TCP the secretin subunit itself, TcpC, is a lipoprotein, and its putative co-chaperone, TcpQ, is non-lipidated. Here we use mutagenesis, cellular fractionation and functional assays to investigate secretin channel assembly in *V. cholerae*. TcpC must be co-expressed with TcpQ to complement a ΔtcpC mutant in an assay of pilus functions, but the reciprocal is not true. TcpQ is necessary for pilus assembly but not for localization of TcpC to the outer membrane, demonstrating that TcpQ is not a co-chaperone for TcpC. The periplasmic domain of TcpC can be expressed on its own, localizes to the outer membrane, and localizes TcpQ at the outer membrane as well, provided TcpC is lipidated. When the periplasmic domain of TcpC is unlipidated it gets degraded and TcpQ accumulates in the periplasm, suggesting that the periplasmic domain of TcpC interacts with TcpQ and localizes it to the outer membrane via its lipid moiety. Our results lead to a model whereby TcpC Cys1 is lipidated at the IM by the Lgt machinery and transported to the OM in complex with TcpQ. TcpC inserts into the OM at two points: via its C-terminal portion, which forms a β-barrel channel with TcpC C-terminal domains of other secretin subunits, and via lipid moiety in its N-terminal domain, which interacts with TcpQ to link the OM channel to periplasmic pilus conduit.
Keywords: secretin; lipoprotein; co-chaperone; type IV pili; localization; channel assembly; *Vibrio cholerae*
Dedication

To my family for their unconditional love and support.

To my mentors, colleagues, and friends for their support and guidance.
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</tr>
<tr>
<td>BAM</td>
<td>β-barrel assembly machinery</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>Type II secretion system</td>
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<tr>
<td>T4P</td>
<td>Type IV pili</td>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline with 0.1% Tween</td>
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<tr>
<td>TCP</td>
<td>Toxin co-regulated pilus</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>UA</td>
<td>Uranyl acetate</td>
</tr>
<tr>
<td>VPI</td>
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<td>WC</td>
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Chapter 1. General introduction

Diarrheal diseases are a major health problem worldwide. In developing countries diarrheal diseases are one of the leading causes of mortality and are responsible for approximately 1.5 million deaths annually for children under five (WHO, 2013). Ingestion of contaminated water, insufficient water for hygiene and poor sanitation facilities are the main factors responsible for diarrheal disease (Black, Morris, and Bryce, 2003). Diarrhea can be caused by viruses, bacteria and protozoans. Amongst diarrheal pathogens, *Vibrio cholerae* is the etiological agent of cholera disease – a severe gastrointestinal disease. There have been 7 pandemics of cholera recorded in human history (Reimer et al, 2011). Due to the global impacts of this pathogen, it is crucial to understand the molecular mechanisms involved with disease pathogenesis to improve upon prevention and treatment to work on vaccinations and anti-microbial drug therapy.

1.1. *Vibrio cholerae*

*V. cholerae* is a curve-rod shaped Gram negative (Figure 1-1). The bacterium was identified by Italian pathologist Filippo Pacini and German scientist Robert Koch in the 1880s (Bentivolgio & Pacini, 1995; Howard-Jones, 1984). In 1854, British physician John Snow was the first to draw the connection between cholera and consumption of contaminated water and he identified the location of water pumps in London (Ghose, 2011; Snow, 1855). The Broad Street water pump was shut off and the number of cholera cases began declining (Snow, 1855). Snow’s work is famous today as the first epidemiology study done. *V. cholerae* is naturally found in marine and freshwater environments (Halpern et al, 2004). Cholera disease is
transmitted by ingestion of *V. cholerae* contaminated food or water. The first step in *V. cholerae* pathogenesis is colonization of the small intestine.

Two key features of *V. cholerae* pathogenesis are the *Vibrio* pathogenicity island (VPI) and the lysogenic cholera toxin bacteriophage (CTXΦ) (Kaper, Morris, and Levine, 1995). The VPI is a virulence gene cluster that contains the genes necessary for the production of the toxin-coregulated pilus (TCP) (Herrington et al, 1988; Pang et al, 2007). Expression of TCP is a pre-requisite for CTXΦ susceptibility as TCP is the receptor for the phage (Faruque, Albert, and Mekelanos, 1998). TCP also play an important role in intestinal colonization by *V. cholerae* as they secrete a colonization factor that is required for successful infection (Pang et al, 2007).

CTXΦ carries the ctxA and ctxB genes which encode for the cholera toxin subunits A and B (Waldor and Mekalanos, 1996). Expression of TCP and cholera toxin are transcriptionally co-regulated by the ToxR regulatory system (Miller and Mekalanos, 1984; Taylor et al, 1987). Cholera toxin is responsible for the voluminous diarrhea, commonly referred to as ‘rice water stool’ (Nelson et al, 2009). This allows the bacteria to disseminate quickly into the environment, contributing to a high disease prevalence (Figure 1-2). Severe cholera without adequate rehydration therapy can lead rapid water and electrolyte loss and result in death (Rafique et al, 2016). Annually, there are 1.3 to 4 million cholera cases estimated worldwide (WHO pg. 433, 2016).

*V. cholerae* is differentiated serologically on the basis of the O antigen of its lipopolysaccharide (LPS) (Nelson et al, 2009). Over 200 O-antigen serogroups
have been identified (Pang et al, 2007; Rafique et al, 2016). Of these, only O1 and O139 serogroups cause pandemic and epidemic cholera (Pang et al, 2007). Pandemic refers to a global outbreak of an infectious disease whereas epidemic means that the disease occurrence is limited within a community and/or area. Within the O1 serogroup, there are two phenotypically distinct biotypes of \textit{V. cholerae} – classical and El Tor (Nelson et al, 2009). The classical biotype caused the first six of the seven cholera pandemics and the El Tor biotype is responsible for the on-going seventh pandemic (Kaper, Morris, and Levine, 1995; Nelson et al, 2009; Rafique et al, 2016). \textit{V. cholerae} O139 emerged in 1992 in epidemic proportions and this serotype is closely related to the El Tor biotype (Faruque et al, 2003). The emergence of \textit{V. cholerae} O139 attracted significant attention as it was the first non-O1 serogroup associated with widespread epidemics of cholera (Ramamurthy et al, 1993).
Figure 1-1 Transmission electron micrograph of *Vibrio cholerae* cells
Rod-shaped, Gram-negative *V. cholerae* cells expressing toxin co-regulated pilus filaments in bundles with their polar flagella found on the apical end of the bacterium. *V. cholerae* wild type strain O395 grown in pilus-inducing conditions and stained with 3% uranyl acetate. Sample preparation done by Navdeep Garcha and imaging done on Hitachi 8100 Transmission electron microscopy by Lisa Craig.
Upon entry into the host, *V. cholerae* attach to intestinal epithelial cells. The bacteria adheres to the small intestine through TCP and adhesins which leads to microcolony formation. Cholera toxin is synthesized and secreted upon adhering to the epithelial cells. Uptake of the toxin results in massive loss of water and electrolytes, resulting in severe diarrhea (Kaper, Morris, and Levine, 1995). Image courtesy of L. Craig, Simon Fraser University.
1.1.1. Cholera toxin

The voluminous and watery diarrhea characteristic of cholera disease is caused by the cholera toxin (CT) and it can result in 10 to 20 litres of fluid loss in a day (Kaper, Morris, and Levine, 1995; Lemichez and Stefani, 2013; Nelson et al, 2009). Upon ingestion of V. cholerae environmental cues within the digestive tract trigger the expression of virulence factors, including cholera toxin and TCP, to facilitate the colonization process.

CT belongs to the AB5 family of toxins. It is an ADP-ribosylating toxin that is secreted into the intestinal lumen by the type II secretion system (Lemichez and Stefani, 2013; Waldor and Mekelanos, 1996). The CT holotoxin consists of an enzymatically active A subunit and a pentameric B subunit that binds to the GM1 ganglioside on intestinal epithelial cells (Holmgren et al, 1975; Lencer et al, 1992; Wolf et al, 1998). This binding facilitates endocytosis of the holotoxin. Upon entering the cell the A subunit undergoes proteolysis and reduction into an enzymatically active A1 domain and inactive A2 domain (Wisnieski and Bramhall, 1981). The A1 domain catalyzes the transfer of ADP-ribose from NAD⁺ to the alpha subunit of the stimulatory G protein, Gₛ, a guanine nucleotide binding regulator protein (Kassis, Hagmann, and Fishman, 1982). The ADP-ribosylated Gₛ protein constitutively activates adenylate cyclase which produces elevated levels of cyclic-AMP (cAMP). cAMP is a broad signaling molecule that activates different cellular factors including the cAMP-dependent protein kinase A (PKA) (Lemichez and Stefani, 2013) which phosphorylates several ion channels (Flach et al 2004). Phosphorylation
opens ion channels causing a rapid efflux of water and electrolytes, resulting in the characteristic watery diarrhea of cholera disease (Figure 1-3). The large loss of electrolytes and water leads to severe dehydration and can be fatal without rapid treatment.

Figure 1-3 Cholera toxin mechanism Cholera toxin is an AB₅ toxin that is secreted by the type II secretion system of *V. cholerae*. The pentameric B subunit binds to the GM1 ganglioside of intestinal epithelial cells and this causes endocytosis of the A subunit which consequently becomes activated in the reducing conditions of the cell. The activated A1 subunit ADP-ribosylates the Gₛ protein which targets adenylate cyclase, subsequently resulting in electrolyte imbalance and water secretion (Kaper, Morris, and Levine, 1995; Lemichez and Stefani, 2013). Image courtesy of Lisa Craig, Simon Fraser University.
1.1.2. The toxin co-regulated pilus

1.1.2.1. Type IV Pili

TCP is another critical virulence factor expressed by *V. cholerae*. TCP is a type IV pilus (T4P), which are filamentous structures expressed on the surface of many Gram-negative bacterium (Giltner et al, 2012). T4P are divided into two subclasses – Type IVa (T4a) and Type IVb (T4b) pili (Figure 1-4) – based on differences in amino acid sequence of their signal peptides and mature protein length (Craig, Pique, & Tainer, 2004; Strom and Lorry, 1993). Genes encoding T4a pili are scattered throughout the genome whereas for T4b pili, these genes are generally clustered onto a single operon (Pellicic, 2008). T4P are thin and long filaments that measure 6-8 nm in diameter and several microns in length (Craig and Li, 2008) (Figure 1-1). These pili facilitate many different functions in different bacterial species including adhesion to host cells, phage transduction, exoprotein secretion and microcolony formation.
Figure 1-4 The tcp operon of the V. cholerae toxin co-regulated pilus
All the genes required for assembly of TCP are found on a single operon which is characteristic of type IVb pilins.
1.1.2.2. Type IV Pilus Assembly

