Minor pilins play a major role in pilus dynamics and functions

by
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in the
Department of Molecular Biology and Biochemistry
Faculty of Science

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Abstract

Type IV pili (T4P) in Vibrio cholerae and enterotoxigenic Escherichia coli (ETEC) represent the simplest of all pilus systems whereby all the proteins needed for pilus assembly are encoded within a single operon. These systems are unusual for their lack of a retraction ATPase and each operon encodes only one minor pilin instead of several. How can pili retract without a retraction ATPase? The only minor pilin in the operon is key to understanding retraction in these systems. V. cholerae T4P, the toxin co-regulated pilus (TCP), produces the minor pilin TcpB which shares N-terminal homology with the major pilin but possess a larger C-terminal domain. TCP can assemble in a ∆tcpB mutant but at much lower levels than the wild type strain. We show that the minor pilin is required for efficient pilus assembly and pilus-related functions. We quantified the stoichiometry between the major and minor pilins and established this ratio is critical to maintaining optimal pilus functions. We show by immunodetection and immunogold electron microscopy that the minor pilins are incorporated into surface-displayed pili at low levels. Moreover, we determined minor pilin incorporation at the base of an assembling filament is necessary to induce pilus retraction and this mechanism is mediated by a conserved glutamate at position 5. This residue is conserved in all major pilins and some minor pilins, and is hypothesized to be critical for stabilizing pilin:pilin interactions by charge complementarity during assembly when new pilins are incorporated at the base of the filament. We characterized the ETEC minor pilins and achieved similar findings. We propose a new model to explain pilus extension and retraction by which the minor pilins have dual functions in priming pilus assembly as the first subunit in assembly and inducing retraction by incorporating into assembling filaments to stall assembly and cause spontaneous depolymerization of the pilin subunits. Our results have implications in understanding pilus dynamics in the more complex T4P systems and in the related bacterial virulence factor, the type II secretion system.

Keywords: minor pilin; type IV pili; pilus retraction; enterotoxigenic Escherichia coli; Vibrio cholerae
Dedication

To my family for their unconditional love.

To my mentors, colleagues, and friends for their support.

To Dr. Gabbott for the inspiration to pursue science.
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<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>Autoagglutination</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>BFP</td>
<td>Bundle-forming pili</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serine albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>Colonization factor</td>
</tr>
<tr>
<td>CFA/III</td>
<td>Colonization Factor Antigen III</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryo-electron microscopy</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CTXφ</td>
<td>Cholera toxin phage</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>Escherichia coli</em></td>
</tr>
<tr>
<td>GC</td>
<td>Gonococcal</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Km</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile toxin</td>
</tr>
<tr>
<td>MM</td>
<td>Molecular marker</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>Rh</td>
<td>Rhamnose</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Sm</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>ST</td>
<td>Heat-stable toxin</td>
</tr>
<tr>
<td>Sup</td>
<td>Culture supernatant</td>
</tr>
<tr>
<td>T2S</td>
<td>Type II secretion</td>
</tr>
<tr>
<td>T4a</td>
<td>Type IVa</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>T4b</td>
<td>Type IVb</td>
</tr>
<tr>
<td>T4P</td>
<td>Type IV pili</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with 0.1% Tween</td>
</tr>
<tr>
<td>TCP</td>
<td>Toxin co-regulated pilus</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UA</td>
<td>Uranyl acetate</td>
</tr>
<tr>
<td>VPI</td>
<td><em>Vibrio</em> pathogenicity Island</td>
</tr>
<tr>
<td>WCC</td>
<td>Whole cell culture</td>
</tr>
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</table>
Chapter 1. General Introduction

Diarrheal pathogens are estimated to cause over 2 million deaths (Kosek, Bern, and Guerrant 2003; Petri et al. 2008; Pruss-Ustun et al. 2014) and infect up to 50% of international travelers each year (Jiang et al. 2002; Kendall et al. 2012; Ericsson 2003). Children under the age of five are most vulnerable to diarrheal diseases; in this age-group the annual mean incidence rate is 3.2 episodes per child and the annual death toll is over 350,000 (Pruss-Ustun et al. 2014). The World Health Organization (WHO) estimates that more than 35% of the world population – 2.5 billion people – lack access to clean water and adequate sanitation facilities and these two factors account for over 50% of the global burden of diarrheal diseases (Pruss-Ustun et al. 2014). The lack of infrastructure to provide clean water and sanitation in many developing countries allow bacterial pathogens, such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC), to thrive and remain endemic in these regions. *V. cholerae* is the etiological agent of the diarrheal disease cholera (Kaper, Morris, and Levine 1995) and ETEC is the leading cause of traveler’s diarrhea and is a re-emerging foodborne pathogen in developed countries like the United States and Canada (Scallan et al. 2011). Given the global implications these bacterial pathogens have on health and economics, there is urgency in understanding their molecular mechanism of pathogenesis to improve upon existing therapeutics and diagnostic assays.

1.1. *Vibrio cholerae*

*V. cholerae* are curved-rod shaped Gram-negative bacteria that cause the severe gastrointestinal disease cholera (Figure 1-1) (Kaper, Morris, and Levine 1995). The bacterium was first identified by Filippo Pacini and Robert Koch in the 1800s. In 1854 English physician John Snow was the first to draw the connection between the prevalence of cholera and the location of water pumps contaminated by the bacteria during an outbreak in London. Pathogenic and non-pathogenic *V. cholerae* are found
naturally in marine and freshwater reservoirs where they attach to algae, crustaceans, insects, planktons, and plants (Butler and Camilli 2005). Cholera disease is transmitted by ingestion of contaminated water with high concentration of \textit{V. cholerae} bacteria, which colonize the small intestinal epithelium, resulting in acute and voluminous watery diarrhea. This allows the bacteria to disseminate back into the environment. Acute diarrhea can lead to rapid and fatal dehydration in the host if not treated in a timely manner. Cholera is endemic in South Asia, Africa, Central and South Americas where clean water and proper sanitation are not readily available. The WHO estimates between 3 to 5 million infections and over 100,000 deaths due to cholera annually on a global scale. Sporadic outbreaks have been reported in the past several years, the most significant being the Haiti 2010 cholera outbreak which followed after a major earthquake in the country. By the end of 2014, there were over 700,000 reported cases and over 8000 deaths caused by cholera. The Haiti government estimates that over US$2.2 billion is needed to eliminate cholera and establish infrastructure to provide clean water and sanitation (Domercant et al. 2015).

\textit{V. cholerae} are classified on the basis of the O antigens of lipopolysaccharide (LPS). Over 200 different serogroups have been identified to date. Most \textit{V. cholerae} serogroups are harmless aquatic microbes and the majority that are pathogenic only cause mild gastroenteritis (Kaper, Morris, and Levine 1995). Two specific \textit{V. cholerae} serogroups – O1 and O139 – have caused pandemic cholera. Within the O1 serogroup there are two biotypes: classical and El Tor. The classical biotype has been responsible for six of the seven recorded cholera pandemics, and the El Tor biotype is responsible for the on-going seventh pandemic (Kaper, Morris, and Levine 1995; Ritchie and Waldor 2009; Slauch, Taylor, and Maloy 1997).
Figure 1-1  Transmission electron micrograph of *Vibrio cholerae* cells

Comma-shaped, Gram-negative *V. cholerae* cells expressing toxin co-regulated pilus filaments in bundles. *V. cholerae* wild type strain O395 grown in pilus inducing conditions, stained with 3% uranyl acetate and imaged on Hitachi 8100 TEM at 200 kEV. Imaging by Lisa Craig and Dixon Ng, Simon Fraser University.
1.1.1. *V. cholerae* pathogenesis

*V. cholerae* must acquire both the *Vibrio* Pathogenicity Island (VPI) and the lysogenic cholera toxin bacteriophage (CTXφ) to be pathogenic. The VPI is a virulence gene cluster that encodes the proteins necessary for production of the toxin co-regulated pilus (TCP) (Herrington et al. 1988; Taylor et al. 1987). TCP is the receptor for the CTXφ, which carries the *ctxA* and *ctxB* genes encoding the cholera toxin. The bacteriophage can integrate into one of the two circular chromosomes in *V. cholerae* to confer pathogenicity to non-pathogenic *V. cholerae* (Waldor and Mekalanos 1996). Expression of TCP and the cholera toxins are co-regulated by the ToxR regulatory system (DiRita et al. 1991). Upon ingestion, environmental cues within the digestive tract trigger the expression of virulence factors to facilitate the colonization process. Cholera toxin is produced and released into the intestinal lumen. (Figure 1-2) (DiRita et al. 1991).

![Figure 1-2 Infection process of *V. cholerae*](image-url)

*V. cholerae* adheres to the intestinal epithelial cells. Environmental cues trigger the expression of virulence factors that facilitate microcolony formation within the small intestinal lumen. Cholera toxin is produced and secreted upon attachment to the small intestinal epithelium. The toxin is taken up by the host, resulting in a massive water-electrolyte imbalance. Image courtesy of Lisa Craig, Simon Fraser University.
1.1.2. **Cholera toxin**

Cholera toxin (CT) is responsible for disrupting the water-electrolyte balance in the intestinal epithelium resulting in the watery diarrhea that is hallmark of the cholera disease. CT is an ADP-ribosylating toxin that belongs to the AB$_5$ family – where the enzymatic A subunit is bound by a pentameric B subunit (Lonnroth and Holmgren 1973; Lospalluto and Finkelstein 1972) – and is secreted by the type II secretion (T2S) system (Davis et al. 2000). The pentameric B subunit interacts with the GM1 gangliosides of the intestinal mucosal cells and facilitate endocytosis of the A subunit (Van Heyningen, Van Heyningen, and King 1976). Within the host, the A subunit is reduced and releases an active A1 domain and an inactive A2 domain; the A1 domain is an ADP-ribosylase that targets the alpha subunit of G$_s$ protein (G$_s$$\alpha$), a guanine nucleotide binding regulator protein. This ribosylation of the G$_s$ protein results in constitutive activation of adenylate cyclase, this elevates levels of cyclic-AMP (cAMP) and in turn causes the activation of cAMP-dependent protein kinase A (PKA) which phosphorylates the cystic fibrosis transmembrane conductance regulator chloride ion channels (Bubien, Jope, and Warnock 1994; Field 1980; Goodman and Percy 2005; Levistre et al. 1995). This phosphorylation opens the channels, resulting in a rapid efflux of fluids and electrolytes causing severe watery diarrhea (Figure 1-3). The loss of electrolytes and fluids can be fatal, water loss can be as high as 20 L per day and death can result in as little as a few hours (Kaper, Morris, and Levine 1995; Levine et al. 1983). Rehydration therapy has a very high success rate for recovery (>80%; WHO) if applied in a timely manner.

1.1.3. **Toxin co-regulated pilus**

Toxin co-regulated pilus (TCP) is another major virulence factor expressed by *V. cholerae*. TCP is a type IV pilus encoded on the *tcp* operon which is found within the *V. cholerae* VPI. These long thin filaments mediate a number of critical roles in *V. cholerae* pathogenesis. TCP self-associate to allow bacterial cells to form protective aggregates or microcolonies (Kirn et al. 2000; Lim et al. 2010; Taylor et al. 1987) and are receptors for the CTXp (Waldor and Mekalanos 1996). Furthermore, the TCP assembly apparatus mediates the secretion of colonization factor TcpF (Kirn, Bose, and Taylor 2003; Megli and Taylor 2013). TCP is essential to *V. cholerae* pathogenesis, as TCP mutants are
unable to colonize the infant mouse model or human volunteers (Aas et al. 2002; Herrington et al. 1988; Kirn et al. 2000; Taylor et al. 1987).

1.1.4. Secreted colonization factor TcpF

The TCP assembly apparatus mediates secretion of a colonization factor TcpF, which has no known function or sequence/structural homology with any known proteins (Kirn, Bose, and Taylor 2003; Megli et al. 2011). TcpF is not required for TCP assembly and a $\Delta tcpF$ mutant can still produce TCP and aggregate via TCP (Kirn and Taylor 2005). However, infectivity in the infant mouse model is much lower in a $\Delta tcpF$ mutant – a five log fold decrease compared to wild type (Kirn, Bose, and Taylor 2003). Furthermore, $\Delta tcpF$ mutants are unable to colonize the intestinal epithelium even when TcpF is complemented from wild type $V.\ cholerae$. This exoprotein is necessary for effective $V.\ cholerae$ colonization and pathogenesis. The precise mechanism by which TcpF is secreted through the TCP assembly apparatus remains unclear.
Cholera toxin mechanism of action

*V. cholerae* produces the AB$_5$ cholera toxin which is secreted by the type II secretion system into the intestinal lumen. The pentameric B subunits facilitate binding to the host epithelial cells through interaction with the GM1 ganglioside and the A subunit is internalized and activated. The activated A subunit is an ADP-ribosylase that targets adenylate cyclase which results in a loss of chloride ions. The water-electrolyte imbalance leads to watery diarrhea. Figure courtesy of Lisa Craig, Simon Fraser University.
1.2. Enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* (ETEC) is a rod-shaped Gram-negative bacterium that is the leading cause of infantile and traveler's diarrhea in developing countries (Figure 1-4) (Okoh and Osode 2008) and is also an under-recognized foodborne pathogen in developed countries (Scallan et al. 2011). The WHO estimates that on a global scale ETEC is responsible for over 200 million cases of diarrhea and up to 400,000 deaths annually (Black 1993). ETEC was first recognized as a diarrheal pathogen in the 1960’s and is estimated to account for close to 20% of all diarrheal related diseases in developing countries (Daniels 2006; Qadri et al. 2005; Sack et al. 1971). The majority of *E. coli* found in the human gastrointestinal tract are harmless, but a small number of these can pick up virulence factors through horizontal transfer of genes. ETEC is one of six pathotypes of diarrheal *E. coli*, the other five are: Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Kaper, Nataro, and Mobley 2004; Palaniappan et al. 2006). Amongst the pathogenic diarrheal *E. coli*, ETEC is the one most commonly found. Pathogenic *E. coli* can also be classified by serogrouping and serotyping, respectively, of the O antigen of LPS (O) and the flagella (H) – there are over 160 known serogroups and 50 serotypes (Nataro and Kaper 1998).

1.2.1. ETEC pathogenesis

The molecular mechanism of ETEC pathogenesis is well studied and similar to that of *V. cholerae*. ETEC gains entry into the host through contaminated water or food sources, and colonizes the host small intestine or bowel. There are over 22 colonization factors (CF) identified in ETEC (Qadri et al. 2005; Gaastra and Svennerholm 1996; Turner et al. 2006) and most of these CFs are fimbrial protein structures that mediate the adhesion to and colonization of the host. Upon adhesion to the host intestinal epithelium, the bacterium produces and secretes one or both of two enterotoxins, heat-labile toxin (LT) and heat-stable toxin (ST). The CFs and enterotoxins are encoded on transmissible plasmids that may also confer antimicrobial resistance in some strains (Qadri et al. 2005).
Figure 1-4  Transmission electron micrograph of Enterotoxigenic *Escherichia coli* expressing CFA/III pili

Enterotoxigenic *Escherichia coli* expressing colonization factor antigen III (CFA/III) pili. ETEC 31-10 wild type strain grown on CFA agar plates. Sample was stained with 3% uranyl acetate and imaged on a Hitachi 8100 TEM at 200 kEV. Imaging by Dixon Ng, Simon Fraser University
1.2.2. Heat-labile and Heat-stable toxins

The heat-labile toxin (LT) is structurally and physiologically similar to the cholera toxin. Both toxins belong to the AB$_5$ family, with a monomeric A subunit bound by a pentameric B subunit, and are secreted by the T2S system (Gill and Richardson 1980). The B subunit facilitates attachment to GM1 ganglioside and the A subunit (LTA) is internalized by endocytosis. Like the cholera toxin A subunit, LTA is activated inside the cell and acts upon Gs$\alpha$ by ADP-ribosylation and this results in constitutive activation of adenylate cyclase. Increased intracellular cAMP stimulates chloride ion secretion in the intestinal epithelium, causing an efflux in the water and electrolyte balance which results in watery diarrhea (Nataro and Kaper 1998).