T4P are protein polymers composed of thousands of pilin subunits (Craig & Li, 2008). Pilin subunits are synthesized in the cytoplasm and translocated via their signal peptide sequence to the periplasm through the general secretion pathway (Sec pathway). On the cytoplasmic side of the IM, the signal peptide is cleaved off by a dedicated pre-pilin peptidase and protein undergoes N-methylation as it is translocating to the periplasm (Strom and Lorry, 1993). Pili are assembled at the inner membrane where pilin monomers are added to the base of the filament. The subunits are held together in the filament through hydrophobic interactions among the hydrophobic N-termini of each pilin subunit (Craig et al, 2004; Craig et al, 2006; Li et al, 2012; Pohlschroder and Esquivel, 2015). Pilus assembly is powered by ATP hydrolysis by the cytoplasmic ATPase, which converts chemical energy into mechanical energy to extrude the pilus outward to allow the incoming pilin subunit to dock (Craig et al. 2006; Mancl et al, 2016; Yamagata and Tainer, 2007). The pili grow from the inner membrane, span the periplasm and cross the outer membrane through the secretin channel (Figure 1-5). The secretin channel is a multimeric channel that is assembled in the outer membrane. Proteins called co-chaperones help facilitate the localization and assembly of this channel (Gu et al, 2012). The assembly machinery of T4P is homologous to the bacterial type II secretion system (T2SS) which suggests that there is an evolutionary relationship between the two systems (Sandkvist, 2001).
Figure 1-5 The Type IV pilus (T4P) and Type II Secretion systems (T2SS) (A) Pilus assembly begins at the base of the inner membrane where pilin subunits are added to the growing filament, energized by ATP hydrolysis by the assembly ATPase. The filament spans the periplasm and cross the outer membrane through the secretin channel to be displayed on bacterial surfaces. The secretin subunits localize and oligomerize in the outer membrane to form a multimeric channel through the help of co-chaperone protein. (B) The type 2 secretion system is a macromolecular complex which spans the periplasm and it is responsible for the secretion of virulence such as the cholera toxin in *V. cholerae*. The T2SS and T4P share structural and functional features: both have outer membrane secretin channels, filamentous structures consisting of pilin or pseudopilin subunits, prepilin peptidases and cytoplasmic ATPases. (Craig et al, 2004; Sandkvist, 2001; Strom and Lorry, 1993)
1.1.2.3. TCP

TCP is one of the simplest Type IV pilus systems known, with all the proteins required for pilus biogenesis encoded on the tcp operon present on the VPI on *V. cholerae*. TCP mediate a number of functions in *V. cholerae* that are crucial to pathogenesis including: autoagglutination, phage transduction, exoprotein secretion and microcolony formation (Giltner, Nguyen, and Burrows, 2012; Wolfgang, Park, et al, 1998; Mattick, 2002).

TCP are critical for *V. cholerae* pathogenicity as TCP mutants are unsuccessful in infecting the infant mouse model (Herrington et al, 1988; Kirn et al, 2000). Deletion of the tcpA gene resulted in loss of colonizing capacity of the bacteria, demonstrating the requirement of the pili for colonization of the human intestine (Herrington et al, 1998). TCP promote bacterial interactions through self-association between the pili of adjacent cells; they retract their pili to form tight aggregates that form microcolonies in the intestine so the bacteria are protected from the host’s immune system (Kirn et al, 2000; Ng et al, 2016). These pili facilitate the secretion of colonization factor TcpF that is required for successful host infection by *V. cholerae* (Figure 1-6) (Kirn, Bose, and Taylor, 2003). TCP also function in CTXΦ phage transduction as they are receptors for the phage (Waldor and Mekalanos, 1996).
Figure 1-6 TCP functions TCP self-associate to form aggregates which are necessary for microcolony formation in the small intestine. The TCP pilus machinery also secrete TcpF, a colonization factor that is required for successful infection. The CTXΦ phage also gets taken up into *V. cholerae* cells through association with the TCP. Upon retraction of the pili, the bacteriophage enters the cytoplasm.
1.1.3. Secreted colonization factor TcpF

*V. cholerae* TCP mediate the secretion of exoprotein, TcpF which is translocated into the periplasm by the Sec system and it is secreted through the outer membrane secretin channel of the pilus assembly apparatus (Kirn, Bose, and Taylor, 2003; Kirn and Taylor, 2005). However, the precise mechanism through which secretion occurs remains unknown. TcpF has no known apparent sequence homology with any known proteins (Kirn, Bose, and Taylor, 2003; Megli et al, 2011). The de novo (i.e. without previous knowledge about the structure) x-ray crystal structure of TcpF reveals that the protein may use the cleft between its N- and C-terminal to bind possible substrates (Megli et al, 2011). The TcpF structure is unique as there no structural homologs to the protein (Megli et al, 2011). TCP do not require TcpF for pilus assembly and a TcpF mutant strain can still produce pili that are capable of self-aggregation but mutants lacking TcpF are severely defective with regards to colonization (Kirn, Bose, and Taylor, 2003; Kirn and Taylor, 2005; Megli and Taylor, 2013). Thus, this exoprotein is necessary for successful *V. cholerae* pathogenesis. Similar to T4P, the bacterial type II secretion system also uses its pilus system to secrete proteins as well.

1.2. Type II Secretion system

Many Gram-negative bacteria use the type II secretion system (T2SS) to translocate folded proteins from the periplasm across the outer membrane and into extracellular milieu (Korotkov, Sandkvist, and Hol, 2012). T2SS are important for
both pathogenic and non-pathogenic species. Some human pathogens with one or more T2SS include *V. cholerae* which secretes the cholera toxin (Sandkvist et al, 1997); ETEC whose T2SS secretes heat-labile enterotoxin (Strozen, Li, and Howard, 2012; Tauschek et al, 2002); *P. aeruginosa* which secretes multiple proteins including the chitin-binding protein and lipase (Bally et al, 1992; Jyot et al, 2011), and *Yersinia enterolitica* (Iwobi et al, 2003). The T2SS forms a macromolecular complex which spans the periplasm and two membranes (Figure 1-5) (Reichow et al, 2011).

1.2.1. **Homology of the T2SS to T4P**

The T2SS consists of at least 12 different proteins, encoded on an operon, which form an assembly apparatus similar to T4P (Figure 1-5). These two systems share structural and functional features: both have secretin channels, filamentous structures consisting of pilin or pseudopilin subunits, prepilin peptidases, and cytoplasmic ATPases (Craig et al, 2006) (Figure 1-5). The T2SS is hypothesized to assemble pseudopili (Korotkov and Hol, 2013; Russell, 1998; Sandkvist, 2001), short periplasmic filaments formed by pilin proteins that grow through the periplasm but do not grow past the outer membrane (Korotkov, Sandkvist, and Hol, 2012). These structures are referred to as pseudopili because their pilin subunits share amino-terminal sequence homology to the type IV pilin proteins (Korotkov, Sandkvist, and Hol, 2012). Similar to the pilin subunits found in T4P, pseudopilin subunits also utilize the Sec pathway for translocation across the inner membrane (Arts et al, 2007; Francetic et al 2007). Preceding translocation, the
pseudopilin subunits are acted upon by a prepilin peptidase that cleaves off their signal peptides and undergo N-methylation (Nunn and Lory, 1991). The pseudopili are predicted to operate through a piston-type mechanism to secrete proteins across the periplasm and outer membrane. The substrates secreted by the T2SS interact and are recognized by the outer membrane secretin protein (Pineau et al, 2014). In particular, it has been demonstrated in the T2SS in *D. danstii* that the periplasmic domains of the secretin play a role in substrate recognition (Pineau et al, 2014). Overexpression of the pseudopilin subunit results in the formation of surface displayed pili in T2SS, consistent with evolutionary origin with T4P (Chang et al, 2017; Sauvonnet et al, 2000).

### 1.3. Secretins and Co-chaperones

Both the T2S and T4P systems have an outer membrane gated channel through which the pilus grows and substrates pass. This channel is formed by secretin subunits. Secretins are integral outer membrane proteins that form large stable multimeric channels typically with 12-14-fold symmetry (Figure 1-7) (Berry et al, 2012; Reichow et al, 2011). The T4P assembly apparatus can be divided into the outer membrane pore complex, the alignment complex that connects the inner and outer membrane components together and the motor complex which includes the inner membrane and cytoplasmic platform proteins (Figure 1-8) (Bischof et al 2016). The N-terminal domains of secretin subunits vary in length and number of domains. These domains extend into the periplasm and form a part of the alignment complex that links the inner and outer membrane sections of the T4P
assembly apparatus together. The C-terminus of secretins have a well-conserved region containing a highly conserved secretin signature sequence, (V,I)PXL(S,G)XIPXXGXL (Bose and Taylor, 2005). This superfamily of secretins is used in T4P and T2SS to translocate pilin subunits and macromolecules across the outer membrane.

Figure 1-7 Secretin Channel Assembly The N domains of secretins are homologous regions arranged in a canonical alpha/beta fold which determines the domain boundary between the N and C termini for secretin. The above picture represents the 7.4 Å cryoelectron microscopy structure of the P. aeruginosa PilQ secretin channel determined by Koo et al, 2017. The channel is separated into three distinct regions: the extracellular cap, the outer membrane, and the periplasmic domains. The outer membrane β-barrel is formed by the oligomerization of secretin subunits (numbered in the bottom panel of the figure) and the PilQ secretin channel exhibits 14 fold symmetry. The height of secretin channels range from 110 Å to 200 Å and their diameter are typically 80 Å on the periplasmic side. (This figure is adapted from Koo et al, 2017 with permission from the publisher).
Figure 1-8 Regions of the T4P assembly apparatus The T4P assembly apparatus can be divided into 3 parts: (1) the OM pore complex which consists of the secretin protein and typically the co-chaperone (2) the alignment complex which interacts with the periplasmic domains of the secretin to link the OM pore complex to the periplasmic and IM proteins (3) the motor complex which actively plays a role in pilus biogenesis and includes the inner membrane and cytoplasmic platform proteins.
1.3.1. Lipoproteins and co-chaperones

A specialized class of small lipoproteins, referred to as either pilotins or co-chaperones, bind the secretin proteins in T2S and T4P systems to facilitate translocation to the outer membrane, oligomerization, insertion and proper assembly of the secretin channel (Berry et al, 2012; Gu et al, 2012). Lipoproteins are macromolecules that contain both protein and lipid portions. They are referred as lipoproteins because of the presence of a “lipobox” consensus sequence (Leu-(Ala/Ser)-(Gly/Ala)-Cys) at their N-termini that is found following their signal peptides (Konovalova & Sihavy, 2015; Okuda and Tokuda, 2009).

1.3.2. Lipoprotein processing and localization

The lipobox consensus sequence of these co-chaperone proteins undergoes processing and maturation in order to become a membrane associated protein, which then aids in secretin channel assembly (Gu et al, 2012; Hoang et al, 2011). The last residue of this consensus sequence, which is a cysteine, becomes the first residue of the mature protein as Cys1 (Goloanov et al, 2006). The signal peptides of these lipoproteins target them for transport from the cytoplasm and across the IM through either the Sec or Tat translocons (Figure 1-9) (Konovalova & Sihavy, 2015). Initially the signal peptide anchors the lipoprotein in the IM. They are processed on the periplasmic side where a diacylglycerol, donated by a phosphatidylglycerol, is added to the sulfhydryl group of the Cys1 residue by phosphatidylglycerol/prolipoprotein diacylglycerol transferase (Lgt) (Sankaran and Wu, 1994). This modification
then signals cleavage of the signal peptide by either LspA or signal peptidase II, leaving the protein anchored in the IM by the diacylglycerol moiety (Dev and Ray, 1984; Yamagata et al, 1983). Following cleavage of the signal peptide, the amino group of the Cys1 is N-acylated by phospholipid/alipoprotein transacylase (Lnt), resulting in the mature lipidated protein (Gupta and Wu, 1991).