In contrast, the heat-stable toxin (ST) is a low molecular weight peptide that is secreted by an outer membrane protein TolC (Yamanaka et al. 1998). The mechanism of action for ST is also different from HT; this toxin targets guanylate cyclase C found in intestinal brush border cells (Schulz et al. 1990) and stimulates cGMP production (Rao 1985), which causes cGMP-dependent protein kinase II to phosphorylate the CFTR channel to release chloride ions. Increased intracellular cGMP can also indirectly activate PKA, leading to further electrolyte imbalance and resulting in acute diarrhea (Vaandrager 2002).

1.2.3. ETEC Type IV pili

Two of the 20+ identified colonization factors (CFs) in ETEC are type IV pili, CFA/III (Colonization Factor Antigen III) and Longus. These filaments mediate attachment to epithelial cells (Mazariego-Espinosa et al. 2010; Taniguchi et al. 2001). CFA/III pili have been shown to adhere to a variety of epithelial cells (Taniguchi et al. 2001) and ETEC expressing CFA/III secrete LT exclusively through the T2S system. Longus pili have been shown to mediate bacterial self-aggregation, antimicrobial resistance, and adherence to intestinal epithelial cells (Mazariego-Espinosa et al. 2010). The majority of ETEC strains producing Longus secrete only ST, but some secrete only LT and others secrete both ST and LT (Gomez-Duarte et al. 1999). The gene clusters that encode the CFA/III and longus proteins are found on large virulence plasmids – 55
kb and 90 kb respectively – that also encode the ETEC toxins (Giron et al. 1997; Giron, Levine, and Kaper 1994; Taniguchi et al. 2001)

1.2.4. Secreted exoprotein CofJ

Like the *V. cholerae* TCP assembly apparatus, the ETEC CFA/III assembly apparatus can also secrete an exoprotein, CofJ (Yuen et al. 2013). CofJ (37kDa) is similar in size to TcpF (36 kDa), and it also has no sequence or structural homolog. The molecular function of this exoprotein is currently not known. The Longus assembly apparatus is hypothesized to secrete LngJ, which shares gene synteny with CofJ and TcpF.
1.3. Type IV pilins and pili

TCP, CFA/III and Longus belong to the family of Type IV pili, which are critical virulence factors expressed on the surface of many bacterial pathogens. Type IV pili are thin and long filaments, about 8 to 10 nm in diameter and measure several microns in length (Craig and Li 2008). These robust filaments can withstand tensile forces up to 100 pN (Merz, So, and Sheetz 2000). Depending on the bacterial species they are expressed in, these filaments facilitate different functions including adhesion to host cells, twitching motility, microcolony formation, bacteriophage and DNA uptake, and exoprotein secretion (Burrows 2005; Craig, Pique, and Tainer 2004).

Type IV pili are assembled from thousands of monomeric pilin subunit. Prior to assembly, the pilin subunits are found in the inner membrane where they are anchored via their conserved N-methylated hydrophobic N-terminal α-helix, α1N, which extends into the C-terminal globular domain and together with a 4- or 5-stranded β-sheet form the canonical pilin fold (Craig, Pique, and Tainer 2004). The C-terminal domain is highly variable with a pair conserved cysteine residues. A number of type IV pilin structures have been solved by x-ray crystallography or nuclear magnetic resonance (NMR). Three full-length pilin structures that have been solved to date: PilE of *N. gonorrhoeae* GC pili (Craig et al. 2006; Parge et al. 1995), PilA of *P. aeruginosa* PAK pili (Craig et al. 2003), and FilmA of *Dichelobacter nodosus* (Hartung et al. 2011). There are also structures for soluble N-terminally truncated pilins: BfpA of EPEC (Ramboarina et al. 2005), PilE of *Francisella tularensis* (Hartung et al. 2011), PilA of *P. aeruginosa* (strain K, strain K122-4, strain Pa110594) (Hazes et al. 2000; Audette, Irvin, and Hazes 2004; Keizer et al. 2001; Nguyen et al. 2010), PilS of *Salmonella enterica* (Xu et al. 2004), TcpA of *V. cholerae* (Craig et al. 2003; Lim et al. 2010), CofA of ETEC (Kolappan et al. 2012), and PilA1 of the Gram-positive *Clostridium difficile* (Piepenbrink et al 2015). The pilin structures reveal a canonical ladle shape shared between T4a and T4b pilins, with the α1 extension supporting 4 or 5-stranded β-sheet that forms the C-terminal globular domain. T4a and T4b pilins differ predominantly in the αβ-loop region between α1 and the β-sheet, the topology of the β-sheet, and the C-terminal D-regions (Craig 2009; Burrows 2012).
All Type IV pilins share sequence homology within the first ~25 residues of the N-terminus and an invariant glutamate at position 5, which is necessary for efficient pilus assembly (Aas et al. 2007; Horiuchi and Komano 1998; Li, Egelman, and Craig 2012; Pasloske and Paranchych 1988; Strom and Lory 1991). Type IV pilins are further classified based on their amino acid sequence and length (Giron et al. 1997; Strom and Lory 1993). *V. cholerae* and ETEC produce pilins of the Type IVb (T4b) subclass which are characterized by their longer signal peptide (25 to 30 amino acids) and larger mature protein (>200 residues). T4b pilins also have a variable hydrophobic residue at the mature N-terminus and a longer region between the conserved cysteines. In contrast, Type IVa (T4a) pilins found in *Neisseria* spp. and *P. aeruginosa* have a short 6 to 8 amino acid signal peptide, and a smaller mature protein with an invariant phenylalanine at the N-terminus. Type IVa pilins are found in a number of pathogens with broad host range, including *N. gonorrhoeae*, *N. meningitidis*, and *P. aeruginosa*. Type IVb pilins are typically found in enteric bacterial pathogens such as *V. cholerae*, *Salmonella enterica*, ETEC, and EPEC.

Despite the differences between Type IVa and Type IVb pilins, all Type IV pili share similar architecture and helical symmetry. The subunits within the filaments are arranged into a helical array with an axial rise of 8 to 10 Å and an azimuthal rotation ~100°. Polymerization via their N-terminus α-helices, which form a hydrophobic core, anchors the pilin subunits with the globular domains loosely packed to form the filament surface. There are currently two pseudo-atomic resolution structures available for Type IV pili. The *N. gonorrhoeae* GC pilus structure was first determined by cryo-electron microscopy. The crystal structure of the GC pilin, PilE, was modeled onto the 12.5 Å resolution cryo-EM structure to generate a pseudo-atomic resolution model (Craig et al. 2006). A similar approach was used to generate a pseudo-atomic resolution model for *V. cholerae* where the crystal structure of the pilin subunit TcpA was fitted into a negative-stained EM structure of TCP (Li, Egelman, and Craig 2012). The GC pilus and TCP structures share similar architecture and helical symmetry, suggesting that they share a similar assembly mechanism.
Figure 1-5  Structure of Type IV pili

(A) Structure of *V. cholerae* Type IV pilin subunit TcpA (PDB Code 1OQV) modelled on the N-terminal α-helix from the *P. aeruginosa* PAK pilin structure (PDB code 1OQW), representative Type IV pilin canonical fold, the N-terminal α-helix, α1, extends into the C-terminal globular domain where it interacts with a 4- or 5-stranded β-sheet. Aβ loop is highlighted in green, D-region in purple, and conserved cysteines are shown in cyan/yellow using ball-and-stick representation. (Figure adapted and used with permission from Craig *et al.*, 2003.) (B) Computational model of the *V. cholerae* toxin co-regulated pilus. This pseudo-atomic resolution structure was obtained by docking the TcpA crystal structure into an EM reconstruction of the TCP filament. Left: side view of filament. Right: top view of the filament, looking into the hydrophobic core formed by α1N. (Figure adapted and used with permission from Li *et al.*, 2012.)
1.4. Type IV pilus assembly and disassembly

The mechanism by which pilus filaments are assembled is not well understood. The more complicated T4P systems require as many as 40 different proteins to assemble pili (Pellicer 2008) while other simpler systems use as little as a dozen different proteins (Taylor et al. 1987; Stone et al. 1996). There are conserved components among all of these systems, summarized in Figure 1-6 which is based on our assembly model for T4P (Craig 2009; Craig and Li 2008; Craig et al. 2006). Pilin subunits are synthesized in the cytoplasm and are translocated to the periplasm via the Sec pathway. The pilins are then processed by a dedicated pre-pilin peptidase, which cleaves the signal peptide and catalyzes the N-methylation process (Kaufman, Seyer, and Taylor 1991; Strom and Lory 1993). The hydrophobic N-terminus of α1N allows the C-terminal globular domains of the pilin subunits to remain anchored on the periplasmic face of the inner membrane where they diffuse freely. In our pilus assembly model, a processed pilin dock at the base of the assembly apparatus and is held there by a charge complementarity interaction with the preceding subunit at the filament base – this interaction is mediated by the conserved negatively charged Glu5 residue and the positively charged N-terminal residue of the terminal subunit (Figure 1-6 B) (Craig and Li 2008; Craig et al. 2006). Pilus assembly is driven by a hexameric assembly ATPase localized at the cytoplasmic face of the inner membrane (Yamagata and Tainer 2007). Upon ATP hydrolysis, the cytoplasmic ATPase undergoes a conformational change which allows the apparatus to extrude the subunit and the filament together a short vertical distance outward (8 to 10 Å), which opens up space for the next pilin subunit to dock. As the filament extends across the periplasm, it passes through the outer membrane secretin channel and onto the cell surface. As illustrated in the model, pilus assembly is processive and is only limited by the supply of pilins in the inner membrane and ATP that powers the assembly apparatus. The core machinery found in T4P assembly systems is also conserved in the structurally and functionally related bacterial type II secretion (T2S) system, which assembles a pseudopilus to facilitate substrate secretion across the outer membrane without forming an extracellular filament (Sandkvist 2001).

Most Type IVa pilus systems, as well the Type IVb EPEC bundle forming pili (BFP), encode a retraction ATPase (Brossay et al. 1994) which coordinates pilus
retraction through a mechanism that is not well understood. Pilus retraction is necessary for a number of key functions, such as twitching motility, DNA uptake, phage transduction, and bacterial dissemination (Giltner, Nguyen, and Burrows 2012; Morand et al. 2004; Wolfgang, Park, et al. 1998; Wolfgang, Lauer, et al. 1998; Mattick 2002); all of these functions require dynamic pilus assembly and disassembly. No retraction ATPase has been identified in the *V. cholerae* and ETEC pilus systems. The T2S system also lacks a retraction ATPase. The T2S pseudopilus is thought to undergo rapid assembly and disassembly to generate a piston-like movement to facilitate secretion. (Reichow et al. 2010; Korotkov and Hol 2008; Korotkov, Sandkvist, and Hol 2012).

The majority of T4P systems also encode multiple minor pilins, which share N-terminal homology with the major pilins but are expressed at much lower levels. These pilin-like proteins are also found in the homologous T2S system. Type IV minor pilins have been shown to incorporate into the pilus filament (Giltner, Habash, and Burrows 2010) and their T2S counterparts can initiate assembly of pseudopili (Cisneros, Pehau-Arnaudet, and Francetic 2012; Cisneros et al. 2012).
**Figure 1-6  Type IV pilus assembly model**

(A) Schematic of the assembly apparatus. Assembly starts with a pilin subunit docking into an open position at the base of the filament. The pilin subunit is stabilized by charge complementarity in the N-terminus. The cytoplasmic assembly ATPase undergoes a conformational change upon ATPase hydrolysis and extrudes the filament upward by about 8 to 10 Å. This stabilizes the newly added subunit by pulling it partly out of the membrane and opening a new gap around the base of the filament for the next pilin subunit to dock. This process is limited only by the supply of pilin subunits and ATP. (B) Charge complementarity interaction proposed to mediate pilin:pilin interactions. The negatively charged glutamate at position 5 on the incoming subunit is stabilized by the positively charged N-terminal amine on the preceding subunit.
1.5. Minor pilins

Minor pilins share N-terminal homology with the major pilin and are aptly named as they are expressed at much lower levels. Most T4P and T2S systems encode several minor (pseudo)pilins that are involved in assembly and functions but their mechanisms are not well understood. The minor pilins in the T4a systems of *P. aeruginosa* and *N. gonorrhoeae* are required for efficient pilus assembly and appear to antagonize the retraction ATPase (Carbonnelle et al. 2006; Giltner, Habash, and Burrows 2010; Nguyen et al. 2015; Winther-Larsen et al. 2005). Winther-Larsen et al. have shown that deletion of the minor pilins in *N. gonorrhoeae* results in a loss of pilus assembly in the wild type background. This phenotype can be suppressed if the retraction ATPase is also deleted, suggesting that the minor pilins facilitate pilus extension or assembly. The *P. aeruginosa* minor pilins, FimU, PilV, PilW, and PilX are thought to form a complex that associates with the adhesion protein PilY1 and have been shown to incorporate into surface-displayed pili at low levels by immunogold transmission electron microscopy (Nguyen et al. 2015). PilW, PilX, and PilY1 are also known to repress *P. aeruginosa* swarming motility by modulating intracellular cyclic nucleotide levels (Kuchma, Griffin, O'Toole 2012). Some T4P systems also encode additional minor pilins that are dispensable in assembly but are required for function, such as *N. meningitidis* PilX and PIV, and *P. aeruginosa* PilE. The *N. meningitidis* minor pilin PilX has been shown to modulate signaling to host cells (Bernard et al. 2014; Brissac et al. 2012) and bacterial aggregation (Helaine et al. 2005; Imhaus and Dumenil 2014).

The ETEC T2S system minor pseudopilins, Gspl, GspJ, and GspK have been crystallized in complex by Koroktov et al. (Korotkov and Hol 2008). The N-terminally truncated structure of each minor pilin has the canonical fold of a Type IV pilin. Of the three, GspK is the largest, with an additional protrusion in its C-terminal domain. It has been hypothesized that GspK is the first subunit in assembly and acts as a large cap that prevents the pseudopilus from exiting onto the cell surface (Korotkov and Hol 2008; Korotkov, Sandkvist, and Hol 2012). In the *P. aeruginosa* T2S system, long pseudopilus filaments are formed when the GspK homolog is deleted and the major pseudopilin is overexpressed (Durand et al. 2005). Hence, the GspK homologs might be the mechanism that halts pseudopilus assembly to initiate disassembly. The GspK family of
proteins share a common trait with the Type IVb minor pilins in that they are significantly larger than the major (pseudo)pilin counterparts. However, the GspK family of proteins do not have an invariant Glu5 in the N-terminus which is found in the Type IVb minor pilins. This lack of the conserved Glu5 might restrict GspK proteins to the tip of the pilus as there is no N1+ charge to neutralize as the primary subunit.
1.6. Retraction in Type IVb pili

The Type IVb pilus systems found in \textit{V. cholerae} and ETEC represent simpler systems, where less than a dozen proteins encoded in one operon are sufficient for pilus assembly and functions (Figure 1-7). In contrast, the more complex Type IVa systems require as many 40 different proteins that are encoded in genes scattered throughout the genome (Pellicic 2008). The TCP, CFA/III and Longus systems lack a separate retraction ATPase and encode a single minor pilin instead of several.

\textbf{Figure 1-7} Alignment of the three T4b pilus operons: tcp, cof, and lng

Genes in the three operons are share synteny and sequence homology. All three operons encode only a single minor pilin (tcp\textit{B}, cof\textit{B}, lng\textit{B}) and they each lack a retraction ATPase typically found in more complicated T4P systems. The genes highlighted are: (cyan) major pilin, (red) minor pilin, (blue) outer membrane secretin, (beige) bitopic accessory proteins, (green) assembly ATPase, (pink) integral inner membrane platform protein, (yellow) secreted exoprotein, and (brown) prepilin peptidase.

\textit{V. cholerae}, TCP mediate three key pathogenic functions: TCP self-associate to allow bacterial aggregation, secrete colonization factor TcpF, and act as receptors for the CTX\textit{f} (Figure 1-8) (Kirn, Bose, and Taylor 2003; Kirn et al. 2000; Lim et al. 2010; Megli and Taylor 2013; Taylor et al. 1987; Waldor and Mekalanos 1996). We propose that all of these functions not only require pilus assembly (extension) but also pilus disassembly (retraction). TCP-mediated aggregation is facilitated by pilus:pilus interactions (Lim et al. 2010) and pilus retraction is hypothesized to draw bacterial cells
into closer together and keep cells tightly bound in microcolonies. TcpF secretion is thought to be facilitated by rapid assembly and disassembly of TCP in a piston-like mechanism similar to one that has been proposed for the T2S system (Korotkov, Sandkvist, and Hol 2012). And lastly, the CTXφ infection cycle requires the bacteriophage to interact directly with the periplasmic protein TolA (Ford et al. 2012) in a process that would require the pilus to retract and bring the bacteriophage into the periplasm. How does the Type IVb pilus system retract its pilus without a retraction ATPase?

Figure 1-8  Retraction is necessary for pilus functions

TCP mediates three critical virulence functions in *V. cholerae*: (1) **bacterial aggregation**, in which the cells form tight aggregates in protective microcolonies; (2) **exoprotein secretion**, the soluble colonization factor TcpF, which is also encode on the *tcp* operon is secreted by the assembly apparatus from the periplasm into the extracellular environment; (3) **uptake of the bacteriophage CTXφ**, TCP mediates the interaction between the bacteriophage and the periplasmic protein TolA to impart infectivity. We propose that TCP is a dynamic structure and each of these three functions require TCP to retract, as indicated by the grey arrows.
1.7. Thesis Objective

Diarrheal bacterial pathogens have serious impact on global health and economics (Domercant et al. 2015; Kosek, Bern, and Guerrant 2003). Most infections are self-limiting if treatment can be administered in a timely manner, but repeated exposures and infections can have negative life-long impacts limiting the health and socio-economic status of the low income population residing in regions endemic to these pathogens. *V. cholerae* and ETEC remains a serious health threat in developing countries and understanding the molecular mechanisms of their pathogenesis can lead to development of new diagnostics and therapeutics. Type IV pili expressed by both *V. cholerae* and ETEC as a virulence factor is an appealing target for vaccine and antimicrobial development. The precise mechanisms for pilus assembly and disassembly remains unclear. The Type IVb minor pilins appear to have a major role in regulating pilus dynamics and functions in the context of pilus extension and retraction. Understanding how these minor pilins function, will provide insight into the molecular machinery of the more complex Type II secretion and Type IVa pilus systems.

The specific aims of this thesis project are:

Aim 1 – Characterize the Type IVb minor pilins of *Vibrio cholerae* and enterotoxigenic *Escherichia coli* in terms of their stoichiometry to the major pilins and their functional role in pilus dynamics.

Aim 2 – Elucidate the molecular mechanism that allows Type IVb pilus systems to retract their pilus without a retraction ATPase.
Chapter 2. *Vibrio cholerae* minor pilin TcpB regulates toxin-coregulated pilus dynamics and functions


Contributions to research: pilin constructs, pilin and pilus expression, electron microscopy, data analysis, and co-wrote manuscript.

2.1. Introduction

*Vibrio cholerae* is a Gram-negative pathogenic bacterium and the etiological agent of the human diarrheal disease cholera (Kaper, Morris, and Levine 1995). Cholera is marked by copious watery diarrhea that can lead to dehydration, shock, organ failure and death in as little as 24 hours after infection. The severe diarrhea is caused by cholera toxin, an ADP-ribosylating toxin that is secreted by the EPS (Extracellular Protein Secretion) Type II secretion (T2S) system and induces water and electrolyte loss from intestinal epithelial cells (Davis et al. 2000). Colonization of the small intestine by *V. cholerae* requires a second virulence factor, the toxin co-regulated pilus (TCP), which self-associates to induce bacterial aggregation and microcolony formation that protect *V. cholerae* from host defenses (Herrington et al. 1988; Taylor et al. 1987). Expression of the cholera toxin and TCP genes is co-regulated by the transcriptional activator ToxT, which is encoded on the tcp operon. The TCP machinery secretes a soluble protein, TcpF, which is also encoded in the tcp operon. The function of TcpF is not known but it is essential for efficient colonization in the mouse cholera infection model (Kirn, Bose, and Taylor 2003). TCP is also the primary receptor for the lysogenic bacteriophage CTXφ, which carries the cholera toxin genes *ctxAB* (Waldor and Mekalanos 1996). CTXφ infection represents an important event in the evolution of pathogenic *V. cholerae*, as
stable incorporation of tandem copies of the phage genome imparted toxigenicity to this previously harmless marine bacterium (Davis and Waldor 2003). The ability of *V. cholerae* to colonize the human intestine, reproduce in massive numbers, and be disseminated into the environment as a consequence of the severe cholera toxin-induced diarrhea confers a tremendous evolutionary advantage to these bacteria.

TCP are Type IV pili (T4P), long thin filaments expressed ubiquitously by Gram-negative and by some Gram-positive bacteria (Berry and Pelicic 2015; Giltner, Nguyen, and Burrows 2012; Melville and Craig 2013). T4P biogenesis can involve as few as 10 and as many as 40 proteins to polymerize their major pilin subunits into surface-displayed pili. T4P perform a number of interrelated functions including adhesion, microcolony formation, DNA uptake, twitching motility, and exoprotein secretion (Craig and Li 2008; Giltner, Nguyen, and Burrows 2012; Pelicic 2008). T4P are polymers of the major pilin subunit, a small protein with a protruding N-terminal hydrophobic α-helix, α1 whose sequence is conserved and includes an invariant glutamate at position 5. α1 extends into the C-terminal globular domain and together with a 4- or 5-stranded β-sheet forms the canonical pilin fold. The C-terminal domain is highly variable in sequence, and the loops connecting α1 with the β-sheet and connecting the β-strands impart structural variability (Craig et al. 2003; Craig et al. 2006; Hartung et al. 2011; Parge et al. 1995). Major pilins are synthesized as prepilins, which are translocated across the inner membrane and are simultaneously processed by a dedicated prepilin signal peptidase that removes the signal peptide, leaving the mature pilins anchored in the inner membrane via their hydrophobic N-terminal α-helices (Nunn and Lory 1991; Strom and Lory 1991). In our T4P assembly model, the pilin subunits dock into the base of a growing pilus and their hydrophobic N-terminal α-helix transitions from the acyl phase of the inner membrane to the hydrophobic core of the pilus where it is surrounded by neighboring α1s (Craig et al. 2006). ATP hydrolysis by the assembly ATPase located on the cytoplasmic side of the inner membrane (Anantha, Stone, and Donnenberg 2000; Tripathi and Taylor 2007; Wall and Kaiser 1999; Wolfgang, Park, et al. 1998) may result in a conformational change in the conserved inner membrane core protein (also called the platform protein) that extrudes the pilus a short distance out of the membrane, stabilizing the newly docked pilin subunit and opening up a gap along the base of the growing pilus for the next subunit to insert (Craig et al. 2006). The conserved Glu5 is
positioned in the assembled filament to neutralize the positively-charged N-terminal amine (N1+) of the adjacent pilin subunit in the hydrophobic core (Figure 1-6B) (Craig et al. 2003; Craig et al. 2006; Li, Egelman, and Craig 2012). The negative charge is necessary for efficient pilus assembly (Aas et al. 2007; Horiuchi and Komano 1998; Kirn, Bose, and Taylor 2003; Li, Egelman, and Craig 2012; Pasloske and Paranchych 1988; Strom and Lory 1991). We have proposed that this charge complementarity drives the docking of each incoming pilin subunit into the growing pilus filament, with Glu5 neutralizing the N1+ of the terminal pilin subunit (Li, Egelman, and Craig 2012; Craig, Pique, and Tainer 2004).

In Gram-negative bacteria T4P filaments grow across the periplasm and through the outer membrane secretin channel where they are displayed on the bacterial surface. Most T4P systems possess a second “retraction ATPase” required to depolymerize or retract the pili by an unknown mechanism. Retraction pulls the bacteria along moist surfaces and can bring bound substances like DNA and bacteriophage into the cell (Aas et al. 2002; Anantha, Stone, and Donnenberg 1998; Burrows 2012; Chiang, Habash, and Burrows 2005). Retraction has been directly demonstrated for Pseudomonas aeruginosa by total internal reflection microscopy (Skerker and Berg 2001), and for Neisseria gonorrhoeae T4P using optical tweezers bead assays (Maier et al. 2002; Merz, So, and Sheetz 2000) and elastic micropillar assay (Biais et al. 2010; Biais et al. 2008). N. gonorrhoeae pili retract at an astounding rate of ~1 µm/sec (Maier et al. 2002), which is equivalent to removing ~1000 pilin subunits per second. Retraction is dependent on the retraction ATPase PilT in N. gonorrhoeae. T4P retract with forces of ~100 pN, making PilT the strongest molecular motor known (Maier et al. 2002).

The V. cholerae TCP are comparatively simple T4P systems with only 10 proteins encoded on a single tcp operon (Figure 1-7), none of which is a retraction ATPase. TCP are not associated with twitching motility or DNA uptake but they do facilitate CTXφ uptake (Ford et al. 2012; Waldor and Mekalanos 1996) and TcpF secretion. Furthermore, although TCP are several microns in length, pilus:pilus interactions cause V. cholerae to form very tight, densely packed aggregates. All of these mechanisms appear to involve pilus retraction, which may occur via spontaneous pilus disassembly.
The T4P system is structurally and functionally related to the T2S system, in which major "pseudopilins" assemble into a pseudopilus that grows from the inner membrane through the periplasm, driven by an assembly ATPase (Burrows 2012; Korotkov, Sandkvist, and Hol 2012; Sandkvist 2001). While T4P are long thin filaments displayed on the bacterial surface, T2S pseudopili remain in the periplasm and extrude substrates across the outer membrane via a secretin channel. The major pseudopilin subunits share the hydrophobic N-terminus and Glu5, as well as a globular C-terminal domain with the Type IV pilins. Secretion is thought to occur via rapid assembly and disassembly of the periplasmic pseudopilin, resulting in a piston-like motion (Korotkov, Sandkvist, and Hol 2012; Reichow et al. 2010), yet T2S systems, like V. cholerae TCP, lack a retraction ATPase.

The T4P and T2S systems each possess several pilin-like proteins that share N-terminal sequence homology and are structurally similar to the major (pseudo)pilins but are much less abundant and are thus called minor pilins. These pilin-like proteins are involved in T4P assembly and T2S functions but their mechanisms are poorly understood. Four core minor (pseudo)pilins in the general secretory pathway (Gsp), GspH, Gspl, GspJ, and GspK have been identified as common to both systems, though their names differ. The T4P GspHIJK homologs in P. aeruginosa, FimU, PilV, PilW, and PilX are required for pilus assembly in an otherwise wild type (WT) background (Alm et al. 1996; Alm and Mattick 1995; Russell and Darzins 1994), but pili are produced in mutants lacking both the minor pilins and the retraction ATPase, PilT (Giltner, Habash, and Burrows 2010; Nguyen et al. 2015). These minor pilins are proposed to form a complex with the adhesion protein PilY1. P. aeruginosa PilW and PilX expression is further linked to repression of flagellar-mediated swarming motility (Kuchma, Griffin, and O'Toole 2012). The N. gonorrhoeae minor pilins are required for pilus assembly to balance the antagonistic activity of the retraction ATPase, PilT (Winther-Larsen et al. 2005). The minor pilins co-purify with the pilus fraction in WT N. gonorrhoeae suggesting they incorporate into the pilus (Winther-Larsen et al. 2005). Low level pilus incorporation of the P. aeruginosa minor pilins was shown by transmission electron microscopy (TEM) using gold-labeled antibodies against the minor pilins (Giltner, Habash, and Burrows 2010). Some T4P systems also possess one or more non-core minor pilins, PilE in P. aeruginosa, and PilX and PilV in Neisseria meningitidis. These proteins are dispensable
for pilus assembly but are required for pilus functions. The *N. meningitidis* minor pilins PilX and PilV are required for T4P functions in bacterial aggregation, adhesion to and signaling of host cells (Bernard et al. 2014; Brissac et al. 2012; Helaine et al. 2005; Helaine et al. 2007; Imhaus and Dumenil 2014; Miller et al. 2014). PilX is structurally similar to the *N. gonorrhoeae* major pilin (Helaine et al. 2005) and was shown by immunogold-TEM to incorporate into surface-displayed pili. However, Dumenil et al. assert that PilX and PilV operate in the periplasm, modulating pilus levels to control pilus functions (Imhaus and Dumenil 2014).

The GspI, GspJ, and GspK minor pseudopilins of the enterotoxigenic *Escherichia coli* (ETEC) T2S system were crystallized in a complex (Korotkov and Hol 2008). Each minor pilin has the canonical Type IV pilin fold comprised of an extended N-terminal α-helix and a β-sheet-containing globular domain. The largest of these minor pseudopilins, GspK, has an unusually large globular domain. GspK caps the complex an may prevent pseudopilus growth through the outer membrane secretin (Korotkov and Hol 2008). Indeed, *P. aeruginosa* mutants lacking the GspK homolog XcpX form long pilus-like pseudopilus on the bacterial surface when the major pseudopilin, XcpT, is overexpressed (Durand et al. 2005). Thus, the minor pilin complex may stall pilus assembly and promote disassembly when it contacts other parts of the periplasmic assembly machinery, producing a piston-like motion in the absence of a retraction ATPase.