Lipidated, cleaved lipoproteins are typically targeted for translocation to the OM unless they have a Lol avoidance signal (Burghout et al, 2004; Golovanov et al, 2006; Tokuda, 2009). The Lol avoidance signal is determined by the amino acid at position 2, following the conserved Cys1 residue (Yamaguchi, Yu, and Inouye, 1988). If the position 2 amino acid is an aspartate the protein remains in the inner membrane; the presence of any other amino acid at position 2 targets the protein for OM localization (Konovalova & Sihavy, 2015; Yamaguchi, Yu, and Inouye, 1988). Lipoproteins that are targeted for the OM are transported by protein in the Lol machinery (Figure 1-9) (Konovalova & Sihavy, 2015). The Lol apparatus consists of five proteins: LolA, LolB, LolC, LolD and LolE (Yakushi et al, 2000). LolC, LolD, and LolE form an IM complex that facilitates the extraction of the lipoprotein from the IM and transfer to the LolA chaperone protein. (Matsuyama, Tajima, and Tokuda, 1995; Yakshi et al, 2000). The lipoprotein is released into the periplasm in complex with LolA. LolA has a large hydrophobic cavity that binds the acyl chains of the lipoprotein to shield them from the aqueous periplasm and it chaperones the lipoprotein to LolB in the
OM (Matsuyama, Yokota, and Tokuda, 1997). LolB is an OM lipoprotein itself and it is a structural homologue of LolA but its hydrophobic cavity has a higher affinity for acyl chains than LolA. Due to this higher affinity, LolB accepts the lipoprotein from LolA via “mouth-to-mouth transfer” and releases the lipoprotein into the OM in a mechanism that is not well understood (Taniguchi, Matsuyama, and Tokuda, 2005). Collectively, the Lol proteins act as a chaperone to localize the mature lipoprotein to the OM.

For non-lipidated proteins that are destined for the OM, the β-barrel assembly machinery (BAM) complex comes into play (Han et al, 2016). The BAM complex is 200 kDa in size and it consists of five proteins: an integral OM protein, BamA and four lipoproteins (BamB, BamC, BamD, and BamE) (Rigel and Silhavy, 2012; Wu et al, 2005). In Gram-negative bacteria, the BAM complex is responsible for folding and inserting nascent β-barrel proteins into the OM (Han et al, 2016). Following translation, OM proteins are translocated to the periplasm through the Sec translocon and from there, they are escorted to the inner leaflet of the OM by chaperone proteins which are non-BAM chaperones (Han et al, 2016; Pugsley, 1993). From there, the BAM complex takes the proteins and integrates them into the OM (Han et al, 2016).
Lipoproteins are translocated across the IM through the Sec or Tat translocons. They stay anchored in the IM through their signal peptides and Lgt adds a diacylglycerol to the sulfhydryl group of Cys1. Following this modification, LspA or signal peptidase II cleaves off the signal peptide which exposes the amino group of Cys1. The amino group of Cys1 undergoes N-acylation by Lnt and the mature lipidated protein is then chaperoned to the OM by the Lol machinery. (This figure is adapted from Tokuda, 2009 with permission from the publisher).
1.3.3. Co-chaperones in pilus systems

The co-chaperone proteins are necessary for the localization and assembly of secretin channels in various T2SS and T4P systems. These proteins are called co-chaperones because the Lol machinery is considered the primary chaperone in these systems. In the case of non-lipidated proteins, the BAM complex is the chaperone and thus, additional proteins that aid in localization are called co-chaperones as well. In the T2SS of *Dickeya dadantii*, the absence of the co-chaperone OutS, results in its secretin, OutD, accumulating in the inner membrane (Gu *et al*, 2012). OutS also was shown to bind tightly to the last 18 residues in the C-terminus of OutD. These 18 residues, which are unstructured prior to binding by the co-chaperone, adopt a helical structure in the secretin/co-chaperone complex (Gu *et al*, 2012). In the T2SS of *Klebsiella oxytoca* the co-chaperone, PulS, is also required for localization of the secretin, PulD to the outer membrane (Guilvout *et al*, 2006). In absence of PulS, PulD mislocalizes to the inner membrane. These data suggest that OutS and PulS may play a role in preventing premature multimerization of the secretin channel (Guilvout *et al*, 2006). In this system T2SS of *K. oxytoca*, there is an equal ratio of secretin to co-chaperone as twelve copies of the co-chaperone co-purify with the secretin dodecamer during purification (Guilvout *et al*, 2006). In the *Neisseria meningitidis* T4P system, the co-chaperone, PilP, plays a key role in secretin (PilQ) channel assembly as in a PilP mutant, there is a significantly reduced amount of secretin channels (Golovanov *et al*, 2006). Furthermore, co-transcription of
PilP and PilQ is necessary for proper pilus assembly in *N. meningitidis* suggesting that defects in PilP transcription, translation or translocation may have downstream effects on PilQ production (Golovanov *et al*, 2006).

1.3.4. Secretins

Secretins are bacterial outer membrane proteins that facilitate the transport of proteins and macromolecular complexes from the periplasm across the outer membrane (Bayan, Guilvout, and Pugsley, 2006). Secretin form gated outer membrane channels composed of 12-14 subunits with a diameter of approximately 50-80 Å to allow for secretion of proteins, pilus passage and DNA uptake (Korotkov, Gonen, and Hol, 2011). They are essential components of pilus systems as they allow for pilus growth across the outer membrane for display on the surface of bacterial cells (Gu *et al*, 2012). The structure of secretin proteins has been investigated through cryoelectron microscopy (EM) EM studies, which reveal ring structures with 12- to 14-fold rotational symmetry (Figure 1-7) (Chami *et al*, 2005; Collins *et al*, 2004; Hodskingson *et al*, 2009; Korotkov, Gonen, and Hol, 2011; Reichow *et al*, 2010). Structural studies using cryo EM on the secretin channel of *K. oxytoca* reveal a cylindrical dodecameric secretin channel with the N-terminal domains located in the periplasm (Chami *et al*, 2005). The T2SS secretin channel of *V. cholerae*, GspD forms a dodecameric channel that measures 200 Å in height with a gated periplasmic vestibule that is 125 Å in height (Reichow *et al*, 2010). The C-terminal domains of the secretin subunits form a β-barrel and each secretin subunit contributes 6
transmembrane \( \beta \) strands to the barrel (Berry et al, 2012). The T4P secretin of *N. meningitidis* forms a channel with 14-fold rotational symmetry that is closed on both ends but the periplasmic region was not resolved though it was expressed (Collins et al, 2003). Recently, the structure of the T4P secretin channel in *P. aeruginosa* was solved at a resolution of 7.4 Å, representing the highest resolution of a T4P secretin channel to date (Figure 1-7) (Koo et al, 2017). This channel is composed of 14 subunits that form a ribbed cylinder with a gated periplasmic side (Koo et al, 2017). It measures 118 Å in height and 110 Å in diameter (Koo et al, 2017). Each secretin subunit contributes 6 \( \beta \)-strands to the OM \( \beta \)-barrel.

Secretins are typically identified based on their amino acid sequence by the presence of a C-terminal secretin superfamily domain (Table 2-3). Secretins show high levels of sequence conservation in their C-terminal portion (Genin and Boucher, 1994). Specifically, amino acid sequence analysis revealed that there are four highly conserved regions that are found across secretins (including the highly conserved secretin signature sequence (V,I)PXL(S,G)XIPXXGXLF) and this sequence conservation is referred to as the secretin superfamily domain (Genin and Boucher, 1994; Hardie et al, 1996). Secretins are classified based on homology with the secretin superfamily domain (Martinez, Ostrosky, and Nunn, 1998). The secretin superfamily domain consists of 18 secretins that all share highly C-terminal sequence homology.
Secretins are grouped into five classes: (1) self-assembling and self-membrane targeting; (2) self-assembling but not self-membrane targeting; (3) self-assembling but inefficiently self-membrane targeting; (4) self-membrane targeting but not self-assembling; and (5) not self-assembling and not self-membrane targeting (Koo, Burrows, and Howell, 2012). Class 1 secretins are lipoproteins themselves, which are targeted to the outer membrane by the Lol machinery where they self-assemble into the multimeric channel (Viarre et al, 2009). An example of a class 1 secretin is the T2SS secretin HxcQ from *P. aeruginosa* (Viarre et al, 2009). Class 2 secretins are capable of self-assembly but they require a co-chaperone protein for localization to the OM and in the absence of the co-chaperone, the secretin mislocalizes to the IM (Hardie et al, 1996). And examples of class 2 secretins include PulD from *K. oxytox*ca and YscC from YscC from *Yersinia enterocolitica* (Burghout et al, 2004; Nouwen et al, 1999) Similar to class 2 secretins, class 3 secretins can self-assemble but they are inefficient at membrane localization without accessory proteins. These include the GspD secretin found in the *V. cholerae* T2SS and ExeD which is the secretin in the T2SS of *Aeromonas hydrophila* (Ast et al, 2002; Strozen et al, 2011). Class 4 secretins localize to the outer membrane but cannot form the secretin channel without their accessory proteins. The T4P secretin, PilQ from the *Neisseria* species is a class 4 secretin (Koo, Burrows, and Howell, 2012; Drake, Sandstedt, and Koomey 1997; Schmidt et al, 2001). Lastly, class 5 secretins need their co-chaperone proteins for both localization and
secretin channel assembly (Koo et al, 2008). In the absence of their co-chaperone proteins, these secretins fail to localize to the OM and form channels. An example of such a secretin is the T4P PilQ secretin from *P. aeruginosa* (Koo, Burrows, and Howell, 2012; Koo et al, 2008).

### 1.3.5. The periplasmic N-terminal domains of secretin subunits

Secretin proteins can be divided into two distinct regions as previously mentioned: an N-terminal periplasmic region and the C-terminal OM region that forms a β-sheet which then oligomerizes with the rest of the subunits to form the β-barrel pore. The N-terminal regions extend into the periplasm and form the sides of the secretin chamber (Berry et al, 2012; Reichow et al, 2010). These N-terminal regions form part of the alignment complex and interact with additional periplasmic proteins that are connected to inner membrane proteins, which are linked to the assembly ATPase on the cytoplasmic side of the pilus assembly machinery to form a trans-envelope complex through which the pilus grows (Figure 1-8) (Salzer et al, 2016). Additionally, some secretin proteins also have β-domains found preceding the N-terminal domains and these β-domains extend into the periplasm as well and play a role in closing the OM channel on the periplasmic side (Berry et al, 2012). The N-terminal regions of secretin proteins play a key role in the T4P assembly apparatus linking the OM complex to the periplasmic alignment complex to form the conduit required for pilus assembly. The periplasm is packed with proteins and the peptidoglycan layer, which would prevent the pilus from growing to the OM. A conduit allows passage of the
pilus through this dense milieu and connects the inner membrane assembly machinery to the OM secretin complex (Figure 1-8) (Koo, Burrows, and Howell, 2011; Korotkov et al, 2009). The secretin N-terminal regions have been expressed separately and characterized with respect to their structure and binding partners.

The structure of the N-terminal region is well-conserved with its core βαββα fold (Berry et al, 2012). The core fold consists of two α-helices packed against a three-stranded β-sheet and it is a conserved fold which determines the end of periplasmic domain in secretin proteins (Berry et al, 2012; Korotkov et al, 2009). Though secretins share C-terminal homology, their N-terminal regions are in fact quite dissimilar, and this canonical fold can be used predict the periplasmic domain organization in other secretins (Berry et al, 2012; Genin and Boucher, 1994).

The crystal structure of the first three N-terminal regions, N0, N1 and N2, of T2SS secretin GspD from ETEC revealed N0 and N1, each containing a conserved secondary structure core fold (βαββα), in a compact lobe, with a second lobe representing the N2 region which appears to link N0/N1 to the C-terminal region (Korotkov et al, 2009). In the EPEC type III secretion system (T3SS), the secretin EscC has two N-terminal βαββα-like domains, N0 and N1, connected by a linker (Spreter et al, 2009).

1.3.6. The V. cholerae secretin complex

The secretin subunit in the V. cholerae T4P system, TcpC, is unusual from most other T2S and other T4P systems as it possesses a lipobox
consensus sequence at its N-terminus (Bose and Taylor, 2005; Parsot et al, 1991). The lipobox is found on the co-chaperone protein in most other T4P and T2S systems as previously discussed (Gu et al, 2012). TcpC is identified as a secretin protein as its C-terminus shares sequence similarity with other secretins (Table 2-3) (Figure 1-11).

TcpC is presumably targeted, like most secretins, to the outer membrane through its C-terminal domain, which has homology to secretin superfamily domains (Figure 1-11). Therefore, it is curious that TcpC has a lipobox to additionally target it to the outer membrane. Parsot et al (1991) generated and expressed a TcpC-PhoA fusion protein and used [$^3$H]-palmitic acid labelling to demonstrate that TcpC is lipidated. Further, they showed by cellular fractionation experiments that lipidated TcpC localizes to the outer membrane. The role of this lipidation on TcpC at its N-terminus has not yet been identified. TcpC appears to fall into Class 1 of secretins as it is a lipoprotein, which means it targets itself to the outer membrane and is likely self-assembling.
Figure 1-10 Domain predictions of TcpC. The N-terminal periplasmic domains of TcpC were predicted by sequence analysis and secondary structure comparison with solved secretin structures. The canonical $\beta_4\alpha_4$βα fold was used to determine the end of the periplasmic domain and the membrane domain contains the secretin superfamily region (not shown in above).

Figure 1-11 TcpC belongs to the secretin superfamily of proteins. Blastp analysis revealed that TcpC shares sequence homology with the other secretin proteins that are a part of the secretin superfamily and thereby.
The *V. cholerae* T4P, like the more complex T4P systems and the T2S systems, has a putative co-chaperone protein, TcpQ which was demonstrated by Bose and Taylor (2005) to be necessary for secretin channel assembly. Yet unlike in most T4P systems, the TcpQ co-chaperone lacks the lipobox targeting it to the outer membrane (Bose & Taylor, 2005). Bose and Taylor (2005) demonstrated using metal affinity column pull down assays that TcpQ and TcpC directly interact with each other. Histidine-tagged TcpQ expressed and captured on a nickel-TNTA column from *V. cholerae ΔtcpC* cell lysates pulled down non-tagged TcpC from the lysate (the reciprocal experiment using histidine-tagged TcpC to pull down TcpQ gave similar results) (Bose and Taylor, 2005). Through ectopic protein expression and membrane fractionation experiments, this group showed that TcpQ localizes to the outer membrane and it is required for TcpC stability and localization to the outer membrane. In the complete absence of TcpQ, TcpC mislocalizes to the IM, suggesting that TcpQ plays a role in chaperoning TcpC to the OM. Since the TcpC is presumably chaperoned to the OM via the BAM system, TcpQ was designated a "co-chaperone". For my thesis project, I sought to investigate the role of TcpC lipidation and the nature of the TcpQ:TcpC interaction with respect to secretin channel localization and pilus assembly.
1.4. Thesis objective

TcpC and TcpQ are both necessary for the assembly of *V. cholerae* TCP. I hypothesize that the lipidation of the *V. cholerae* subunit at Cys1 anchors the N-terminus of the secretin into the outer membrane. TcpQ associates with the periplasmic domains of TcpC to form a conduit through which TCP can grow through the periplasm and be displayed on the cell surface.

The specific aims of this thesis project are:

Aim 1 – Determine the role of the periplasmic protein, TcpQ, in secretin channel localization and assembly of a functional pilus biogenesis machine.

Aim 2 – Determine the role of TcpC Cys1 in secretin channel localization and pilus assembly.
Chapter 2. Methods

2.1. Plasmids and strain list

Bacterial strains, plasmids and primers are listed in Table 2.1. *V. cholerae* strains were grown with streptomycin (as *V. cholerae* is naturally resistant to streptomycin) and ampicillin for plasmid selection. Final concentrations for antibiotics: 100 µg ml\(^{-1}\), ampicillin (Ap) and 100 µg ml\(^{-1}\), streptomycin (Sm). *V. cholerae* strains O395, YG005 (ΔtcpC), ΔtcpQ, TcpA-6 and TcpQ rabbit antibody were gifts from Ronald Taylor (Giesel School of Medicine).

2.2. Inducing auto-agglutination in vitro

*V. cholerae* cells were grown overnight on LB-Sm agar plates at 37 °C. Single colonies were picked and used to inoculate 2 ml of lysogeny broth (LB) (Bioshop) and grown for 2 hours at 37 °C. To normalize each inocula, cells were diluted to an optical density measurement of 600 nm (OD\(_{600}\)) of 0.6 then 3 ul was transferred to 3 ml LB at a starting pH of 6.5 and grown for 17 hours at 30 °C with aeration. In the Craig lab, we define “pilus-inducing conditions” as growing *V. cholerae* in LB at a starting pH of 6.5, at 60 rpm with aeration at 30 °C for a period of 17 hours to induce the cells to enter a state where they are growing pili *in vitro*. These conditions have been calibrated through trial and error by ourselves and our collaborators at Ron Taylor’s lab.
Table 2-1 List of bacterial strains, plasmids and primers.

<table>
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<tr>
<th>Strain</th>
<th>Description</th>
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<td><em>V. cholerae</em> O395</td>
<td>Wild-type classical strain</td>
<td>Ron Taylor</td>
</tr>
<tr>
<td><em>V. cholerae</em> O395 ΔtcpC (YG5)</td>
<td>Knockout strain; lacks tcpC gene</td>
<td>Ron Taylor</td>
</tr>
<tr>
<td><em>V. cholerae</em> O395 ΔtcpQ</td>
<td>Knockout strain; lacks tcpQ gene</td>
<td>Ron Taylor</td>
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<tr>
<td><em>E. coli</em> (DH5α)</td>
<td>F− endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK− mK+), λ−</td>
<td>Life Technologies</td>
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<tr>
<th>Plasmid</th>
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<td>Ng et al, 2017</td>
</tr>
<tr>
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<td>tcpC inserted into pJMA10.1 at BamHI and HindIII restriction sites</td>
<td>This work</td>
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<tr>
<td>pJMA10.1-ttcpC-236-H6</td>
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<td>This work</td>
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<tr>
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<td>Gene fragment encoding tcpC&lt;sub&gt;C1S&lt;/sub&gt; (periplasmic domain) residues 1-236 with a C-terminal 6-His purification tag inserted into pJMA10 at BamHI and HindIII restriction sites</td>
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<tr>
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<tr>
<td>pJMA10.1-ttcpQC-C1S-236-H6</td>
<td>Gene fragment encoding tcpC&lt;sub&gt;236C1S&lt;/sub&gt; (periplasmic domain) truncated at residue 236 with a C-terminal 6-His purification tag</td>
<td>This work</td>
</tr>
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</table>
inserted into pJMA10.1 at BamHI and HindIII restriction sites

<table>
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<tr>
<th>Primer</th>
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<td>Gene fragment encoding tcpQ inserted into pJMA10.1 at BamHI and HindIII restriction sites</td>
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<tr>
<td>VC-TcpC-R-HindIII</td>
<td>5’ – GAT CCA AGC TTC TAG TCA TAT ATA TTT ACT CTA TTA – 3’</td>
<td>This work</td>
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<tr>
<td>VC-TcpC-236-H6-R-HindIII</td>
<td>5’ – GAT CCA AGC TTT CAG TGG TGG TGG TGG TGG CTC TCA TTG ATC AAC TTG TC – 3’</td>
<td>This work</td>
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<tr>
<td>VC-TcpC-C1S-F-Sap1</td>
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<td>This work</td>
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<tr>
<td>VC-TcpC-C1S-R-Sap1</td>
<td>5’ – AAA GCT CTT CAA CTA CCG GCA ACC AAA CCG ATA A – 3’</td>
<td>This work</td>
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</table>
2.3. Construction of ptcpC, ptcpQ and ptcpQC.

Vectors expressing the secretin protein TcpC and the periplasmic protein TcpQ were derived from the expression vector pJMA10.1 (Ng et al. 2017), which contains an ApR marker. Genes for TcpC and TcpQ were PCR amplified with Q5 polymerase from V. cholerae wild type strain O395 genomic DNA using primers tcpC-F-BamH1/R-HindIII and TcpQ-F-BamH1/R-HindIII. PCR products were purified, digested and ligated into pJMA10.1 at the BamH1/HindIII restriction sites using T4 DNA ligase. All constructs were verified through DNA sequencing and transformed into V. cholerae strains for complementation testing. Protein expression was induced using rhamnose at specified concentrations.

2.4. Construction of ptcpQC_{C1S}, ptcpC_{236-H6}, ptcpC_{C1S-236-H6}, ptcpQC_{236-H6}, and ptcpQC_{C1S-236-H6}.

Vectors expressing the secretin protein TcpC and the periplasmic protein TcpQ were derived from the expression vector pJMA10.1, which contains an ApR marker. A single amino acid substitution was made in the ptcpQC construct in which residue cysteine 1 was mutated to serine 1 using a PCR stitching method adapted from Ko and Ma (2005). Briefly, two sets of primers were used to introduce the C1S mutation. The two individual fragments were then ligated together to form the complete ptcpQC_{C1S} construct. TcpC_{236} was generated using primers TcpC-F-BamH1/R-236-H6-HindIII. This C-terminally truncated TcpC comprises only the predicted periplasmic domain of TcpC with a hexa-histidine tag at the C-terminus (Figure 2-1). Both wild-type, the periplasmic domain and the C1S mutant TcpC constructs were cloned both independently and with the tcpQ gene on pJMA10.1.
All the genes were PCR amplified with Q5 polymerase and purified, digested and ligated into pJMA10.1 at the BamH1/HindIII restriction sites using T4 DNA ligase. All constructs were verified through DNA sequencing and transformed into *V. cholerae* strains for complementation testing. Protein expression was induced using rhamnose at specified concentrations.