GspK and XcpX belong to the GspK superfamily of proteins. These minor pseudopilins are all substantially larger than the other minor pseudopilins encoded in their respective gene clusters, and they lack the position 5 glutamate seen in the minor and major (pseudo)pilins. This difference might restrict GspK proteins to the tip of the pilus filament, where there is no existing N1+ charge in which to neutralize. The T4P minor pilins all include a GspK-like protein encoded in the last gene of each cluster (eg. PilX in *P. aeruginosa*, PilK in *Neisseria* spp.). In contrast to the GspK family minor pseudopilin, the GspK minor Type IV pilins are typically comparable in size to their associated minor pilins, but importantly they also lack a position 5 glutamate, suggesting that they may also be tip-associated.
The *V. cholerae* TCP represents a simplified T4P system in which to study pilus dynamics. TCP assembly requires only 10 proteins encoded by genes clustered in a single operon (Taylor et al. 1987), and lacks a retraction ATPase. Unlike the T2S system and most T4P systems, TCP has only a single minor pilin, TcpB, which is encoded immediately downstream of the major pilin, TcpA. We show here that TCP are in fact produced in a \( \Delta tcpB \) mutant but at much reduced levels compared to the *V. cholerae* wild type strain O395, supporting a role for TcpB in pilus assembly. However, the pili they do assemble in the absence of TcpB are nonetheless non-functional. We show that TcpB incorporates into surface-displayed pili and its ability to incorporate into the assembling filament via the invariant Glu5 is critical for TCP-mediated functions. Our findings have important implications for understanding assembly and retraction in the more complex T4P systems and the T2S system.
2.2. Materials and Methods

2.2.1. Plasmids and strain list

Bacterial strains, plasmids, and primers are listed in Table 2.1. *V. cholerae* strains were grown with streptomycin and antibiotics appropriate for plasmid selection. Final concentrations for antibiotics: 100 μg ml\(^{-1}\), ampicillin (Ap) and 100 μg ml\(^{-1}\), streptomycin (Sm). *V. cholerae* strains O395, SJK70 (ΔtcpA), YG003 (ΔtcpB), YG005 (ΔtcpC), RT4225 (TcpA\(_{H181A}:\text{toxT}\)), RT4236 (ΔflaA:toxT), plasmid pJMA10, and TcpA-6 and TcpB (61-78) polyclonal rabbit antibody were gifts from Ronald Taylor (Geisel School of Medicine).
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description or nucleotide sequence</th>
<th>Source / Reference</th>
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**Strains**

<table>
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<tr>
<th>Strain</th>
<th>Description</th>
<th>Source / Reference</th>
</tr>
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<tbody>
<tr>
<td><em>V. cholerae</em> O395</td>
<td>Classical O1, Ogawa, Str&lt;sup&gt;r&lt;/sup&gt;, derivative</td>
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<td>ΔtcpA, major pilin deletion strain</td>
<td>Kirn et al. (2000)</td>
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<td><em>V. cholerae</em> YG003</td>
<td>ΔtcpB, minor pilin deletion strain</td>
<td>Kirn et al. (2000)</td>
</tr>
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<td><em>V. cholerae</em> YG005</td>
<td>ΔtcpC secretin deletion strain</td>
<td>Kirn et al. (2000)</td>
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<td><em>V. cholerae</em> RT4225</td>
<td>TcpA&lt;sub&gt;H181A&lt;/sub&gt;:toxT</td>
<td>R.K. Taylor</td>
</tr>
<tr>
<td><em>V. cholerae</em> RT4236</td>
<td>Flagella minus strain, pMT5:toxT</td>
<td>DiRita et al. (1996)</td>
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<tr>
<td>ETEC 31-10</td>
<td>Wild-type ETEC, LT, CFA/III</td>
<td>Honda et al. (1989)</td>
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<td><em>E. coli</em> DH5α</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; 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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<td><em>E. coli</em> SHuffle® T7 Express</td>
<td>MiniF lysY (Cam&lt;sup&gt;R&lt;/sup&gt;) / fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal λatt::pNEB3-r1-cDsbC (Spec&lt;sup&gt;R&lt;/sup&gt;, lacI&lt;sup&gt;q&lt;/sup&gt;)ΔtrxB sulA11 R(mcr-73::miniTn10--Te&lt;sup&gt;e&lt;/sup&gt;)2 [dcm] R(zgb-210::Tn10 -- Te&lt;sup&gt;e&lt;/sup&gt;) endA1 Δgor Δ(mcrC-mrr)114::IS10</td>
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<td><strong>Plasmid</strong></td>
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<td>pJMA10</td>
<td>pBAD22 derivative; <em>araC</em> replaced with PrhaB. Bla, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>R.K. Taylor</td>
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<td>pJMA10.1</td>
<td>pJMA10 with <em>NcoI</em> site removed</td>
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<td><em>ptcpA</em></td>
<td>pJMA10.1 containing the <em>tcpA</em> gene</td>
<td>This study</td>
</tr>
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<td>This study</td>
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<td><em>ptcpB</em>&lt;sub&gt;E5D&lt;/sub&gt;</td>
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<td>This study</td>
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<td><em>ptcpB</em>&lt;sub&gt;E5Q&lt;/sub&gt;</td>
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<td><em>ptcpB</em>&lt;sub&gt;E5V&lt;/sub&gt;</td>
<td><em>ptcpB</em> with Val&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>Reagent</td>
<td>Description or nucleotide sequence</td>
<td>Source / Reference</td>
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<td>TcpB-R-238-XbaI</td>
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<td>L1:CofJ2-F</td>
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<td>CofJ2:L2-R</td>
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<td>TcpA1:L1:CofJ2-R</td>
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<td>CofJ2:L2:TcpA2-F</td>
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<td>TTG GGC AAC CAC CAC CAC TCG AGG GAG CGA TAG ATT TGA TCA CAC CAA CGC TGT TCT CA</td>
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<tr>
<td>CofJ2S2:L2:TcpA2-F</td>
<td>TGA AGG CTT GTG CTG GTA GTG GAG CTT TAG ATC TAA CGA ACA TCA CTC ACG TTG AG</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.2.2. Pilus inducing condition

*V. cholerae* cells were grown overnight on LB-Sm agar plates at 37 °C. Individual colonies were inoculated in 2 ml of lysogeny broth (LB) (Bioshop) and grown for 2 hours at 37 °C and normalized to an optical density measurement at 600 nm (OD$_{600}$) of 0.6. The cells were inoculated 1/100 in 2 mL LB (starting pH 6.5) and grown overnight at 30 °C with aeration.

2.2.3. Generation of complementation vector pJMA10.1

Plasmid pJMA10 is derived from pBAD22 (Guzman et al. 1995) which is an expression vector containing the $P_{BAD}$ promoter and its regulator *araC*. Expression of the vector insert can be modulated over a wide range of arabinose concentrations and had been used by the Taylor lab (Geisel School of Medicine) in complementation studies of deletion strains in *V. cholerae* (Kirn, Bose, and Taylor 2003). However, arabinose concentrations above 0.01% (w/v) can interfere with pilus-mediated aggregation phenotype in *V. cholerae* (Marles and Taylor, unpublished). The *araC* promoter in pBAD22 was excised and replaced with the $P_{rhaB}$ promoter from the genome of *E. coli* strain BL21 (New England Biolabs) to generate pJMA10, a rhamose-inducible promoter system based on the pBAD22 backbone. Rhamnose supplementation at various concentrations (0.001%, 0.01%, and 0.1% (w/v)) showed no negative impact on *V. cholerae* aggregation phenotype, making it a suitable inducer to test for gene complementation in deletion strains. Replacement of *araC* with *PrhaB* rendered the EcoRI restriction site in the multiple cloning site (MCS) unusable and the Shine-Dalgano sequence was aligned with the next available restriction site, Ncol, in the MCS. The Ncol cut site (CCATGG) was problematic because it introduced a start codon creating a frameshift mutation. Thus the Ncol restriction site was removed and the Kpnl restriction site was shifted up to be aligned with the Shine-Dalgano sequence. Primers pJMA10-NcoDEL-F-Nhel and pJMA10-NcoDel-R-Kpnl were used to PCR amplify a 1.7 kb fragment of pJMA10 upstream of the MCS that contains a unique Nhel restriction site using the Q5 DNA polymerase (New England Biolabs). The fragment downstream of the MCS was directly digested from the purified plasmid. These fragments were restriction
digested with NheI and KpnI (New England Biolabs) and ligated together using T4 DNA ligase (New England Biolabs) to generate the corrected plasmid pJMA10.1. The vector was verified by DNA sequencing (Genewiz) and transformed into storage strain *E. coli* DH5α. Complementation tests with *tcp* genes in their corresponding deletion strains using pJMA10.1 was successful in restoring wild type expression and functionality.

### 2.2.4. Construction of *ptcpA* and *ptcpB*

Vectors expressing the major pilin TcpA and the minor pilin TcpB were derived from the expression vector pJMA10.1, which contains an Ap<sup>R</sup> marker. Genes for TcpA and TcpB were PCR amplified with the Q5 DNA polymerase from *V. cholerae* wild type strain O395 genomic DNA using primers TcpA-F-KpnI/-R-XbaI and TcpB-F-KpnI/-R-XbaI. PCR products were purified, digested, and ligated into pJMA10.1 at the KpnI/XbaI restriction sites using T4 DNA ligase. All constructs were verified by DNA sequencing and transformed into *V. cholerae* strains for complementation testing. Pilin expression was induced using rhamnose at indicated concentrations.

### 2.2.5. Generating minor pilin Glu5 mutants

The conserved Glu5 residue in the minor pilin TcpB was changed to Asp, Gln, and Val on *ptcpB* to generate *ptcpB<sub>E5D</sub>*, *ptcpB<sub>E5Q</sub>*, and *ptcpB<sub>E5V</sub>* respectively. Forward primers VC-tcpB(E5D)-F-KpnI, VC-tcpB(E5Q)-F-KpnI, and VC-tcpB(E5V)-F-KpnI were used individually with reverse primer TcpB-R-XbaI to PCR amplify the *tcpB* gene fragment from *ptcpB* containing the corresponding amino acid substitutions E5D, E5Q, and E5V. PCR products were purified and ligated into pJMA10.1 at the KpnI/XbaI restriction sites using T4 DNA ligase. All constructs were verified by DNA sequencing. Plasmids were transformed into *V. cholerae* ∆*tcpB* and cells were grown on LB-Sm/Ap plates.

### 2.2.6. Construction of pilin-mimic constructs

Vectors expressing truncated minor pilin TcpB were derived from the *ptcpB* plasmid. Genes for *tcpB*(1-142), *tcpB*(1-217), *tcpB*(1-228), and *tcpB*(1-238) were PCR
amplified with the Q5 DNA polymerase with forward primer TcpB-F-KpnI and reverse primers TcpB-R-142-Xbal, TcpB-R-217-Xbal, TcpB-R-228-Xbal, or TcpB-R-238-Xbal. PCR products were purified and ligated into pJMA10.1 at the KpnI/XbaI restriction sites using T4 DNA ligase. All constructs were verified by DNA sequencing. Plasmids were transformed into \( V. \text{cholerae} \ \Delta tcpB \) and cells were grown on LB-Sm/Ap plates.

Constructs expressing the hybrid pilin TcpA:CofJ were derived from the \( ptcpA \) plasmid. Primers L1:CofJ2-F and CofJ2:L2-R were used to PCR amplify a portion of the \( cofJ \) gene fragment (encoding residues 28 – 314) from ETEC 31-10 genomic DNA using the Q5 DNA polymerase; the primers also encode linkers for the fragment at the 5’ end (SSGG) and the 3’ end (GSGA). This fragment was subcloned into \( tcpA \) in a region that encodes for a surface exposed loop, either between residue 139 – 147 (s1) or 169 – 174 (s2), using an overlap extension PCR approach (Heckman and Pease 2007). These constructs were completed with help from G. Yang and S. Luongo. All constructs were verified by DNA sequencing. Plasmids were transformed into \( V. \text{cholerae} \ \Delta tcpB \) strain and cells were grown on LB-Sm/Ap plates.

### 2.2.7. Autoagglutination assay

\( V. \text{cholerae} \) cells were grown under pilus inducing conditions in 2 mL LB (starting pH 6.5) and visually inspected for the aggregation phenotype after allowing the cultures to stand at RT for 15 minutes. Scoring is done by inspecting clarity of the culture supernatant and the size of the bacterial aggregates at the bottom of the culture tube. \( V. \text{cholerae} \) wild type strain O395 is used as a positive control and its aggregation phenotype is scored as ++++. \( V. \text{cholerae} \ \Delta tcpA \) strain is used as negative control and its phenotype is scored as a –.

### 2.2.8. Pilus isolation and immunoblotting of TcpA and TcpB

\( V. \text{cholerae} \) cells were grown overnight under pilus-inducing conditions in 2 mL LB (starting pH 6.5) and let stand at RT for 15 minutes. Pilus function is first assayed by visual inspection for autoagglutination. Cells were resuspended by vortexing for 30 seconds and surface-displayed pili were sheared from the cells using an IKA® ULTRA-
TURRAX® T8.01 disperser (IKA-Werke) at maximum setting for 20 seconds (Lim et al. 2010). This whole cell culture (WCC) fraction was used to assess total protein levels. Cell debris was removed by centrifugation at 13,000 x g for 30 minutes at RT using a bench top microfuge. The culture supernatant containing the sheared/shed pili (SSUP) was separated by pipette and 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 prior to loading in 15% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane at 4 °C in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) with a wet transfer apparatus (Bio-Rad). The membrane was blocked for 1 hour at RT with BLOTTO (5% (w/v) nonfat dried milk in Tris-buffered saline with 0.1% Tween (TBST)). Proteins were detected with rabbit polyclonal antisera raised against TcpA (peptide 174-199) (Sun et al. 1991) and TcpB (peptide 64-78). 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 TcpB in the SSUP fraction where the SuperSignal West Femto chemiluminescent substrate (Fisher Scientific) was used. Blot images were digitized using the FujiFilm LAS 4000 imager (FujiFilm). Major and minor pilin expression levels were assayed by immunoblot of the WCC fraction. Pilus assembly was assayed by comparative analysis of the TcpA band in the SSUP fraction using ImageJ (Schneider, Rasband, and Eliceiri 2012) to determine the density of the protein bands. V. cholerae O395 wild type strain is used as the comparative standard at 100% for TCP assembly.

2.2.9. TcpF secretion assay

V. cholerae cells were grown overnight under pilus-inducing conditions in 2 mL LB (starting pH 6.5). Cells were centrifuged at 3000 x g for 10 minutes at RT and the 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% hand-cast 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 WCC and the filtered supernatant (FSUP) 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). Immunoblots were digitized using the FujiFilm LAS4000 imager. The presence of TcpF in the FSUP fraction indicates secretion by the TCP assembly apparatus.

2.2.10. Immunogold labelling and transmission electron microscopy analysis

For negative-stained transmission electron microscopy (TEM) analysis, 20 µL of whole cell culture were applied to formvar carbon-coated grids (Electron Microscopy Sciences) or grids were placed directly onto cells grown on LB agar plates. Samples were washed with PBS and stained with 3% uranyl acetate. For immunogold labelling TEM analysis, samples were applied to formvar carbon-coated grids and fixed for 1 hour at RT by placing the grid in a 15 µL drop of fixative (2% paraformaldehyde, 0.5% glutaraldehyde, in 50 mM sodium cacodylate, pH 7.4). The grid was washed with Tris-buffered saline with 0.1% Tween (TSBT) and blocked for 1 hour in TBST with 1% bovine serine albumin (BSA) (Bioshop). The sample was then incubated with primary antibody (1:50 dilution in TBST 1% BSA), washed in TBST, and then incubated for 30 minutes with the gold-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) at 1:60 dilution. A final wash in TBST and the samples were stained with 3% uranyl acetate. Proteins were probed with rabbit polyclonal antisera raised against TcpA (peptide 174-199) (Sun et al. 1991) and TcpB (peptides 286-299). Goat-anti-rabbit secondary antibodies conjugated to either 12 or 18 nm colloidal gold (Jackson ImmunoResearch) were used to bind the primary antibody. All samples were imaged on a Hitachi 8100 TEM at accelerating voltage of 200 kEV.

2.2.11. Expression and purification of ∆N-TcpA and ∆N-TcpB for quantification of pilin stoichiometry

Expression and purification of ∆N-TcpA displaying an N-terminal hexahistidine tag (6His) and linker was described elsewhere (Craig et al. 2003). Purified ∆N-TcpB was kindly provided by S. Kolappan. A gene fragment encoding ∆N-TcpB (residues 25-423)
was PCR amplified from *V. cholerae* wild type strain O395 and cloned into expression vector pet15b (Novagen). The construct was transformed into Shuffle® T7 Express lysY competent *E. coli* cells (New England Biolabs) by heat shock. Cells were grown in LB supplemented with ampicillin to OD$_{600}$ of 0.1 in shaking incubator at 250 rpm at 37 °C. Protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration 0.1 mM) and the temperature was reduced to 14 °C; cells were grown for a further 20 hours and harvested by centrifugation at 5000 x g for 30 minutes at 4 °C. The cell pellet was resuspended, cells were lysed by sonication, and the cell debris was removed by centrifugation at 40,000 x g for 1 hour at 4 °C. Purification was carried out by loading the filtered cell lysate onto a Ni-NTA column (Qiagen, Bio-Rad), washed and eluted at 250 mM imidazole. The N-terminal His-tag was cleaved off of the bound TcpB by thrombin digestion (GE Healthcare) and eluted. The protein was further purified by size exclusion chromatography using a Sephacryl S-100 column (GE Healthcare) and concentrated to 10 mg ml$^{-1}$ using a stirred cell concentrator (Millipore). Purity was assayed by SDS-PAGE analysis and estimated to be >95%.