**Figure 2-1 Periplasmic construct of TcpC** The N-terminal periplasmic domains of TcpC were predicted by sequence analysis and secondary structure comparison with solved secretin structures. The membrane domain was predicted using the domain cutoff for the periplasmic domain (see Section 3.1 for details). Based on these predictions, an N-terminally truncated construct of TcpC expressing only the periplasmic domain with a C-terminal hexa-histidine tag was generated.
2.5. Autoagglutination assay

*V. cholerae* cells were grown ON under pilus inducing conditions in 3 mL LB (at initial pH 6.5) and inspected for the aggregation phenotype after allowing the cultures to stand at RT for 15 minutes. Scoring is done by measuring the optical cell density at 600nm (OD$_{600}$) of the supernatant after the cell aggregates fall to the bottom of the tube. A lower OD$_{600}$ measurement corresponds to a greater amount of cell aggregates formed and a higher reading translates to poor or no autoagglutination. *V. cholerae* wild type strain O395 is used as a positive control and *V. cholerae* ΔtcpC and ΔtcpQ are used as negative controls. The OD measurements of the wild type O395 strain represent auto-autoagglutination and the higher OD measurements of the ΔtcpC and ΔtcpQ represent a lack of autoagglutination.

2.6. Immunoblotting of TcpC and TcpQ

*V. cholerae* cells were grown overnight under pilus-inducing conditions in 3 mL LB (starting pH 6.5) and let stand at RT for 15 minutes. After measuring OD$_{600}$ cells were resuspended by vortexing for 30 seconds and 1 mL of the whole cell culture was centrifuged at 7,000 x g for 10 minutes at RT to pellet the cells. The culture supernatant (SUP, 750 ul) containing secreted proteins was mixed with Laemmli sample buffer (60 mM Tris pH 6.8, 5% 2-mercaptoethanol, 2% SDS, 10% glycerol, 0.02% bromophenol blue) and boiled for 10 minutes. The whole cell (WC) pellet was resuspended in 1 mL in phosphate-saline buffer (PBS) and was mixed with Laemmli sample buffer and boiled for 10 minutes. For each of the boiled samples, 25 µL were loaded into 15% SDS-PAGE, 15 lane 1.5 mm gels and electrophoresed
at 130 V for 1 hour 45 minutes in 1X SDS buffer. The gels were removed from their glass racks and rinsed briefly with ddH₂O. The proteins were transferred from the gel onto polyvinylidene difluoride (PVDF) membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) with a wet transfer apparatus (Bio-Rad) cooled with ice blocks. The transfer was done for 1 hour 10 minutes at 100 V. The membrane was blocked overnight at 4 °C with BLOTTO (5% nonfat dried milk in Tris-buffered saline with 0.1% Tween [TBST]). Protein bands were detected with rabbit polyclonal antisera raised against TcpC (this study) and TcpQ (Ron Taylor) (Table 2-2). Goat-anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch) were used to bind the primary antibody. Immunoblots were visualized by enhanced chemiluminescence (ECL). All ECL detection was carried out with the SuperSignal West Pico chemiluminescent substrate (Fisher Scientific) except for the detection of TcpQ in the WC fraction where the more sensitive SuperSignal West Femto chemiluminescent substrate (Fisher Scientific) was used. Blot images were digitized using the FujiFilm LAS 4000 imager (FujiFilm). Secretin and periplasmic expression levels were assayed by immunoblot of the WC fraction. *V. cholerae* O395 wild type strain is used as the comparative standard for TCP assembly.
**Table 2-2 List of antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcpC</td>
<td>Monospecific antibody generated against C-terminal sequence of TcpC residues 335-354; primary antibody Cys-SSIE TTKDNTDEE TRTVK</td>
<td>This study</td>
</tr>
<tr>
<td>TcpQ</td>
<td>Affinity purified primary antibody; raised against internal TcpQ peptide sequence (residues 29-47)</td>
<td>Bose and Taylor, 2005</td>
</tr>
<tr>
<td>TcpF</td>
<td>Mouse monoclonal antisera; primary antibody</td>
<td>Megli et al, 2011</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Goat-anti-rabbit secondary antibody conjugated to Horseradish Peroxidase (HRP)</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Mouse</td>
<td>Goat-anti-mouse secondary antibody conjugated to Horseradish Peroxidase (HRP)</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Strep</td>
<td>StrepTactin and horseradish peroxidase (HRP) conjugate.</td>
<td>BioRad</td>
</tr>
</tbody>
</table>

**Table 2-3 Protein accession numbers.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcpC</td>
<td>P29481 (UniProt)</td>
</tr>
<tr>
<td>TcpQ</td>
<td>P29490 (UniProt)</td>
</tr>
<tr>
<td>Secretin Superfamily</td>
<td>C127726 (NCBI)</td>
</tr>
</tbody>
</table>
| Hypothetical *V. cholerae* OM protein | AK078198 (NCBI)  
- cross reacts with TcpC antibody  
- this protein has a predicted molecular mass of 53.4 kDa, which is close to the XX kDa for mature TcpC |
Hypothetical protein EN12_24210 [Vibrio cholerae]
Accession number: AKO78198.1

Peptide Sequence
MLLINKGINLHTKRNSSEVQKRWLEGKRFAYAPIGMGLKITDNDEEIVIDFVDPNSEEA
TRTVSKRKARGDYVPLIEINECNSLELTSEDEVLRIIVVKEGIRIRANKNTVASR
RKEEESIKYNVANNIPLDGMGIFAGGGTDFHAGHEGFELSGLSTVRFRAINDPDCIE
NLATNVHHVFSRSVLESIDLLNVNGALPKLNNLLKLTPPCTDASKAGKAKKGNKFE
TSKTAHLIYYSQIIERTNPAYIVENVPSFQNEVSFHLLRALLESWEYKIHRLVINSHSE
GFSLETRERMFLVAESAGLDSGFDDLHVCPFERKGVGVEIDLDDVPLDDCWKSKQG
LIEKEKRDEAGKGFRMQIFNEESTYIGTIRAQYTSGSDPIKHPEKDMFRLINKEE
HCRLKSLPESFVEGWPETTAKHFLGNGLDGAMTAESITFELGRCLGILNSREAANQQP
EPQAA

TcpC antibody peptide: C-SSIETTKDTNTDEETRTVK
D N E TRTV

Hypothetical protein EN12_24210: DPNSEEA TRTV

Figure 2-2 Sequence alignment of the hypothetical cross-reactive protein Blastp analysis of the peptide sequence used to generate the TcpC antibody revealed that there is a hypothetical antibody that cross-reacts with the TcpC antibody. This protein has a predicted molecular mass of 53.4 kDa which explains why a band at the expected molecular weight of TcpC is seen in the immunoblots for ΔtcpC.
2.7. TcpF secretion assay

*V. cholerae* cells were grown overnight under pilus-inducing conditions in 3 mL LB (starting pH 6.5). Cells were centrifuged at 7,000 x g for 10 minutes at RT and culture supernatant containing the secreted TcpF was filtered through a 0.22 µm syringe-drive filter (Pall) to remove remaining cells. Samples were mixed with Laemmli sample buffer and boiled for 10 minutes prior to being loaded onto 15% SDS-PAGE gels. Proteins were transferred onto PVDF membrane for immunoblotting as previously described. Mouse monoclonal antisera for TcpF (Megli et al, 2011) was used to detect TcpF in the WC and the filtered supernatant (SUP) fractions. Goat-anti-mouse secondary antibodies conjugated to HRP (Jackson ImmunoResearch) were used to bind the primary antibody. Immunoblots were visualized by ECL with the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and digitized using the FujiFilm LAS4000 imager. The presence of TcpF in the SUP fraction indicates secretion by the TCP assembly apparatus.

2.8. Transmission electron microscopy analysis

For negative-stained transmission electron microscopy (TEM) analysis, 20 µL of whole cell culture (grown under pilus-inducing conditions) were applied to Formvar carbon-coated grids and incubated on the grid for 5 minutes (Electron Microscopy Sciences). Samples were washed twice with PBS and stained with 3% uranyl acetate for 10 minutes. Following staining, grids were air dried for 10 minutes on filter paper. Samples were imaged on a Hitachi 8100 TEM at accelerating voltage of 200 kEV.
Safety Precautions – 3% Uranyl Acetate

Uranyl acetate (UA) is the acetate salt of uranyl and is a yellow-green crystalline solid composed of yellow-green rhombic crystals and it has a slight acetic color. According to the material safety data sheet (MSDS) for UA, it is classified as a level 4 health hazard. This compound is both radioactive and toxic. UA is extremely toxic if ingested, inhaled as a dust or by skin contact if skin is cut or abraded. The high toxicity is a combination of chemical toxicity and mild radioactivity. There are elevated dangers resulting from cumulative effects due to long term exposure which include but are not limited to: genetic defects, cancer, organ damage, fertility damage and damage to unborn child.

Prior to working with UA, it is crucial to receive specific training for handling and using radioactive compounds offered by Simon Fraser University (or given institution). Furthermore, when working with UA in the laboratory, proper personal safety equipment must be worn at all times including: lab coat, safety googles, radioactive badge (issued by the radioactive safety program to monitor background exposure – this is necessary by federal law in Canada), nitrile gloves and proper footwear. All wastes (including gloves, pipette tips, filter paper, tubes, etc) that are contaminated with UA, even it is a minor amount, must be placed in a plastic bag and disposed of according to low level solid waste guidelines for radioactive waste. Following completion of the experiment(s), swipe tests must be completed on the area and equipment where UA was used to measure the background radioactivity and ensure that there has been no unknown spillage of UA and that the area has been appropriately cleaned.
2.9. Cellular fractionation through differential centrifugation

Cells were grown in pilus-inducing conditions and harvested by spinning the culture at 6,700 x g at 4 °C for 10 minutes. The cell pellet was washed with a 10 mM Tris-base pH 7.5, 100 mM NaCl buffer and centrifuged at 6,708 x g at 4 °C for 10 minutes. The step was repeated to remove any residual culture supernatant and the cells were incubated in 1 mL of polymixin B lysis solution on ice for 10 minutes, which makes the OM leaky, and then were centrifuged at 10,000 x g at 4 °C for 10 minutes to remove the periplasmic fraction. The cells were sonicated for 1 minute and 15 seconds (15 seconds ON and 45 seconds OFF) at 30% amplitude with a Branson Digital sonicator to release cytoplasmic contents and ultracentrifuged at a g force of 108,000 x g at 4 °C for 10 minutes to separate the cytoplasm from the membrane pellets. The membrane pellet was resuspended in 2% Triton 100-X, 10 mM Tris-HCl pH 7.5 100 mM NaCl and incubated at RT for 30 minutes to solubilize the inner membrane as the outer membrane is resistant to solubilisation by 2% Triton-100X. The solution was then centrifuged at 108,000 x g at 4 °C for 10 minutes and supernatant, containing the inner membrane, was removed (protocol adapted from Sandrini et al., 2014). The remaining pellet, containing the outer membrane was resuspended in PBS. All fractions were mixed with Laemmli sample buffer and boiled for 10 minutes prior to being loaded onto 15% SDS-PAGE gels for immunoblot analysis.
2.10. Replicates and standard error in experiments

All experiments were performed in triplicate. For autoagglutination studies, the averages for the OD$_{600}$ readings were calculated and plotted along with the standard error of the mean (SEM).

2.11. Prediction algorithms

Signal Peptide Prediction

The settings for the SignalP 4.1 Server included Gram negative bacteria (organism group), default (D-cutoff value), standard (output format), and input sequence my included transmembrane region (method).

Secondary Structure Prediction

The PSIPRED Protein Sequence Analysis Workbench setting used to generate the TcpC secondary structure prediction was the PSIPRED v3.3 prediction method. This algorithm works by taking the sequence of the inputted protein and comparing it to solved protein structures in the Protein Data Bank (Berman et al, 2000; Buchan et al, 2013; Jones, 1999) to predicted secondary structure.
Chapter 3. Results

3.1. Domain and secondary structure predictions of TcpQ and TcpC

The *V. cholerae* secretin channel TcpC is encoded on the *tcp* operon immediately downstream of the gene encoding TcpQ, a predicted periplasmic chaperone protein required for pilus biogenesis (Bose and Taylor, 2005). Based on the [SignalP 4.1 Server](http://www.cbs.dtu.dk/services/SignalP), TcpC is predicted to have a 16 amino acid signal peptide and a 473 amino acid mature protein (52 kDa) (Petersen et al, 2011). TcpC has a lipobox consensus sequence (Val-Ala-Gly-Cys1) at the end of its signal peptide and has been shown to undergo lipidation (Figure 3-1A, 3-1B) (Parsot et al, 1991). The presence of a lipobox in the secretin subunit is unusual as secretin superfamily proteins contain a canonical (i.e. conserved) outer membrane-interacting secretin domain in their C-terminus (Salzer et al, 2016). The TcpC secretin domain was identified using blastp analysis as spanning residues 344 to 438 in the mature protein (Figure 3-1D).

A secondary structure prediction of TcpC was performed using the [PSIPRED Protein Sequence Analysis Workbench](http://bioinf.cs.ucl.ac.uk/psipred/) (Buchan et al, 2013; Jones, 1999). A βαβαβα fold for residues 177-236 was identified as an N domain, upstream of the secretin domain (Figure 3-1C) (Berry et al, 2012). A domain boundary was predicted at residue 236. Thus, we predicted that the periplasmic domain spans residues 1-236 and the OM domain comprised the remaining 234 amino acids.

The putative secretin co-chaperone, TcpQ, has a 23 amino acid signal peptide, predicted by SignalP 4.1 Server and a 127 amino acid mature protein (15
TcpQ is predicted to be a periplasmic protein and is required for TCP biogenesis (Bose and Taylor. 2005).

Figure 3-1 Vibrio cholerae tcp operon and comparison of the secretin subunit, TcpC, and the predicted periplasmic protein, TcpQ.
Figure 3-1 *Vibrio cholerae* tcp operon and comparison of the secretin subunit, TcpC, and the predicated periplasmic protein, TcpQ. (A) tcp operon with the secretin tcpC gene colored in with purple and the tcpQ gene in green. The tcpC gene is immediately downstream of the tcpQ gene. (B) Schematic of the TcpQ and TcpC proteins. TcpC has a 16 residue signal peptide, indicated by the black portion. The mature TcpC protein begins at the Cys1 residue. The secretin superfamily domain in TcpC is a conserved domain that is seen in secretin proteins from various T2S and T4P systems. (C) The predicated secondary structure of the mature TcpC protein generated by the PSIPRED Protein Sequence Analysis software. TcpC contains a canonical (i.e. conserved) N domain based on analysis of the structure of known secretins including the T2SS of *V. cholerae* GspD (Salzer et al, 2016). (D) The protein blast (blastp) analysis of the TcpC gene sequence revealed that TcpC shares the highest sequence similarity to the secretin superfamily between residues 350-454 (though some similarity is seen starting at residue 309) (also see Figure 1-11). The secretin superfamily consists of secretin proteins that share sequence similarity in their C-terminus.
3.2. TcpC does not require TcpQ for expression.

It was previously shown through ectopic protein expression and localization experiments that TcpQ is required for stable expression of TcpC and for localization of TcpC in the OM (Bose and Taylor, 2005). We sought to investigate the interaction between TcpC and TcpQ and their roles in TCP assembly. Consistent with these published findings, TcpC was not detected in the whole cell fraction of a ΔtcpQ V. cholerae deletion strain using an antibody directed to a peptide (residues 335-354) on TcpC (Fig. 3-2A). While a faint band at the mass corresponding to TcpC does appear, this band is also present in the ΔtcpC mutant and appears to represent a protein other than TcpC that cross-reacts with the anti-TcpC antibody. Protein blast (blastp) analysis of the peptide against which the antibody was raised reveals some similarity to a peptide in a hypothetical OM V. cholerae protein with a mass of 53 kDa, which is very close to that of TcpC (53.4 kDa, Figure 2-2) (Table 2-3).

Next, TcpC was expressed ectopically in the ΔtcpC strain, where it was detected at levels greater than wild-type (Figure 3-2A). Surprisingly though, this complemented strain does not form a functional assembly machinery, as no pili are made based on the autoagglutination assay, which tests for the presence of functional pili, and by direct imaging of the bacteria using transmission electron microscopy (TEM) (Figure 3-2B, 3-2C). Thus, overexpression of TcpC in the presence of endogenous levels of TcpQ is not sufficient to rescue pilus assembly. This result contradicted that of Bose and Taylor (2005), who demonstrated that pilus assembly is restored when TcpC is ectopically expressed in a ΔtcpC deletion
strain. This is despite both studies employing the same ΔtcpC strain. Our results suggest that TcpQ must be co-expressed with TcpC in a 1:1 stoichiometry to form a functional pilus assembly apparatus.

Figure 3-2 TcpC is stably expressed in a ΔtcpC mutant but pili are not formed (continued on next page).
Figure 3-2 TcpC is stably expressed in a ΔtcpC strain but pili are not formed

(A) Immunoblotting of TcpC in whole cells (WC) shows that TcpC undetectable in a ΔtcpQ strain and that TcpC can be stably overexpressed in a ΔtcpC strain. The top numbers (0.001, 0.01, and 0.1) represent the percentage of rhamnose induction

(B) (Left) TEM image of negatively-stained *V. cholerae* wild type strain O395 showing abundant TCP bundles. (Right) No TCP bundles are observed in the ΔtcpC + ptcpC strain. Pili are indicated with red arrows and flagella with blue.

(C) Auto-agglutination is a visual assay for production of functional pili. Cells expressing functional TCP self-aggregate and fall to the bottom of the tube. The level of auto-agglutination in a strain can be quantified by measuring optical density (600 nm). The lower the OD_{600} reading the better the auto-agglutination. Expressing TcpC in a ΔtcpC strain does not rescue the autoagglutination phenotype.
3.3. Ectopic expression of TcpC requires TcpQ co-expression for efficient pilus assembly.

Since ectopic expression of TcpC in a ΔtcpC mutant is unable to rescue pilus assembly despite endogenous TcpQ being expressed, and since the tcpC gene is located immediately downstream of the tcpQ gene, we hypothesized that both proteins must be co-expressed in a 1:1 ratio to form a functional complex (Figure 2-1A). Thus, TcpQ and TcpC were co-expressed in ΔtcpQ and ΔtcpC strains, which restored pilus assembly, as shown by TEM (Figure 3-3A). Furthermore, co-expression restored pilus functions of TcpF secretion and auto-agglutination (Figure 3-3B, 3-3C). TcpF secretion was determined by examining the whole cell (WC) and filtered supernatant (SUP) fractions by SDS-PAGE and immunoblotting with anti-TcpF antibody. *V. cholerae* ΔtcpC + ptcpQC and ΔtcpQ + ptcpQC strains secrete TcpF at levels comparable to the wild-type strain O395. TcpF typically runs as two species in WT *V. cholerae*, and this is observed for the complemented strains as well. The 37 kDa band represents full length TcpF, and the lower band, at approximately 35 kDa, is a processed form of TcpF which is enriched in the SUP fraction (Figure 3-3B). The ΔtcpQ and ΔtcpC mutant strains do not secrete TcpF, which accumulates in its unprocessed form in the WC fraction. Furthermore, the ΔtcpQ and ΔtcpC mutants are unable to auto-agglutinate but co-expression of TcpQ and TcpC partially restores auto-agglutination (Figure 3-3C).
Figure 3-3 Co-expression of TcpQ and TcpC is necessary for efficient pilus assembly (figure continued on next page).
Figure 3-3 Co-expression of TcpQ and TcpC is necessary for efficient pilus assembly (A) When TcpQ and TcpC are co-expressed in ΔtcpC and ΔtcpQ strains, high levels of TCP are made as demonstrated by the presence of thick bundles. Pili are indicated with red arrows and flagella with blue. (B) TcpF secretion into the culture supernatant (SUP) is impaired in ΔtcpC and ΔtcpQ strains. When TcpQ and TcpC are ectopically co-expressed in these strains, TcpF is detected in the supernatant by SDS and immunoblotting with an anti-TcpF antibody. The WC fraction shows an accumulation of TcpF in the strains which fail to secrete the exoprotein. (C) Co-expression of TcpQ and TcpC partially rescues the auto-agglutination phenotype (percentages represent rhamnose induction levels for the strain).
3.4. TcpC does not require TcpQ for localization to the outer membrane

As co-expression of TcpQ with TcpC was shown to be necessary for pilus assembly and functions, we wanted to determine if TcpQ was necessary for localization of TcpC to the OM. TcpC was expressed both with and without TcpQ in ΔtcpC mutant strains and the cells were into their subcellular components by differential centrifugation in combination with membrane solubilisation. Using SDS-PAGE and immunoblotting with an anti-TcpC antibody, the different fractions were examined for the presence of TcpC (Figure 3-4). TcpC was detected in the OM fraction in very concentrated amounts when expressed alone and when co-expressed with TcpQ in a ΔtcpC strain. These results imply that while co-expression of TcpC with TcpQ is crucial for pilus assembly, TcpC does not require TcpQ for localization to the OM. However, since endogenous TcpQ is present in the ΔtcpC mutant, we further tested localization of overexpressed TcpC in a ΔtcpQ mutant and found that TcpC is both stably expressed and localizes to the OM in the complete absence of TcpQ (Figure 3-5), confirming that TcpQ is not required for TcpC stability or OM localization.