2.2.12. **Quantification of total TcpA and TcpB stoichiometry in whole cell culture**

ΔN-TcpA and ΔN-TcpB concentrations were determined by UV absorbance. Known amounts of each protein were loaded onto a 15% hand-cast SDS-PAGE gel. Proteins were transferred onto PVDF membrane for immunoblotting. The corresponding bands on the immunoblots were scanned with a FujiFilm LAS4000 imager and analyzed using ImageJ. The density of each band was plotted against its amount and the plot was used to extrapolate the amount of native TcpA or TcpB present in the whole cell lysate of *V. cholerae* wild type strain O395.
2.3. Results

2.3.1. *V. cholerae* TcpB is a pilin-like protein

TcpB is encoded on the *tcp* operon immediately downstream of the *tcpA* gene, which encodes for the major pilin subunit, TcpA. These are the only two pilin-like protein encoded on this operon. TcpB is predicted to have a 7-residue signal peptide, which is considerably shorter than the 25-residue TcpA signal peptide, and a 423 amino acid mature protein (46.1 kDa), which is much larger than the 199-residue TcpA. TcpB shares amino acid homology with TcpA in the N-terminal polymerization domain, including the conserved Glu5, but differs from TcpA in the C-terminal region (Figure 2-1). Importantly, *tcpA* gene has a putative rho-independent transcription termination site at its 3’ end (Brown and Taylor 1995), suggesting that it is transcribed at much higher levels than *tcpB* and the other genes within the *tcp* operon.
Figure 2-1  Comparison of the *V. cholerae* minor pilin gene and gene product, TcpB with that of the major pilin, TcpA

(A) *tcp* operon with the single pilin-like protein gene colored red. (B) Amino acid sequence alignment between the signal peptide and N-terminal 30 residues of TcpB and TcpA. (C) Schematic of TcpB and TcpA pre-proteins.
2.3.2. Pilus assembly is disrupted in *V. cholerae* O395 ∆tcpB strain

It was previously reported that a *V. cholerae* ∆tcpB strain was deficient in pilus assembly (Kirn, Bose, and Taylor 2003) but a more detailed analysis reveals that this strain can assemble TCP but at much lower levels than the WT O395 strain. To quantify the pilus levels in *V. cholerae* ∆tcpB relative to the WT strain, overnight cell cultures were homogenized to mechanically shear the pili off the surface of the cell (Lim et al. 2010). Cell debris was removed by centrifugation and the sheared pilus fraction, which includes the culture supernatant, was analyzed by SDS-PAGE and immunoblotting (Figure 2-2, 2-3). Total TcpA levels in the whole cell cultures (WCC) are comparable for the WT and the ∆tcpB strains, whereas the sheared pilus fraction (Sup) has reduced TcpA levels for the ∆tcpB strain, consistent with our electron microscopy observations. TCP production is increased to closer to WT levels when tcpB is expressed on a plasmid. Shearing pili from the cells introduces TcpA contamination from *V. cholerae* membranes, as shown in the ∆tcpC strain (Figure 2-3), which lacks the outer membrane secretin channel and cannot assembly pili (Kirn, Bose, and Taylor 2003). Nonetheless, the results of the shearing assay are consistent with those observed by TEM and serve as a crude method for quantifying pilus assembly. The requirement for TcpB in establishing WT levels of TCP production suggest a role for this minor pilin in initiating pilus assembly.
**Figure 2-2**  Expression of minor pilin TcpB impacts TCP production and TCP-mediated functions

Expression of the minor pilin in the wild type strain O395 (WT) and in the minor pilin deletion strain (ΔtcpB). The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB, the major pilin TcpA, and the secreted colonization factor TcpF in whole cell cultures (WCC), representing total protein, and in the culture supernatant (Sup). In the case of TcpB and TcpA, the culture supernatant is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. In the case of TcpF, which is secreted by the TCP apparatus, the unhomogenized cells are removed from the whole cell culture by centrifugation and the culture supernatant is further filtered to remove residual cells. TcpB expression in cells transformed with ptcpB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. The amount of TcpA in bands in the culture supernatant was quantified by densitometry using ImageJ (Schneider, Rasband, and Eliceiri 2012). Autoagglutination (AA) phenotype is indicated in red. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.
2.3.3. Low level but not high level expression of tcpB restores pilus functions

A V. cholerae ΔtcpB mutant is deficient in TCP-mediated functions, including autoagglutination and secretion of the colonization factor TcpF (Kirn, Bose, and Taylor 2003). We confirm here that TcpB is required for efficient TcpF secretion and autoagglutination as both functions are abrogated in the ΔtcpB strain (Figure 2-3). We further show that TcpF secretion and autoagglutination are restored in the ΔtcpB mutant when TcpB is expressed ectopically. However, the TcpB expression level is critical to pilus functions: low levels of expression using 0.001% rhamnose (w/v) for induction restores TcpF secretion (Figure 2-3A) and autoagglutination (Figure 2-3B) to almost WT levels, but higher rhamnose concentrations disrupt these functions in a dose-dependent manner. Notwithstanding TCP assembly levels are partially recovered with increasing expression of TcpB, as indicated by the immunoblots of TcpA in the sheared culture supernatant (Figure 2-3A) and by TEM. These results are mirrored in V. cholerae WT strain O395 overexpressing TcpB ectopically (Figure 2-2). Even though TCP assembly levels are not affected, increasing expression of TcpB negatively impacts TcpF secretion and autoagglutination. These results suggest that a precise TcpA:TcpB stoichiometry is required for optimal pilus functions.
Figure 2-3  TCP functions in a ∆tcpB strain are rescued by low but not high levels of ectopic TcpB expression.
(Figure-2-3 figure legend cont’d from previous page)

(A) Expression of the minor pilin in a ΔtcpB strain. The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB, the major pilin TcpA, and the secreted colonization factor TcpF in whole cell cultures, representing total protein, and in the culture supernatant (Sup). In the case of TcpB and TcpA, the culture supernatant is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. In the case of TcpF, which is secreted by the TCP apparatus, the unhomogenized cells are removed from the whole cell culture by centrifugation and the culture supernatant is further filtered to remove residual cells. TcpB expression in cells transformed with ptcpB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. The amount of TcpA in bands in the culture supernatant was quantified by densitometry using ImageJ (Schneider, Rasband, and Eliceiri 2012). (B) Autoagglutination of V. cholerae strains. Cell cultures are shown after 12 hours of growth on a ferris wheel rotator at 30 °C followed by 15 minutes of stationary incubation at room temperature. A qualitative measurement is indicated in on the top right corner of each panel. For (++) autoagglutination, as seen in WT V. cholerae, the cells form macroscopic aggregates that fall to the bottom of the tube leaving the supernatant almost clear. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.
2.3.4. *V. cholerae* TcpB is a low abundance pilin

To determine the relative amounts of the major pilin, TcpA, and the minor pilin, TcpB, we quantified their expression levels in *V. cholerae* wild type strain O395 by SDS-PAGE and immunoblotting. As antibodies to TcpA and TcpB likely differ in their respective antigen affinities, we quantified the total TcpA and TcpB levels in whole cell cultures by comparing the densities of their respective immunoblot bands with those of known amounts of purified recombinant N-terminally truncated TcpA and TcpB, respectively (Figure 2-4). Known amounts of ΔN-TcpA and ΔN-TcpB were subjected to SDS-PAGE and immunoblotting with their respective antibodies and the resulting protein bands were analyzed by densitometry and plotted using the image processing program ImageJ (Schneider, Rasband, and Eliceiri 2012). The plots were extrapolated to determine the amounts of native TcpA and TcpB in immunoblots of O395 whole cell culture. TcpA bands were detected with the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) whereas the less abundant TcpB was detected with the more sensitive Femto chemiluminescent substrate. The molar ratio of the major:minor (TcpA:TcpB) pilins in whole cell culture is ~800:1, confirming that TcpB is indeed a minor pilin of the TCP system.

**Figure 2-4**  Quantification of TcpA and TcpB in *V. cholerae* whole cell culture

Specified amounts of recombinant N-terminally truncated TcpA (ΔN-TcpA) and TcpB (ΔN-TcpB), both having a hexahistidine tag plus a linker at their N-terminus, were analyzed by SDS-PAGE and immunoblotting using antibodies against TcpA and TcpB, respectively. The TcpA blot was detected using the SuperSignal West Pico chemiluminescent substrate whereas the TcpB blot was detected using the more sensitive Femto substrate. The amount of protein in each band was quantified by densitometry using ImageJ (Schneider, Rasband, and Eliceiri 2012). Plots of band density versus protein amounts were used to determine the total amount of native TcpA and TcpB in *V. cholerae* O395 whole cell culture (WCC). The molar ratio of TcpA:TcpB is ~800:1.
2.3.5. TcpB is incorporated at low levels into surface-displayed pilus

TcpB is readily detected in the whole cell culture of the *V. cholerae* WT strain by immunoblotting but is barely detectable in the sheared pilus fraction even when the more sensitive Femto chemiluminescence substrate is used (Figure 2-5). Some of the TcpB detected in this fraction may result from membrane contamination as a faint band is also observed in the ΔtcpC mutant that does not assemble pili (Figure 2-5). A more intense TcpB band is observed in a *V. cholerae* strain with a mutation encoding a His181Ala change in the major pilin, TcpA, and over-produces TCP due to ectopic expression of ToxT (Kirm et al. 2000; Li, Egelman, and Craig 2012). Thus, the levels of TcpB in the WCC correlates with the levels of TCP. To directly demonstrate incorporation of TcpB in the pilus filament, we performed immunogold TEM of *V. cholerae* O395 culture using anti-TcpB antibodies. Many of the TCP bundles observed by TEM had one or more gold clusters associated (Figure 2-6A, B & C), indicating low levels of TcpB incorporation into the pili. Labeling of the pilus bundles from the ΔtcpB mutant was rarely observed. Importantly, TcpB localizes at the pilus tips in some of the filaments from WT *V. cholerae*, consistent with its function as an initiator of pilus assembly.

Interestingly, TcpB appears in high levels in the culture supernatant when overexpressed (Figure 2-2 and 2-3A). Immunogold TEM of *V. cholerae* overexpressing TcpB using anti-TcpB antibodies revealed thick densely labelled filaments that are distinct from the pilus bundles (Figure 2D, E & F). Two types of filaments were present: irregular filaments that may be encased in a membrane and have a diameter of ~30 nm, and filaments with a diameter of ~65 nm comprised well-delineated TcpB subunits. Since these filaments are substantially thicker than TCP filaments, which are ~9 nm in diameter, we wondered if they were exported via a different mechanism than the TCP assembly apparatus, possibly by cell lysis. However, export of TcpB to the pilus fraction requires functional pilus assembly as a ΔtcpA mutant is incapable of exporting TcpB (Figure 2-7). While the TcpB filaments are clearly artifacts of overexpressing a protein that is normally present in the bacteria at very low levels, they indicate that TcpB is able to polymerize, most likely via its N-terminal segment, which is homologous to the TcpA polymerization domain (Craig et al. 2003; Li, Egelman, and Craig 2012).
Figure 2-5  TcpB is incorporated into the pilus

TcpB is detected in the sheared pilus fraction. The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB and the major pilin TcpA in whole cell cultures, representing total protein, and in the culture supernatant (Sup), which is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. TcpB expression in cells transformed with ptcpB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. Immunoblots of V. cholerae strains showing low levels of TcpB in the sheared pilus fraction (Sup) when TcpB is detected with a sensitive SuperSignal West Femto chemiluminescent substrate. TcpB levels in this fraction increase in V. cholerae strain RT4225 that makes high levels of pili (H181A). Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.
Figure 2-6  Immunogold transmission electron microscopy showing TcpB incorporation

(A) Immunogold transmission electron microscopy (TEM) images of *V. cholerae* wild type strain O395 probed with anti-TcpB primary antibody and 12 nm colloidal gold secondary antibody. Gold clusters associated with pilus bundles are indicated by red arrows. Samples were stained with 3% uranyl acetate and imaged on a Hitachi 8100 TEM at 200 kEV.
(Figure 2-6 cont’d from previous page)

(B, C) Immunogold TEM images of *V. cholerae* wild type strain O395 probed with anti-TcpB primary antibody and 12 nm colloidal gold secondary antibody. Gold clusters associated with pilus bundles are indicated by red arrows. Samples were stained with 3% uranyl acetate and imaged on a Hitachi 8100 TEM at 200 kEV. (D, E, F) Immunogold TEM images of *V. cholerae* wild type strain O395 overexpressing TcpB showing TcpB filaments alongside TCP. The 65-nm filament appears to be comprised of TcpB whereas the 30-nm filaments may be membrane bound.
**Figure 2-7**  TcpB is exported only when TCP is assembled

Immunoblot comparing TcpB export when the minor pilin is overexpressed in ΔtcpA and ΔtcpB strains. The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB and the major pilin TcpA, in whole cell cultures, representing total protein, and in the culture supernatant (Sup), which is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. TcpB expression in cells transformed with ptcpB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. *V. cholerae* TCP biogenesis mutant (ΔtcpA) overexpressing TcpB show that TcpB is only present in significant amount in the sheared culture supernatant when pili are assembled. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.
2.3.6. **V. cholerae TCP are retractile**

We have proposed that *V. cholerae* TCP functions in autoagglutination, TcpF secretion, and CTXφ phage uptake are likely dependent on both pilus extension and retraction (Ford et al. 2012). TCP retraction allows cells to stay tightly bound in protective aggregates or microcolonies (Lim et al. 2010); rapid pilus extension and retraction can facilitate TcpF secretion in a piston-like manner (Korotkov, Sandkvist, and Hol 2012); and CTXφ must be retracted into the periplasm to co-localize with TolA for function (Ford et al. 2012). Since *V. cholerae* ΔtcpB produces some pili but is impaired in autoagglutination and TcpF secretion, we considered that TcpB might have a direct role in mediating pilus retraction. We worked with Dr. Nicolas Biais (Brooklyn College, CUNY) to first determine whether wild type *V. cholerae* TCP can retract using a micropillar displacement assay which has been used to quantify pilus retraction in *N. gonorrhoeae* (Biais et al. 2008). The assay is based on an array of evenly spaced micropillars seeded with cells (Figure 2-8). These micropillars are fabricated from elastic hydrogel and are sensitive to attachment by pilus filaments, which causes them to displace. This displacement is correlated with the force exerted by the pilus and can be used to detect pilus retraction. Working with *V. cholerae* wild type strain O395 and a flagella minus strain, Dr. Biais was able to demonstrate TCP-mediated retraction, which was absent in the *V. cholerae* ΔtcpB strain. These results establish TCP to be retractile, despite the lack of a retraction ATPase. Furthermore, retraction appears to be mediated by TcpB, but the lack of retraction in the ΔtcpB mutant could also be an indirect consequence of the reduced pilus expression levels in this strain.
Figure 2-8  Micropillar displacement assay demonstrates TCP retraction

(A) Schematic of a micropillar displacement assay. Cells are seeded on top of the pillars and nonspecific attachment by TCP can be observed by the displacements of the pillars. Displacements (d) are recorded by video and can be correlated with the force generated by the pilus during attachment to the pillar. Micropillars are fabricated from elastic hydrogels which can be varied in stiffness, allowing a precise range of force measurements. (B) Time-lapse of *V. cholerae* wild type strain O395 in a micropillar assay. The images indicate micropillar displacements over time, note that the pillars are pulled closer together at 160 sec and pushed apart again at 320 sec. This type of displacement is not observed in a *V. cholerae* ΔtcpB strain.