Although no TcpC is detected in the whole cell fraction of the ΔtcpQ mutant (Fig. 3-2A), it is detected in the more concentrated outer membrane fraction of a ΔtcpQ mutant (Figure 3-4A), indicating that it is somewhat stable in this strain. Overexpressed TcpC is also readily detected in the OM fraction in a ΔtcpQ strain (Figure 3-4B). Thus, TcpC is relatively stable in the absence of TcpQ and localizes to the OM, suggesting that TcpQ is not a co-chaperone for TcpC.
Figure 3-4 TcpC does not require TcpQ for OM localization (A) TcpC was expressed both independently and with TcpQ in ΔtcpC mutant strains and the cells were subjected to differential centrifugation. The resulting fractions were analyzed through immunoblotting using an anti-TcpC antibody. TcpF represents the periplasmic fraction and the anti-Strep is an inner membrane marker used to differentiate the different cellular fractions and ensure that there was no contamination of the cellular components. The OM band observed in the ΔtcpC strain represents the hypothetical protein that is detected by the TcpC antibody due to cross-reactivity as mentioned earlier. (B) Immunoblotting the OM and IM fractions of the ΔQ + ptcpC strain revealed that TcpC is stably expressed in the complete absence of TcpQ and it localizes to the OM. These results contradict those of Bose and Taylor (2005) who demonstrated that TcpC mislocalizes to the IM in the complete absence of TcpQ. Our results show that TcpC does not require TcpQ for OM localization, negating TcpQ’s role as chaperone for TcpC.
3.5. TcpQ does not require co-expression of TcpC for rescue of pilus assembly in a \( \Delta tcpQ \) mutant

As demonstrated earlier, in our hands TcpC is only able to rescue pilus assembly in a \( \Delta tcpC \) mutant when it is co-expressed with TcpQ, suggesting that these proteins must fold and/or assemble together in a 1:1 stoichiometry as they are synthesized. To test whether the reciprocal is true, that TcpQ requires TcpC co-expression in order to rescue pilus assembly in a \( \Delta tcpQ \) deletion strain, we expressed TcpQ in a \( \Delta tcpQ \) strain and assayed for pilus assembly. In contrast to our results with the \( \Delta tcpC \) rescue (Figure 3-2B), ectopic expression of TcpQ alone is sufficient to at least partially rescue pilus assembly, as the \( \Delta tcpQ + ptcpQ \) strain auto-agglutinates at 60% of WT on average (Figure 3-5A) and secretes TcpF at levels comparable to wild-type (Figure 3-5B). Both TcpQ and TcpC are stably expressed in the \( \Delta tcpQ + ptcpQ \) strain (Figure 3-5C, 3-5D), indicating that ectopically expressed TcpQ can restore TCP assembly by utilizing the endogenous TcpC present in the \( \Delta tcpQ \) cell. TcpQ migrates as two bands in the Western blot analysis, at approximately 20 and 15 kDa (Figure 3-5C). The predicted molecular mass of TcpQ is 15 kDa and the antibody used to detect this protein recognizes an internal TcpQ peptide sequence (residues 29-47, Bose and Taylor, 2005). Hence, the higher mass band may correspond to a post translationally modified TcpQ but this has not been investigated.
Figure 3-5 TcpQ does not require co-expression of TcpC for pilus assembly (figure continued on next page).
Figure 3-5 TcpQ does not require co-expression of TcpC for pilus assembly

(A) Expressing TcpQ in a ΔtcpQ strain rescues auto-agglutination as a lower OD600 reading is observed in the complement strain in comparison to the negative controls. The ΔtcpQ + ptcpQ strain retrieves the auto-agglutination phenotype by approximately 50%.

(B) TcpF is detected in the filtered supernatant fraction. The indicated strains were grown in pilus-inducing conditions with increasing concentrations of rhamnose (measured in percentages) for the complement strains.

(C) TcpQ is detected in levels comparable to wild type in the whole cell fraction as detected by immunoblot analysis.

(D) TcpC is stably expressed in this complement strain as well when the whole cell fraction is probed with an anti-TcpC antibody.
3.6. TcpC chaperones TcpQ to the outer membrane

We found it interesting that ectopically expressing TcpQ in a ΔtcpQ strain restored pilus functions yet ectopic expression of TcpC in a ΔtcpC failed to do so. Our results suggested that TcpC might act as a chaperone for TcpQ, localizing it to the OM. This interaction might require that TcpQ be translated and translocated in close proximity to TcpC. If TcpC is not in close proximity to TcpQ when TcpQ is released from the inner membrane via signal peptidase I, TcpQ may be released into the periplasm and TcpC would translocate in the OM without TcpQ. When TcpQ is ectopically expressed in a ΔtcpQ strain, there is sufficient amount of TcpQ that it is capable of associating with the endogenous TcpC and therefore, pilus functions are restored in this complemented strain. Thus, we hypothesized that TcpC may be associating with TcpQ during the translation and translocation to the periplasm and that it acts as a chaperone for TcpQ.

To test this, I looked for localization of TcpQ in a ΔtcpC + ptcpC strain (Figure 3-6). No TcpQ was detected in this complemented strain. It may be that when TcpC is ectopically expressed in a ΔtcpC mutant, it gets translated, translocated, and targeted to the OM with or without TcpQ. If TcpQ is not co-expressed with TcpC, it will not associate with TcpC and will be degraded in the periplasm following translocation. These results demonstrate that TcpQ requires TcpC for OM localization, suggesting that TcpC is a chaperone for TcpQ.

60
TcpQ is not detected in the complemented $\Delta tcpC + ptcpC$, which suggests that TcpQ requires TcpC as a chaperone to localize it to the OM.

**Figure 3-6 TcpQ requires TcpC for OM localization** TcpQ is not detected in the complemented $\Delta tcpC + ptcpC$, which suggest that TcpQ requires TcpC as a chaperone to localize it to the OM.
3.7. TcpC Cys1 is required for formation of a functional secretin channel

TcpC is a lipoprotein that undergoes lipid modification upon translocation into the periplasm (Parsot et al, 1991). To validate that this lipidation site is necessary for pilus assembly, a single amino acid substitution was introduced using site-directed mutagenesis to change the lipobox Cys1 to a serine residue. This is the most conservative change as the Cys1 sulfhydryl group, which undergoes lipidation is replaced with a hydroxyl group on the Ser1 which will not be lipidated (Figure 3-7A). We then co-expressed TcpC<sub>C1S</sub> with TcpQ in ΔtcpC mutant strains and assessed its ability to rescue pilus assembly in comparison to the wild-type complement strain. TEM analysis showed that the strains expressing TcpC-C1S do not rescue pilus assembly (Figure 3-7B), demonstrating that the TcpC Cys1 is necessary for pilus assembly and function, presumably because it is lipidated.
Figure 3-7 TcpC requires Cys1 for pilus assembly (A) Cysteine has a sulfhydryl group in its R chain that is the site of lipidation on the residue. When cysteine is mutated into a serine residue, the sulfhydryl group is replaced by a hydroxyl group. The hydroxyl group is incapable of undergoing lipidation as the oxygen is highly electronegative and thus tends not to release the hydrogen, which is necessary to facilitate the lipidation of the residue. Thus, the C1S mutant is not lipidated. (B) V. cholerae strains were imaged by transmission electron microscopy. High levels of pili are produced in the TcpQC complement, as shown by the large pilus bundles (red arrows) whereas no pili were observed for the TcpQC_{C1S} complement. Polar flagella are also indicated (blue arrows).
3.8. TcpC Cys1 is not necessary for stability or localization of TcpC to the outer membrane

Since TcpC Cys1 is necessary for formation of a functional secretin channel, we wanted to determine if the lipidation of Cys1 contributes to localizing TcpC to the OM. TcpC \textsubscript{C1S} was co-expressed with TcpQ in \textit{ΔtcpC} mutant strains. Cell fractions were separated and analyzed by SDS-PAGE and immunoblotting with an anti-TcpC antibody. Both TcpC\textsubscript{WT} and TcpC\textsubscript{C1S} localize to the OM (Figure 3-8). This result implies that lipidation of TcpC Cys1 is not crucial for targeting the secretin protein to the OM despite Cys1 being critical for pilus functions. Secretin subunits form multimeric channels in the outer membrane, anchored by their C-terminal domains, which are predicted to form a \(\beta\)-barrel (Bose & Taylor, 2005; Korotkov et al, 2009). TcpC has a large C-terminal region that localizes the protein to the OM; and though removal of the Cys1 lipidation site has a profound effect on the localization of a lipoprotein, the effect is negated here because TcpC has a transmembrane domain. Thus, TcpC Cys1 is not necessary for the localization of full-length TcpC to the OM.
**Figure 3-8 TcpC does not require Cys1 for localization to the OM** To evaluate the role of the TcpC Cys1 lipidation site on secretin localization, we used site-directed mutagenesis to change the cysteine to serine, expressed the proteins in Δ tcpC mutant strains, and then subjected the strains to cellular fractionation. Immunoblot analysis with an anti-TcpC antibody shows that the TcpC-C1S, like WT TcpC localizes to the OM, demonstrating Cys1 is not necessary for OM localization.
3.9. Localization of the N-terminus of TcpC to the outer membrane requires Cys1

The TcpC C-terminal region has a transmembrane β-barrel domain which is homologous to other secretin proteins and this domain targets the protein for insertion into the OM (Bose and Taylor, 2005; Korotkov et al, 2009). However, lipidation of TcpC at Cys1 implies that the N-terminal periplasmic region is also anchored in the outer membrane (Parsot et al, 1991 and this study). To test if Cys1 lipidation targets the N-terminus of TcpC to the OM, I expressed the N-terminal periplasmic portions of TcpC, residues 1-236 (TcpC(1-236)), with its wild type sequence and as a C1S variant, replacing the C-terminal secretin domain (residues 237-473) with a histidine tag, and examined their cellular localization. Both TcpC\textsubscript{236} and TcpC\textsubscript{C1S,236} were co-expressed with TcpQ in the ΔtcpC strain and subjected to cellular fractionation and immunoblot analysis using an anti-His antibody (Figure 3-9). While TcpC\textsubscript{236} is present in all fractions it predominantly localizes to the OM whereas TcpC\textsubscript{C1S,236} was not detected in any of the cell fractions. These results imply that TcpC Cys1 is essential for anchoring the periplasmic domain of the secretin protein into the OM; without that lipidation, C-terminally truncated TcpC (TcpC\textsubscript{C1S,236}) is unstable and is degraded.
Figure 3-9 C-terminally truncated TcpC requires Cys1 for localization to the OM and for stabilization The N-terminal periplasmic portion of TcpC, both WT and a C1S variant with a C-terminal histidine tag was co-expressed with TcpQ in the *V. cholerae* ΔtcpC deletion strain. The overnight cell cultures were fractionated and examined by immunoblot analysis using an anti-His antibody. While the WT form localizes to the OM the TcpC<sub>C1S_236</sub> construct is not detected, suggesting that it is unstable when not attached to the OM.
3.10. TcpQ is not required for localization of the TcpC periplasmic domains to the OM

In Section 3.4 we showed that TcpQ is not required for the localization of the full-length secretin protein to the outer membrane, but that pilus assembly is disrupted in the absence of TcpQ. To test whether TcpQ plays a role in the localization of the periplasmic domain of TcpC I expressed TcpC236 in a ΔtcpQ strain and looked for protein localization and expression. The N-terminally truncated TcpC236 is expressed stably and localizes to the outer membrane in the absence of TcpQ as detected by immunoblotting with an anti-His antibody (Figure 3-10). This result demonstrates unambiguously that TcpQ is not a co-chaperone for TcpC. However, TcpQ is necessary for formation of a functional pilus assembly apparatus. Thus, it seems likely that TcpQ associates with TcpC, most likely via its periplasmic domain, which links TcpC to the alignment complex and the inner membrane complex of the pilus machine.
Figure 3-10 The periplasmic domain of TcpC localizes to the OM in the absence of its secretin domain When the histidine-tagged periplasmic domain of TcpC (TcpC$_{236}$-H$_6$) is expressed in a ΔtcpQ strain under pilus inducing conditions, the truncated secretin still localizes to the outer membrane. The domain is C-terminally histidine tagged so that it can be detected using an anti-histidine antibody as the TcpC antibody we generated in this study is targeted for the C-terminal domain.
3.11. The lipidated periplasmic region of TcpC localizes TcpQ to the OM

To test whether lipidation of TcpC is a requirement for its interaction with TcpQ to the OM I looked at localization of TcpQ in the ΔtcpC + ptcpQC and ΔtcpC + ptcpQC\textsubscript{C1S} strains. TcpQ localizes to the outer membrane in both strains (Figure 3-11A). The loss of the lipidation site does not affect the localization of TcpQ. To determine whether the lipidated periplasmic domain of TcpC is sufficient to localize TcpQ to the OM I looked at localization of TcpQ when co-expressed with the periplasmic domain of TcpC, wild type and C1S, in a ΔtcpC background (Figure 3-11B). Results demonstrated that TcpQ localizes to the outer membrane with the periplasmic domain of TcpC but is mostly periplasmic in the TcpQC\textsubscript{C1S236} strain. Collectively, these results suggest that TcpQ localizes to the OM by binding to the periplasmic region of TcpC, but correct OM insertion of this TcpC region is also necessary for formation of a functional pilus assembly machinery.
TcpQ interacts with TcpC prior to membrane insertion (A) TcpQ localizes to the outer membrane when it is co-expressed with both the wild-type and C1S variant of TcpC. This suggests that two proteins interact with each other prior to membrane insertion. Furthermore, these results demonstrate that the loss of the lipidation site on TcpC does not affect localization of TcpQ but it does affect pilus assembly as TcpC C1S fails to form pili even when co-expressed with TcpQ. (B) TcpQ localizes to the outer membrane when it is co-expressed with the periplasmic domain of TcpC. When the periplasmic domain of TcpC is unlipidated, TcpQ accumulates in the periplasm. These results suggest that TcpQ interacts with the periplasmic domain of TcpC to form the alignment complex required for pilus assembly.
Chapter 4. Discussion

Overall, our findings show that TcpC does not require TcpQ for protein stability or localization. When TcpC is expressed in a ΔtcpQ strain, TcpC is stably expressed and localizes to the OM. This informs us that TcpC does not require TcpQ for localization and these results contradict previous findings done by Bose and Taylor (2005) who demonstrated that TcpC mislocalizes to the IM in the absence of TcpQ. A possible explanation for this discrepancy in data could be attributed to differences in antibody detection methodology. In the Craig lab, we use very sensitive developing reagents (see chapter 2) which allow us to detect proteins very easily and readily. Furthermore, my work also looked at the localization of the periplasmic domains of TcpC which also showed that TcpC localizes to the OM. This further substantiated that TcpQ is not a chaperone for TcpC and these experiments were not investigated by Bose and Taylor (2005).

Ectopic expression of TcpC in a ΔtcpC fails to restore function and this is surprising as though TcpC is over-expressed and it localizes to the outer membrane, the strain is not functional despite endogenous TcpQ present in the ΔtcpC strain. However, when TcpC is co-expressed with TcpQ in ΔtcpC, pilus assembly is restored. This suggests that TcpC requires TcpC in a 1:1 stoichiometric ratio to produce a functional secretin channel (Figure 3-3). Through metal affinity column pull down assays, Bose and Taylor showed a direct interaction between TcpC and TcpQ as his-tagged TcpQ was able to pull down TcpC from cell lysates and the reciprocal experiment yielded the same results (Bose and Taylor, 2005). With this, it is reasonable to hypothesize that TcpQ and TcpC are required in a 1:1 stoichiometric ratio.
Surprisingly, when the reciprocal experiment is done with TcpQ ectopically expressed in a \( \Delta tcpQ \) strain, pilus assembly is restored with the endogenous TcpC present in the strain. The endogenous TcpC present in the \( \Delta tcpQ \) strain is sufficient to interact with TcpQ and restore pilus assembly. The requirement of TcpC being co-expressed with TcpQ in order to restore pilus assembly but not vice versa suggests that TcpC chaperones TcpQ to the OM (Figure 3-6). TcpC is made in the cytoplasm and translocated across the IM via the Sec machinery, then lipidated by the Lgt and Lnt proteins and translocated by the Lol system to the OM with or without TcpQ. Thus, if TcpC is not expressed simultaneously with TcpQ, it will go straight to the OM. TcpQ is also translocated across the IM via the Sec pathway but it must interact with TcpC for stability. If the two proteins are not co-expressed, TcpQ is not transported to the OM and therefore cannot interact with TcpC. TcpQ requires TcpC as a chaperone to target it the OM as otherwise, TcpQ is not targeted to the OM.

TcpQ likely plays a role in completing the pilus assembly by linking the outer membrane portion of the pilus apparatus to the periplasmic and inner membrane complex (Figure 4-1). TcpC does not require TcpQ for membrane localization, similar to how the secretin PilQ of \textit{Neisseria meningitidis} does not require its lipoprotein PilW for localization but requires it for pilus assembly (Carbonnelle et al, 2005).

We show here that TcpQ of the \textit{V. cholerae} TCP system is required for forming a functional pilus apparatus. TcpC is stably expressed and localizes to the outer membrane without dependence on TcpQ but fails to form a functional pilus apparatus. When TcpC is co-expressed with TcpQ, it is able to restore pilus assembly. Our results have important implications of the interaction between TcpQ and TcpC with regards to forming a
complete alignment complex necessary for pilus assembly. TcpC acts as a chaperone for localizing TcpQ to the outer membrane and aiding TcpQ in interacting with the alignment complex. Based on our results, we reclassify TcpC as a Class 4 secretin as it is able to localize to the OM on its own but it cannot form a functional pilus channel without its accessory protein, being TcpQ (Koo, Burrows, and Howell, 2012; Schmidt et al, 2001). We classify TcpQ as an alignment protein as it is not a lipoprotein and nor does it play a role in chaperoning TcpC to the OM but without, we cannot have pilus assembly occur.

This is novel as in many bacterial secretion systems including the T2SS of _V. cholerae_ a, the secretin protein requires a chaperone or pilotin protein to assist with secretion channel assembly and localization. For example, in the T4P of _N. meningitidis_, the levels of secretin channels formed is significantly reduced in the absence of the co-chaperone (Golovanov et al, 2006). Similarly, in the T2SS of _K. oxytoca_, the absence of the co-chaperone, PulS, results in mislocalization of the secretin, PulD, to the inner membrane and consequently, no pilus functions (Guilvout et al, 2006)

We also show here that the secretin TcpC Cys1 is not required for localization of the full-length secretin subunit. In the TcpC Cys1Ser (C1S) mutant, the full length protein is stably expressed and localizes to the outer membrane. This finding is very surprising and novel. Without undergoing lipidation, TcpC_{C1S} remains embedded in the IM through its signal peptide and it is shocking that we show the mutant protein localizing to the OM. The localization of the C1S mutant of full-length TcpC can be attributed to the fact that the protein has a large membrane domain which targets it to the OM. It is possible for the protein to undergo proteolysis at the signal peptide cleavage site by proteases in the IM which would then free the protein from the IM. From there, the mutant TcpC is
translocated to the OM, mostly likely with assistance from other chaperoning systems such as the BAM complex. However, when this C1S strain is expressed in ΔtcpC under pilus-inducing conditions and assayed for pilus assembly, we see that no pili are formed through TEM analysis. This suggests that the TcpC Cys1 plays an important role in forming the pilus assembly conduit by anchoring the periplasmic domains of the secretin into the outer membrane for proper orientation of the secretin channel.

To evaluate this, we generated a C-terminally truncated TcpC containing only the periplasmic domains and expressed it in ΔtcpC strains with TcpQ. The wild-type periplasmic TcpC236 localizes to the OM, whereas the C1S variant appears to be degraded as we are unable to detect the protein in any of the fractions. These results demonstrate that the TcpC Cys1 is crucial for stability and localization of the periplasmic domains of the secretin protein, and for correct formation of the pilus assembly apparatus. Without the Cys1, the periplasmic domains of the secretin protein does not undergo lipidation and consequently fails to localize to the outer membrane.

As demonstrated in this work, full-length TcpC does not require TcpQ for localization or stability as it localizes to the OM in the absence of TcpQ. To further test whether TcpQ is required for the localization of the periplasmic domains of TcpC, we expressed ptcpC236 in ΔtcpQ strain under pilus inducing conditions and looked for localization. TcpC236 localizes to the outer membrane in the absence of TcpQ, negating any role of TcpQ as a co-chaperone in the V. cholerae TCP system.

In conclusion, the TcpC Cys1 plays a vital role in localizing the periplasmic domains of the secretin protein in the outer membrane. This localization is necessary for optimal pilus functions as the C1S variant is deficient in forming pili. Collectively, these
results suggest that the TcpC Cys1 is necessary to anchor and orient the periplasmic domains of the secretin properly in the pilus alignment complex (Figure 4-1). Our results with TcpC<sub>236</sub> confirm that the N-terminal domain interacts with TcpQ to complete the alignment complex necessary for pilus assembly. We propose that anchoring of the N-terminal region of TcpC correctly orients TcpQ to interact with its partners in the alignment complex.

It is also a reasonable possibility that TcpQ is needed for the assembly of the TcpC secretin channel as we demonstrated that TcpQ is not needed for expression and localization of TcpC but not channel assembly. Purification and crystallization of TcpC from the OM in the complete absence of TcpQ would be an avenue of exploring this potential role of TcpQ. It maybe that TcpQ does not play a role in the alignment complex and simply is responsible for the assembly of the TcpC channel; however, our findings in this work strongly support a model in which TcpQ is an alignment protein required for forming the conduit needed for pilus assembly.

Future investigation of this interaction between the periplasmic domains of TcpC and TcpQ will provide crucial insight into TCP pilus dynamics and possible targets for anti-microbial treatments. Overall, our findings have important implications for other bacterial secretion systems where the secretin protein may have a dual role as well where it forms the outer membrane channel and chaperones pilus assembly proteins to the outer membrane as well.
Our findings indicate that TcpC Cys1 localizes the periplasmic portion of TcpC to the OM which is necessary for pilus assembly. TcpQ is critical for pilus assembly and must be co-expressed with TcpC, suggesting a 1:1 stoichiometry. We propose a model in which Cys1 (in red) anchors the periplasmic portion of TcpC into the OM and the periplasmic domains (in purple circles) of TcpC interact with TcpQ which interacts with pilus accessory proteins to form a fully functional pilus apparatus.

Figure 4-1 Proposed Model of the TcpQ-TcpC interaction complex
References


Hodskingson, J., Horsely, A., Stabat, D., Simon, M., Johnson, S., Fonseca, P., Morris,


Koo, J., Tammam, S., Ku, S. Y., Sampaleanu, L. M., Burrows, L. L., & Howell, P. L.


Megli, C. J., & Taylor, R. K. 2013. “Secretion of TcpF by the *Vibrio cholerae* Toxin-
Coregulated Pilus Biogenesis Apparatus Requires an N-terminal Determinant" 
*Journal of Bacteriology*, 195(12), 2718-2727

Megli, C. J., Yuen, A. S. W., Kolappan, S., Richardson, M. R., Dharmasena, M. N., 


Ng, D., Harn, T., Altindal, T., Kolappan, S., Marles, J. M., Lala, R., Spielman, I., Gao, Y., 


Household Contacts of Cholera Patients (CHoB17 Trial)" Frontiers in Microbiology. 7(1635).