2.3.7. TCP assembly but not functions require TcpB-Glu5

The highly conserved Glu5 found in all Type IV pilins is required for efficient TCP assembly in *V. cholerae* (Li, Egelman, and Craig 2012). TcpB has a glutamate at position 5, as do most minor pilins and minor pseudopilins, with the exception of the GspK family members. The ETEC Type II secretion minor pseudopilin GspK is much larger than the other minor pseudopilins and the major pseudopilin GspG, and is thought to form the tip of the pseudopilus (Korotkov and Hol 2008; Yanez et al. 2008b). This first subunit in the (pseudo)pilus filament would not require Glu5 as there is no preceding
subunit with a N1+ to neutralize, but every new subunit that is added to a growing (pseudo)pilus would require Glu5 to complete that charge complementarity interaction with the preceding subunit (Figure 1-6). Our data suggest that TcpB both initiates pilus assembly, as a \( \Delta tcpB \) strain assembles less pili than wild type (Figure 2-2), and induces pilus retraction, possibly by incorporating randomly into the growing pilus filament. Therefore, we hypothesized that TcpB initiation of pilus assembly would not require Glu5 but inducing retraction would. To test this hypothesis, we introduced several different Glu5 substitutions into the plasmid-encoded \( tcpB \) gene and tested the ability of these mutants to rescue pilus assembly and functions in a \( \Delta tcpB \) mutant. Pilus assembly was restored to wild type levels with \( tcpB \) expression for each of the TcpB-Glu5 variants. In contrast to wild type TcpB overexpression, the levels of TcpB in the sheared pilus fraction of TcpB-Glu5 variants are lower, suggesting that they cannot be exported out of the cell as TcpB filaments. Although TCP assembly was restored for each of the TcpB-Glu5 variants (Figure 2-9), TcpF secretion and autoagglutination were both impaired. The degree of functional impairment depended on the Glu5 substitution: the most conserved change, to the negatively charged aspartate (E5D) showed a slight reduction in TcpF secretion compared to the WT strain, and only very low levels of autoagglutination; the Glu5Gln (E5Q) substitution, which results in loss of the negative charge but retains polarity, shows poor TcpF secretion and autoagglutination; and the Glu5Val results in a hydrophobic side chain, was unable to rescue either TcpF secretion or autoagglutination (Figure 2-9). Thus, Glu5 is essential for pilus functions but not pilus assembly.

We analyzed a \( V.\ cholerae \) TcpB\(_{E5V} \) strain to verify whether the functional defects in a TcpB-Glu5 change are a result of non-retractile TCP. Like the \( V.\ cholerae \) \( \Delta tcpB \) mutant expressing TcpB-Glu5Val ectopically, this strain does not autoagglutinate nor secrete TcpF. Preliminary data based from micropillar displacement assays show that TCP retraction is significantly impaired in \( V.\ cholerae \) TcpB\(_{E5V} \). These results are consistent with our hypothesis that TcpB is critical for efficient retraction and that the invariant Glu5 is central to this process, yet not required for initiation of pilus assembly.
Figure 2-9  TcpB-Glu5 is necessary for pilus functions but not for assembly

(A) Immunoblots of *V. cholerae* ΔtcpB complemented with WT tcpB gene of TcpB-Glu5 mutants. Pilus assembly is restored with WT pTCPB and with each of the mutants whereas pilus functions are impaired in the mutants. Autoagglutination phenotypes (AA) are indicated in red. The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB, the major pilin TcpA, and the secreted colonization factor TcpF in whole cell cultures, representing total protein, and in the culture supernatant (Sup). In the case of TcpB and TcpA, the culture supernatant is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. In the case of TcpF, which is secreted by the TCP apparatus, the unhomogenized cells are removed from the whole cell culture by centrifugation and the culture supernatant is further filtered to remove residual cells. TcpB expression in cells transformed with pTCPB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. Protein levels can be
compared across each panel which represents a single blot; while vertical lines delineate strains. Autoagglutination (AA) phenotype is indicated in red. (B) Immunogold TEM images of V. cholerae $\Delta tcpB$ strain expressing TcpB$_{ESV}$ show similar TCP levels to V. cholerae wild type strain O395. TCP bundles are indicated on images. TcpB$_{ESV}$ expression in cells transformed with pltcpB is induced with rhamnose (w/v). Samples were stained with 3% uranyl acetate and imaged on a Hitachi 8100 TEM at 200 kEV.

### 2.3.8. Pilin mimics cannot restore pilus functions

One significant difference between the major and minor pilins is their relative size. The minor pilin TcpB is 244 residues larger than the major pilin TcpA. To determine if this size difference contributes to the functional characteristic of each pilin, we generated two types of ‘pilin mimic’ constructs: major pilins with a conjugated globular domain to mimic the larger minor pilin, and minor pilins with truncated C-terminal domain to mimic the smaller major pilin. Atomic resolution crystal structure are available for the major pilin TcpA (Craig et al. 2003) and the CFA/III exoprotein CofJ (Yuen et al. 2013). We selected CofJ to be inserted into a surface-displayed loop in TcpA because it is comparable in size to the predicted C-terminal domain of TcpB, and it is ideal for insertion within another protein because its N- and C-termini are brought together by a disulfide bond. The TcpA:CofJ constructs are expressed and detected in the whole cell fraction but not the sheared pilus fraction, and neither TCP assembly nor TCP-mediated functions are restored (Figure 2-10). This suggests that the hybrid pilins cannot initiate TCP assembly or facilitate TcpF secretion and autoagglutination.

Overexpression of TcpB in either WT or $\Delta tcpB$ strains resulted in proteolysis of TcpB, producing a ~27 kDa fragment (Figure 2-12). This fragment represents an N-terminal fragment as it was detected with a TcpB antibody against an N-terminal peptide (residue 64-78) but not a C-terminal peptide (residues 258-272). This result suggested that the proteolytically resistant N-terminal fragment might represent the N-terminal pilin domain (residues 1 to ~230). Using secondary structure prediction as a guide we introduced stop codons into the tcpB gene in pltcpB to generate truncated forms of the minor pilin: TcpB(1-142), TcpB(1-217), TcpB(1-228), and TcpB(1-238). We tested the ability of these mutated plasmids to rescue pilus assembly and functions in the $\Delta tcpB$ strain. Based on immunoblotting, both TcpB(1-142) and TcpB(1-217) were not expressed presumably because these pilins were truncated within the pilin domain and
consequently did not fold correctly (not shown). The other two constructs were expressed stably, with TcpB(1-228) expressing at WT level and TcpB(1-238) at much lower level. The slightly larger TcpB(1-238) expressed in the \( \Delta tcpB \) strain neither restored pilus assembly nor TCP-mediated functions (Figure 2-10B).

Interestingly, expression of TcpB(1-228) in the \( \Delta tcpB \) strain increased TcpA levels in the sheared pilus fraction and the truncated pilin is also detected in this fraction at the highest expression level (Figure 2-11). However, no pili are observed by TEM when TcpB(1-228) is expressed at low levels and only small but well defined pilus bundles are observed when expressed at high levels. We speculate these are filaments predominantly comprised of truncated TcpB pilins, an artifact that is also observed when the full length minor pilin is overexpressed at high levels. We were unable to confirm the composition of these filaments by immunogold TEM as our N-terminally-directed antibody does not detect the fully folded protein. Nonetheless, TcpF secretion and autoagglutination are not restored by this construct, suggesting that the N-terminal pilin domain itself cannot initiate pilus assembly nor mediate pilus functions.
Figure 2-10  Minor pilin mimics do not restore TCP assembly or functions

Immunoblots of *V. cholerae* ΔtcpB strain complemented with minor pilin mimic constructs. Constructs TcpA:CofJ(s1) (site 1, residues 139-147) and (s2) (site 2, residues 169-174) do not restore pilus assembly nor pilus functions. The lower panel are schematics comparing the constructs to the major pilin TcpA and the minor pilin TcpB. The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB, the major pilin TcpA, and the secreted colonization factor TcpF in whole cell cultures, representing total protein, and in the culture supernatant (Sup). In the case of TcpB and TcpA, the culture supernatant is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. In the case of TcpF, which is secreted by the TCP apparatus, the unhomogenized cells are removed from the whole cell culture by centrifugation and the culture supernatant is further filtered to remove residual cells. TcpB expression in cells transformed with ptcpB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains. Autoagglutination (AA) phenotype is indicated in red.
Figure 2-11   Major pilin mimics do not restore TCP assembly or functions

The TcpB C-terminal domain is necessary for pilus assembly. Truncated minor pilin constructs (major pilin mimic constructs) TcpB(1-228) and TcpB(1-238) are shown. Neither constructs restored TCP functions and Tcp(1-238) did not restore assembly to WT levels. The lower panel are schematics comparing the constructs to the major pilin TcpA and the minor pilin TcpB. Only TcpB(1-228) and TcpB(1-238) were expressed stably in the cell. By immunoblot, TcpB(1-228) appears to assemble TCP at wild type levels at low level expression and beyond wild type levels at high level expression. TEM analysis shows no pili in ΔtcpB mutant
strains expressing TcpB at low level expression. The indicated strains were grown in pilus inducing conditions. Immunoblots of the minor pilin TcpB, the major pilin TcpA, and the secreted colonization factor TcpF in whole cell cultures, representing total protein, and in the culture supernatant (Sup). In the case of TcpB and TcpA, the culture supernatant is prepared by shearing pili off the cells by homogenizing the whole cell culture, then removing intact cells by centrifugation. In the case of TcpF, which is secreted by the TCP apparatus, the unhomogenized cells are removed from the whole cell culture by centrifugation and the culture supernatant is further filtered to remove residual cells. TcpB expression in cells transformed with ptcpB is induced with rhamnose (w/v). The loading control is an unknown ~60 kDa protein detected with the secondary antibody. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains. Autoagglutination (AA) phenotype is indicated in red.
Figure 2-12  N-terminal domain pilin domain is resistant to proteolysis

Overexpression of the minor pilin TcpB in either wild type strain O395 or ΔtcpB strain resulted in proteolysis of TcpB, producing a ~27 kDa fragment (residues 1 to ~230). This fragment represents an N-terminal fragment as it was only detected by a TcpB antibody against an N-terminal peptide (residues 64-78) but not with a C-terminal peptide (residues 258-272).
2.4. Discussion

We show here that the minor pilin TcpB is involved in both pilus assembly and pilus functions. Previous analysis of *V. cholerae* ∆tcpB concluded that the minor pilin was required for pilus biogenesis as TCP was not detected by TEM analysis and the ∆tcpB strain was deficient in all TCP-mediated functions: TcpF secretion, autoagglutination, and phage transduction (Kirn, Bose, and Taylor 2003). We show here by electron microscopy and immunoblotting that the pilus assembly defect in ∆tcpB is not absolute, as some pili are observed in this strain but at very low levels, indicating that TcpB is involved in but is not essential for pilus biogenesis.

Optimal pilus functions can only be restored in a ∆tcpB strain by low level expression of TcpB on a plasmid. This is consistent with our quantification of the TcpA: TcpB stoichiometry in the whole cell lysate where we show that only 1 TcpB subunit is produced for 800 TcpA subunits. Both pilin subunits share amino acid sequence homology in their N-terminal ~30 residues (20% identity, 47% homology) and this suggests that TcpB is capable of incorporating into the growing pilus filament. Likewise, the multiple minor pilins in the *P. aeruginosa* T4P also share high-degree of N-terminus homology with their major pilin and were shown to be incorporated along the pilus filament by immunogold TEM (Giltner, Habash, and Burrows 2010). Indeed, we show by immunoblot and immunogold TEM that surface-displayed pili possess low levels of TcpB in *V. cholerae* wild type strain O395. TCP filaments are several microns in length and have an axial rise of 10.5 Å per TcpA subunit, or just under 1000 TcpA subunits per micron (Li, Egelman, and Craig 2012). If the TcpA: TcpB stoichiometry is maintained in the pilus filament as it is in the whole cell lysate, there should be approximately 1 TcpB subunit per micron length of a pilus, or a few TcpB molecules per filament. Even lower levels were observed by immunogold TEM imaging, with only a few gold clusters per pilus bundle, and in some cases no labeling at all. Since it seems unlikely that only some TCP would incorporate TcpB, it may be that many of the incorporated TcpB molecules are not detected due to inaccessibility of the antibody epitope in the pilus bundles or even in individual filaments.
TCP functions appear to require a dynamic filament capable of rapid assembly and disassembly. It is not clear how TcpB controls diverse TCP functions of TcpF secretion, autoagglutination, and phage uptake, particularly when this protein is present at such low levels. The reduced pilus assembly in addition to the abrogated functions in *V. cholerae ΔtcpB* suggest that TcpB may be involved in both pilus assembly and disassembly. One explanation is that the minor pilin initiates pilus assembly by forming the primary subunit in a growing pilus, and another minor pilin stalls pilus assembly to initiate retraction by its subsequent and random incorporation at the base of the assembling filament. (Figure 2-12) Thus, TcpB has a role in efficient initiation of pilus assembly and is absolutely required for pilus-mediated functions. This dual functionality is perhaps not surprising since TcpB is the only minor pilin in the *V. cholerae* TCP system, whereas most other T4P systems possess 4 or 5 minor pilins to accomplish the same tasks. Such a mechanism would restrict TcpB to the tip of surface-displayed TCP and is consistent with our observed TcpA:TcpB stoichiometry. The immunogold TEM imaging identified gold clusters throughout the pilus bundles, but it is not clear whether these associations were specific with the tips of individual filaments within the bundle. The multiple minor pilins in other T4P systems have been shown to be required for pilus biogenesis (Alm and Mattick 1995; Carbonnelle et al. 2006; Russell and Darzins 1994). The minor pseudopilins in the related T2S system found in enterotoxigenic *E. coli* form a complex (Korotkov and Hol 2008) that is hypothesized to initiate pseudopilus assembly as a ternary cap that interacts with the major pseudopilin (Yanez et al. 2008a, 2008b). In the *Klebsiella oxytoca* T2S system, minor pseudopilins are essential for initiating pseudopilus assembly (Cisneros et al. 2012). Heterologous expression of the major pseudopilin in an *E. coli* K-12 strain capable of assembling T4P shows that pseudopilus assembly can be initiated by the cognate type IV minor pilins, suggesting functional parallels between the minor (pseudo)pilins found in the T4P and the T2S systems. Hence, it is reasonable to hypothesize that TcpB is the priming subunit for TCP assembly.

Although the atomic structure of TcpB has not been determined, structures are available for a number of other minor pilins and pseudopilins (Helaine et al. 2007; Korotkov and Hol 2008; Nguyen et al. 2015; Yanez et al. 2008a, 2008b). All of the structures reveal canonical pilin globular domains, with an α-helical spine embedded in a
β-sheet (reviewed in Giltner/Nguyen/Burrows, 2012). Though the N-terminal ~28 residue segment is absent in each of these structures, it is predicted by sequence homology to form the protruding half of the N-terminal α-helix, as it does in the full length structures of the major pilins from *N. gonorrhoeae*, *P. aeruginosa*, and *Dichelobacter nodosus* (Parge et al. 1995; Craig et al. 2003; Craig et al. 2006; Hartung et al. 2011). Thus, the minor pilins are expected to incorporate into the growing pilus in addition forming a cluster that caps the pilus. While most of these minor pilins are comparable in size to their corresponding major pilins, GspK, which forms the tip of the ETEC T2S minor pilin cap, is considerably larger and has a second 1860-residue α-helical domain protruding from its pilin domain (Korotkov and Hol 2008). This minor pilin also lacks glutamate at residue 5. GspK belongs to a larger GspK family, members of which are present in most T2S and T4P systems. *V. cholerae* TcpB, like ETEC GspK, is much larger than its corresponding major pilin, TcpA (Reyss and Pugsley 1990; Bleves et al. 1998). Whereas the lack of Glu5 may restrict GspK to the pseudopilus tip, the Glu5 on TcpB may allow it to be incorporated at the tip to initiate pilus assembly and within the growing filament to initiate retraction. Our results with the TcpB-Glu5 variants is in agreement with this hypothesis. We show here that each of the TcpB-Glu5 variants can initiate and restore pilus assembly in *V. cholerae* ∆tcpB, yet pilus-mediated functions remain impaired. The most conserved change to the negatively charged aspartate (E5D) resulted in a decrease in TcpF secretion and very poor autoagglutination; the change to the neutral but polar glutamine (E5Q) did not restore TcpF secretion and only minimal autoagglutination was observed; and the change to the hydrophobic valine (E5V) was unable to rescue TcpF secretion or autoagglutination. These TcpB-Glu5 variants are also detected at lower levels in the sheared pilus fraction than the wild type pilin, suggesting that they are primarily exported out of the cell as a tip-associated component. Furthermore, preliminary data from a *V. cholerae* strain expressing TcpB$_{E_{5V}}$ shows a significant reduction in retraction to corroborate our hypothesis. The TcpB-Glu5 variants demonstrate the minor pilins need the N1+ and Glu5 charge complementarity interaction to exert their functional role, suggesting that they can incorporate at the base of the filament. It remains unclear whether the minor pilin incorporates and is extruded along an assembling filament to induce retraction, or simply incorporates at the base of a growing filament to induce retraction without extrusion.
In conclusion, we show here that the single minor pilin of the *V. cholerae* TCP system, TcpB, is involved in both pilus assembly and pilus retraction. This protein is present at much lower levels than that of the major pilin TcpA and this stoichiometric balance between the two is critical for optimal pilus functions. A crystal structure and further characterization of TcpB will allow us to test our mechanistic model for TcpB function, which have important implications for other more complex T4P systems and related T2S system.

**Figure 2-13**  TcpB initiates assembly and retraction of TCP

Model illustrating the dual functionality of the *V. cholerae* minor pilin TcpB. (A) The minor pilin is required to initiate pilus assembly, acting as a cap for the assembling filament. (B) During assembly, the major pilin is incorporated more often than the minor pilin, given their 800:1 stoichiometric ratio, as denoted by the larger red arrow. The minor pilin – because of its N-terminal homology and conserved Glu5 residue is also able to incorporate at the filament base. This incorporation stalls assembly and prevents other subunits from being added. This leads to a spontaneous disassembly at the base of the filament and results in the pilus ‘retracting’ back into the cells and the pilins diffusing back within the inner membrane.
Chapter 3. Minor pilin CofB, the initiator of CFA/III pilus assembly in enterotoxigenic Escherichia coli

Adapted and expanded from: Subramania Kolappan*, Dixon Ng*, Guixiang Yang, Tony Harn, and Lisa Craig. 2015, manuscript submitted.

*Equal contributions

Contributions to research: generating pilin deletion strain, pilin constructs, pilin and pilus expression, electron microscopy, data analysis, and co-wrote manuscript.

3.1. Introduction

Bacterial Type IV pili (T4P) are long thin polymers of the major pilin subunit. This small protein has an extended ~53-amino acid α-helix, of which the C-terminal half, α1C, is embedded in the globular C-terminal domain of the pilin, and the N-terminal half, α1N, forms a hydrophobic stalk that both anchors the subunit in the inner membrane prior to pilus assembly and holds the subunits together in the assembled pilus filament. Within the hydrophobic α1N is an invariant acidic residue, Glu5. Vibrio cholerae and enterotoxigenic E. coli (ETEC) produce Type IV pilins of the IVb (T4b) class, which are distinguished from those of the Type IVa (T4a) pilins by having longer signal peptides (25-30 amino acids) and mature proteins (>200 amino acids) and a variable hydrophobic amino acid at their mature N-terminal position (Craig, Pique, and Tainer 2004). T4b pilins also have long D-regions, segments that lie between a pair of conserved disulfide bonds in the C-terminal region of the globular domain. In contrast, the T4a pilins of Neisseria gonorrhoeae, Neisseria meningitidis and Pseudomonas aeruginosa have a 6-8 amino acid signal peptide, a ~150 amino acid mature pilin and an N-terminal phenylalanine. The T4a and T4b pilins differ primarily in the αβ-loop region between α1 and the 4- or 5-stranded β-sheet, between their C-terminal D-regions, and in the
connectivity of the β-sheet itself. The T4b pilus machinery is much simpler, requiring less than a dozen proteins all encoded on the same gene cluster, whereas the T4a assembly machinery utilizes 40 or more proteins, the genes of which are distributed throughout the genome (Pellicic 2008). Despite these differences, all Type IV pilins share the canonical ladle-shaped pilin structure and helical arrangement within the pilus filament in which the N-terminal α-helices form a hydrophobic core and subunits are related by an axial rise of 8-10 Å and an azimuthal rotation of ~100°. Importantly, the conserved Glu5, which is critical for efficient pilus assembly (Aas et al. 2007; Horiuchi and Komano 1998; Li, Egelman, and Craig 2012; Pasloske and Paranchych 1988; Strom and Lory 1991) is positioned in this hydrophobic core to neutralize the positively-charged N-terminal amino group of its neighboring subunit (Craig et al. 2003; Li, Egelman, and Craig 2012).

Type IV pilus (T4P) assembly occurs in the inner membrane where the pilin subunits are anchored via their hydrophobic N-terminal α-helix, α1N. Subunits are thought to add to the growing pilus at its base, with the filament growing through the periplasm and across the outer membrane via the secretin channel. Pilus assembly requires a core assembly machinery comprised of the major pilin subunit, a prepilin signal peptidase that removes the signal peptide and adds a methyl group to the N-terminal amine of residue 1 (Kaufman et al. 1993; Strom and Lory 1991; Zhang, Lory, and Donnenberg 1994), a cytoplasmic assembly ATPase that powers the addition of each subunit to the growing pilus, an inner membrane core protein of unknown function, and an outer membrane secretin channel. This core assembly machinery is also conserved in the bacterial Type II secretion (T2S) system, which assembles a periplasmic “pseudopilus” that extrudes protein substrates from the periplasm across the outer membrane to the extracellular space without itself forming an extracellular filament. The genes encoding the T2S machinery, like those of the T4b pilus systems, are encoded on a single operon (Sandkvist 2001). Most Type IVa pili, as well as enteropathogenic E. coli (EPEC) bundle forming pilin (BFP) of the Type IVb class, also utilize a second “retraction” ATPase that catalyzes filament disassembly. Retraction is necessary for Type IV pilus functions such as twitching motility, DNA uptake, phage transduction and bacterial dissemination (Giltner, Nguyen, and Burrows 2012; Morand et al. 2004). No such retraction ATPase has been identified for V. cholerae and ETEC Type IVb pili and these pili have not been shown to mediate twitching motility or DNA uptake.
The T2S systems also lack a retraction ATPase. Type II secretion thought to occur via a piston-like movement of the pseudopilus (Korotkov and Hol 2008; Reichow et al. 2011; Reichow et al. 2010), a mechanism that is apparently independent of a retraction ATPase. The T4P and T2S systems are structurally and functionally related as several T4P systems have secretory functions. *V. cholerae* toxin co-regulated pili (TCP) apparatus secretes a protein, TcpF, which is required for colonization of the infant mouse (Kim, Bose, and Taylor 2003), and CFA/III secretes CofJ (Yuen et al. 2013).

In addition to the major pilin, which is the structural unit for the Type IV pilus filament, all Type IV pilus systems possess one or more minor pilins, which share the N-terminal α-helix with the major pilins but are expressed in much lower levels. The *V. cholerae* TCP and ETEC CFA/III and longus pilus systems, all of the T4b class, each possess a single minor pilin encoded on the pilus operon immediately following the major pilin gene (Figure 1-7). In contrast, the EPEC BFP T4b pilus system has three minor pilins that are encoded at the end of the bfp operon. This arrangement is also seen for the T2S systems, which have 4 minor pilins. The more complex T4a pilus systems have multiple minor pilins, typically encoded on their own gene clusters. A similar arrangement is also seen in the Type IV pilus systems of some Gram-positive *Clostridia* species (Melville and Craig 2013). Most minor pilins are similar in size to their respective major pilins and share the position 5 glutamate. However, the T2S and T4P systems typically have a distinct minor pilin with a hydrophobic residue at position 5; in the T2S systems this protein is much larger than its corresponding major pseudopilin. These proteins are classified as GspK family members (Bleves et al. 1998) and include ETEC GspK, *Klebsiella oxytoca* PulK and *P. aeruginosa* XcpX from T2S systems and *P. aeruginosa* PilX and *N. meningitidis* PilK from T4a pilus systems. The single minor pilins of the *V. cholerae* and ETEC T4b pilus systems, TcpB and CofB, respectively, have a Glu5, but like the T2S GspK proteins, are substantially larger than their respective major pilins.

Minor (pseudo)pilins play roles in pilus assembly and functions but their precise functions have been challenging to identify due to their multiplicity and functional redundancy and, in the case of the T4P, the presence of retraction ATPases. The minor pilins of *N. gonorrhoeae* and *P. aeruginosa* are required for wild type levels of T4P
assembly, but assembly can proceed at reduced levels in minor pilin mutants that also lack the retraction ATPase (Carbonnelle et al. 2006; Giltner, Habash, and Burrows 2010; Nguyen et al. 2015; Winther-Larsen et al. 2005). In addition to their role in pilus assembly, minor pilins act in adherence to and signaling of host cells (Bernard et al. 2014; Brissac et al. 2012), autoaggregation (Helaine et al. 2005; Helaine et al. 2007) and swarming motility (Kuchma, Griffin, and O’Toole 2012).

Several minor (pseudo)pilin structures have been solved, all lacking their N-terminal α1N segment. They resemble the structures of their respective major (pseudo)pilins, suggesting they can incorporate into the (pseudo)pilus, but some possess additional features. The _N. meningitidis_ T4a minor pilin PilX is similar in structure to the _N. gonorrhoeae_ major pilin, PilE, but with a 2-turn α-helix instead of a β-hairpin in the D-region (Helaine et al. 2007). This feature would be exposed on the pilus surface, and immunogold transmission electron microscopy (TEM) demonstrated a low level of PilX incorporation into _N. meningitidis_ Type T4a pili. PilX is involved in pilus-mediated autoaggregation and adhesion to host cells (Helaine et al. 2005; Helaine et al. 2007). The _P. aeruginosa_ minor pilins PilV, PilW and PilX along with a non-pilin protein PilY, are proposed to form a priming complex that is connected via minor pilins PilE and FimU to the major pilin subunits in the _P. aeruginosa_ pilus shaft (Giltner, Habash, and Burrows 2010; Nguyen et al. 2015). PilE and FimU have pilin-like structures but FimU has a second β-sheet that lies between α1 and the conserved β-sheet (i.e. in the αβ-loop) (Nguyen et al. 2015).

A ternary structure of the ETEC T2S minor pseudopilins Gspl, GspJ and GspK provides critical insight into the role of these proteins in (pseudo)pilus assembly (Korotkov and Hol 2008). While Gspl is similar in structure to major pseudopilins, GspJ is larger, with 2 β-sheets in the globular domain similar to _P. aeruginosa_ FimU. GspK is the largest of the three, having a discrete pilin domain with an N-terminal α-helix and a β-sheet, but with a large insert between strands 1 and 2 of the β-sheet, that bulges from the globular domain and contains 10 short α-helices. These proteins are staggered with respect to one another, similar to the arrangement of the _N. gonorrhoeae_ Type IVa pilins GC pilus, with GspK located at the tip. These minor pilins protein are predicted to cap the pseudopilus. Indeed the globular domain of GspK is too large to fit anywhere but at
the tip of the pilus, and may serve as a steric block to prevent the pseudopilus from growing across the outer membrane secretin (Korotkov and Hol 2008). In support of this idea, the minor pseudopilins of the *K. oxytoxa* Pul T2S system are required for efficient pseudopilus assembly (Cisneros et al. 2012), as are the Xcp T2S minor pseudopilins in *P. aeruginosa* (Durand et al. 2005). Furthermore, the Type IVa and T2S minor (pseudo)pilins are functionally interchangeable to some degree, as *E. coli* K12 minor pseudopilins initiate *K. oxytoxa* pseudopilus assembly but not secretion (Cisneros, Pehau-Arnaudet, and Francetic 2012) and *P. aeruginosa* minor pseudopilins restore T4a pilus assembly in a minor pilin deletion strain when the retraction ATPase is absent (Nguyen et al. 2015).

The complexity of the T2S and T4a pilus systems, with multiple minor (pseudo)pilins and, in the case of the T4P, a retraction ATPase, make it challenging to decipher the molecular mechanism by which the minor pilins influence pilus assembly and functions. The *V. cholerae* and ETEC T4b pilus systems represent comparatively simple systems with only a single minor pilin and no retraction ATPase. We show here that the ETEC minor pilins CofB and LngB mediate CFA/III and Longus pilus assembly, respectively.
3.2. Materials and Methods

3.2.1. Plasmids and strain list

Bacterial strains, plasmids, and primers are listed in Table 3.1. *E. coli* strains were grown with antibiotics appropriate for plasmid selection. Final concentrations for antibiotics: 100 μg ml⁻¹, ampicillin (Ap); 20 μg ml⁻¹, chloramphenicol (Cm); 100 μg ml⁻¹, streptomycin (Sm); 45 μg ml⁻¹, kanamycin (Km). Enterotoxigenic *Escherichia coli* strains 31-10 and 31-10P were gifts from Ronald Taylor (Geisel School of Medicine), and strain E9034A was provided Fred Cassel (Walter Reed Army Institute of Research). *E. coli* strain MC4100 was obtained from the Coli Genetic Stock Center (CGSC) at Yale University. Plasmids for gene deletions using the λRED recombinase were gifts from Michael Donnenberg (University of Maryland). Plasmid pcof, which contains the entire CFA/III producing cof operon in the backbone of pACYC184, and the derivatives containing deletions of the cofA, cofD, and cofJ genes were generated by Alex Yuen (Yuen et al. 2013).
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3.2.2. Expression of pcof in MC4100

The vector pcof contains the entire cof operon inserted into the cloning vector pACYC184 (ATCC) between restriction enzyme sites EcoNI and EagI-HF (Yuen et al. 2013). pcof was electroporated into electrocompetent E. coli MC4100 cells and selected with Cm. To induce CFA/III pilus expression, cells were grown overnight at 37 °C on CFA (1% casamino acids, 0.15% yeast extract, pH 7.4) agar-Cm (Evans, Evans, and Tjoa 1977).

3.2.3. Construction of pcofA, pcofB, pcofB259, plngA, and plngB

Vectors expressing the major pilin CofA, the minor pilin CofB, and the truncated minor pilin CofB259 were derived from a plasmid with a pBAD22 backbone where the arabinose-inducible araC promoter has been replaced with the rhamnose-inducible PrhaB (pJMA10.1). pJMA10.1 contains an ApR marker. Genes for cofA, cofB, and cofB(1-259) were PCR amplified from ETEC 31-10 genomic DNA with the Q5 DNA polymerase (New England Biolabs) using primers CofA-F-KpnI/-R-HindIII, CofB-F-KpnI/-R-XbaI, and CofB-F-KpnI/-R-BamHI, respectively. PCR products were purified, digested, and ligated into pJMA10.1 at the corresponding restriction sites using T4 DNA ligase (New England Biolabs). All constructs were verified by DNA sequencing. Plasmids were transformed into MC4100-pcof and –pcof deletion strains and cells were grown on LB-Cm/Ap plates. Genes for lngA and lngB were PCR amplified from ETEC E9034A genomic DNA using primers LngA-F-KpnI/-R-HindIII and LngB-F-KpnI/-R-HindIII, respectively. PCR products were purified, digested, and ligated into pJMA10.1 at the KpnI/HindIII restriction sites using T4 DNA ligase. All constructs were verified by DNA sequencing (Genewiz). Plasmids were transformed into ETEC E9034A, -ΔlngA, -ΔlngB deletion strains and cells were grown on LB-Ap plates.

3.2.4. Generating minor pilin Glu5 mutants

The conserved Glu5 residue in the minor pilin CofB was changed to Ala, Asp, and Leu on pcofB to generate pcofB_{E5A}, pcofB_{E5D}, pcofB_{E5L}, respectively. Forward primers ET-CofB(E5A)-F-KpnI, ET-CofB(E5D)-F-KpnI, or ET-CofB(E5L)-F-KpnI were used with reverse primer CofB-R-XbaI to PCR amplify the cofB gene fragment from
pcotB encoding the corresponding amino acid substitutions. PCR products were purified and ligated into pJMA10.1 at the KpnI/XbaI restriction sites using T4 DNA ligase. All constructs were verified by DNA sequencing. Plasmids were transformed into MC4100-pcotΔcotB strain and cells were grown on LB-Cm/Ap plates.

3.2.5. Expression of major and minor pilin constructs

All vectors expressing the major pilins CofA and LngA, minor pilins CofB and LngB, and minor pilins derivatives CofB259, CofB_E5A, CofB_E5D, and CofB_E5L, were generated from the backbone of pJMA10.1. This plasmid has the rhamnose-inducible promoter PrhaB (see Chapter 2) and pilin expression was induced using rhamnose at the indicated concentrations.

3.2.6. Deletion of minor pilin genes

The cotB gene was disrupted in pcot using the λRED recombinase system (Datsenko and Wanner 2000) used to generate the pcotΔcotA construct (Yuen et al. 2013). To avoid polar effects on the cot operon, a portion of the cotB gene was targeted for deletion (nucleotides encoding residues 45 – 462) using the CofB-RED-For/-Rev primers to PCR amplify the kanamycin cassette in the plasmid pKD4 using Q5 DNA polymerase. PCR products were purified using the QIAquick PCR purification kit (Qiagen). E. coli DH5α pcot cells were transformed with pKD46 encoding the RED recombinase and clones were prepared to express the recombinase and made competent to take up the kanamycin cassette PCR product. Cells with successful integration of the kanamycin cassette were selected by plating on LB-Cm-Km, and subsequently incubated 30 °C and further screened for sensitivity to ampicillin corresponding to the loss of pKD46. Ap^S, Km^R, Cm^R colonies were screened by PCR to confirm insertion of kanamycin cassette and pcot-cotB::Km was purified by plasmid miniprep (Qiagen). The intermediate construct pcot-cotB::Km was transformed into competent E. coli SW105 cells expressing the FLP recombinase which recognizes and cleaves the FRT sequence flanking the kanamycin cassette. The FLP recombinase is activated and colonies were screened for loss of the kanamycin cassette on LB plates with and without Km. Km^S clones were screened for deletion of the cotB gene fragment.
by PCR amplification using primers CofB-F-KpnI/-R-XbaI for the 5’ and 3’ ends of cofB and confirmed by DNA sequencing. A positive pcofΔcofB plasmid was amplified in E. coli DH5α, purified and transformed into electrocompetent E. coli MC4100 cells.

IngA and IngB genes were disrupted in ETEC E9034A with the same λRED recombinase system used to disrupt the cofB gene (Datsenko and Wanner 2000). The Cm resistance-encoding cat cassette was PCR amplified from pKD3 with primer sets LngA-RED-For/-Rev and LngB-RED-For/-Rev to replace, respectively, the IngA gene fragment (encoding residues 57 to 155) and the IngB gene fragment (encoding residues 80 to 381). The cassettes were electroporated into ETEC E9034A expressing RED recombinase and clones were screened for resistance to Cm and sensitivity to Ap. Positive clones were made electrocompetent and transformed with pCP20 which encodes the genes for the FLP recombinase. Positive clones were screened for sensitivity to Cm and PCR amplified with LngA-For-KpnI/-Rev-HindIII or LngB-For-KpnI/-Rev-HindIII to confirm gene disruption. The gene deletions were also confirmed by DNA sequencing. These deletions were completed with help from G. Yang.

3.2.7. Accessing pilus assembly and CofJ secretion in ETEC and MC4100 -pcof

ETEC 31-10 and E. coli MC4100 pcof cells were grown overnight under CFA/III inducing conditions on CFA agar plates. Cells were overlayered with 5 mL of phosphate saline buffer (PBS, 10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl) with gentle agitation on a rocking unit for 15 minutes. Cells were gently washed off the plates and the optical density at 600 nm (OD600) was measured. Cell suspensions were normalized to OD600 of 0.1; this mixture constitutes the whole cell culture (WCC) fraction used to assess total protein levels. Cells were removed by centrifugation at 3000 x g for 10 minutes and the supernatant was further filtered through a 0.22 µm syringe-drive filter (Pall). This filtered supernatant (Sup) fraction was used to assess CofA within the sheared/shed CFA/III pili as well as CofJ secreted by the CFA/III assembly apparatus. Samples were 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 prior to being loaded onto 15% hand cast SDS-PAGE gels. Proteins were
transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for immunoblotting (see Chapter 2). Proteins were detected by rabbit polyclonal antisera raised against N-terminally truncated CofA (Kolappan et al. 2012), CofB (N-terminal peptide 61-74; C-terminal peptide 403-419) and CofJ (peptide 164-185) (Yuen et al. 2013) (Pacific Immunology). Goat-anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch) were used to detect primary antibody. Immunoblots were visualized by enhanced chemiluminescence (ECL) with the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) for CofA and CofJ or the SuperSignal West Femto chemiluminescent substrate (Thermo Scientific) for CofB. A Fujifilm LAS4000 imager (FujiFilm) was used to capture images of the immunoblots.

3.2.8. Assessing pilus assembly and aggregation in ETEC E9034A strains

ETEC E9034A cells were grown overnight on LB agar plates at 37 °C. Single colonies were inoculated in 2 mL of terrific broth (TB) (1.2% tryptone, 2.4% yeast extract, 1.7 mM KH$_2$PO$_4$, 7.2 mM K$_2$HPO$_4$, pH 7.2) and grown for 4 hours in an upright position on a shaking incubator at 250 rpm and 37 °C. Cells were inoculated 1/100 in 2 mL TB and grown for a further 2 hours, and visually inspected for aggregation phenotype after allowing the cultures to stand at RT for 10 minutes. The whole cell culture fraction (WCC) was used to assess total protein levels. Cells were removed by centrifugation at 3000 x g for 10 minutes and the supernatant (Sup) was used to assess LngA within the sheared/shed longus pili. Samples were mixed with Laemmli sample buffer and boiled for 10 minutes prior to being loaded onto 15% hand cast SDS-PAGE gels. Proteins were transferred onto PVDF membrane membrane (Bio-Rad) for immunoblotting. Since the longus pilins and the CFA/III pilins share a high degree of sequence identity, LngA and LngB could be detected by rabbit polyclonal antisera raised against N-terminally truncated CofA (Kolappan et al. 2012) and CofB (N-terminal peptide 61-74; C-terminal peptide 403-419) (Pacific Immunology), respectively. Goat-anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch) were used to detect primary antibody. Immunoblots were visualized by ECL with the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) for LngA and the
Super Signal West Femto chemiluminescent substrate (Thermo Scientific) for LngB. A Fujifilm LAS4000 imager was used to digitize the immunoblots.

3.2.9. **Immunogold labelling and transmission electron microscopy analysis**

For negative-stained transmission electron microscopy (TEM) analysis, 20 µL of the whole cell culture were applied to formvar carbon-coated grids (Electron Microscopy Sciences) or grids were placed directly onto cells grown on CFA agar plates. Samples were washed with PBS and stained with 3% uranyl acetate. For immunogold labelling TEM analysis, samples were applied to formvar carbon-coated grids. Samples were fixed for 1 hour at RT by placing the grid in a 15 µL drop of fixative (2% paraformaldehyde, 0.5% glutaraldehyde, in 50 mM sodium cacodylate, pH 7.4). The grid was washed with Tris-buffered saline with 0.1% Tween (TSBT) and blocked for 1 hour in TBS with 1% bovine serine albumin (BSA) (Bioshop). The samples were incubated with primary antibody (1:50 dilution in TBS 1% BSA), followed by wash in TBS, and a 30 minute incubation with the 12 nm or 18 nm colloidal gold-conjugated anti-rabbit secondary antibody at 1:60 dilution (Jackson ImmunoResearch). After wash in TBS, the samples were stained with 3% uranyl acetate. Proteins were probed with rabbit polyclonal antisera raised against N-terminally truncated CofA (Kolappan et al. 2012) and CofB (1:1 mixture of antisera raised against peptides 258-272 and 403-419) (Pacific Immunology). All samples were imaged on a Hitachi 8100 TEM at accelerating voltage of 200 kEV.
3.3. Results

3.3.1. Crystal structure of ETEC minor pilin CofB

A 2 Å resolution x-ray crystal structure of the ETEC minor pilin CofB was determined by our research associate, S. Kolappan. Recombinant CofB, lacking its 24-residue N-terminus corresponding to α1N of the major pilin, was expressed and purified and crystals were grown. CofB is an extended protein with 3 discrete domains connected by flexible linkers: an N-terminal "pilin domain", a central β-repeat domain with two tandem 3-stranded anti-parallel β-sheets, and a C-terminal elongated β-sandwich domain (Figure 3-1A). The pilin domain is similar to the globular domain of the major pilin CofA and has the canonical Type IV pilin fold (Figure 3-1B); the C-terminal end of the α-helix leads into the αβ loop, which is followed by a 5-stranded β-sheet. The CofB αβ loop is almost twice the length of the one found in CofA. Strand β5 in CofA would have been the C-terminus, but in CofB, it extends into a flexible linker connected to two successive β-repeats. The second β-repeat leads into another linker that is connected to the C-terminal domain which begins with a β-hairpin that folds into an elongated 6-stranded β-sandwich. There are 8 cysteines, forming four disulfide bonds, in CofB. The first bond is found in the pilin domain, the second and third are separately found in each of the two β-repeats, and the fourth is located in the C-terminal domain.
Figure 3-1  X-ray crystal structure of ETEC CofB

(A) Cartoon representation of CofB, residues 25 – 518 (PDB ID 4QS4). The pilin domain is colored blue, the central β-repeat domain is orange, and the C-terminal β-sandwich domain is green. The eight cysteines participating in disulfide bonds are labeled and shown in stick representation with sulfurs colored yellow. Residues 1 – 24 of CofB is expected to form the N-terminal half of α1, α1N, which both anchors the pilin subunits in the inner membrane and acts as the polymerization domain in the assembled pilus. Figure courtesy of Lisa Craig and Subramania Kolappan, Simon Fraser University.  

(B) Comparison of the major pilin CofA and the minor pilin CofB, both modeled on full length PAK pilin structure (Craig et al. 2003). The overall size and volume of the CofB pilin domain is very similar to that of CofA, the major differences being the bulkier αβ-loop in CofB and the relative positions of the disulfide bonds (indicated in cyan). Figure adapted from Tony Harn, Simon Fraser University, and used with permission.
3.3.2. The ETEC minor pilin CofB is required for CFA/III pilus assembly

The ETEC minor pilin CofB is encoded on the cof operon immediately downstream of the gene encoding the major pilin, CofA, the structural subunit for the CFA/III Type IVb pilus (Kolappan et al. 2012). CofB is a 518 amino acid protein with a predicted signal peptide of 5 residues (Figure 3-2A). The CofB signal peptide contrasts to the 30-amino acid signal peptide of CofA, which is likely processed by the prepilin peptidase CofP encoded on the cof operon. However, the N-terminal 26 amino acids of the mature CofB protein shares sequence similarity with CofA, including Glu5. This region corresponds to the α1N, the inner membrane anchor and polymerization domain of the major pilins. Beyond the N-terminus, CofB has no obvious homology with CofA. CofB is more than twice the length of the 208-residue CofA. CofB is, however, highly similar to LngB, the minor pilin for the ETEC Longus pilus, with 78% sequence identity between the two proteins (Figure 3-2B). Both proteins have 8 cysteines, 1 of which is located in each of the two ~30-residue tandem repeats (Figure 3-2C).

The role of the minor pilin CofB in CFA/III assembly was examined using a heterologous E. coli CFA/III expression system, which allows for manipulation of genes within the cof operon. In ETEC strain 31-10, the cof operon is located on a 55 kb virulence plasmid (Taniguchi et al. 2001) that is unstable and not amenable to genetic manipulation (Yuen et al. 2013). The cof operon was cloned into vector pACYC184 producing pcof, which was transformed into several E. coli expression strains (DH5α, HB101, and MC4100) (Yuen et al. 2013). These strains express CFA/III and secrete the soluble protein CofJ, also encoded on the cof operon, in a pilus-dependent manner (Yuen et al. 2013). To test the role of CofB in CFA/III pilus assembly, the cofB gene in pcof was deleted using the λRED recombinase system (Datsenko and Wanner 2000) and pcofΔcofB was transformed into E. coli MC4100. Cells were grown on CFA agar plates (Evans, Evans, and Tjoa 1977) then washed off the plates with phosphate buffered saline (PBS) and their numbers were normalized. This mixture, referred to as the whole cell culture fraction (WCC) was analyzed by SDS-PAGE and immunoblotting using anti-CofA antibody (Kolappan et al. 2012) to determine total CofA levels in comparison to “wild type” MC4100-pcof strain. Additional controls included the strain lacking the major pilin, CofA (MC4100-pcofΔcofA), wild type ETEC 31-10, and ETEC 31-
10P, which lacks the 55 kb virulence plasmid. To determine CFA/III pilus assembly levels, cells were removed from the WCC by centrifugation and filtration and the amount of CofA in the supernatant fraction (Sup) was analyzed by SDS-PAGE and immunoblotting. Total CofA levels in the WCC fraction of MC4100-PCOF∆cofB are comparable to those of the positive controls: ETEC strain 31-10 and MC4100-PCOF (Figure 3-3A). As expected, no CofA is present in negative control strains ETEC 31-10P and MC4100-PCOF∆cofA. However, whereas CofA levels in the Sup fraction, representing CFA/III pili, are comparable for ETEC 31-10 and MC4100-PCOF, no CofA is present in this fraction for MC4100-PCOF∆cofB, suggesting that CofB is required for pilus assembly.

To confirm that the loss of CFA/III in MC4100-PCOF∆cofB is due to disruption of the cofB gene and not a downstream effect, cofB was cloned into expression vector pJMA10.1 (see chapter 2.2.3). This vector is derived from pBAD22, with the araC promoter replaced by the rhamnose-inducible promoter PrhaB. The new pcofB vector was transformed into MC4100-PCOF∆cofB. Pilus assembly is rescued to approximately wild type levels when no rhamnose is added, presumably due to leaky expression (Figure 3-3A). However, induction with rhamnose, even at very low levels (0.001%) results in reduced levels of CofA in both the WCC and Sup fraction. These results suggest that CofA expression and stability of CFA/III assembly are optimal when CofB is expressed at low levels. Total CofA levels are also reduced at the highest CofB expression level (0.1% rhamnose). This observation cannot be explained by competition for the signal peptidase as we see no accumulation of unprocessed CofA under these conditions.
Figure 3-2  CofB amino acid sequence and alignment with CofA and LngB.

(A) Alignment of the signal peptides (shaded grey) and N-terminal 30 residues of the minor pilin CofB and the major pilin CofA from the ETEC CFA/III pilus system. (B) Sequence alignment of CofB with the minor pilin LngB from the ETEC Longus pilus system. The disulfide bond connectivity is indicated and regions of CofB are colored based on the crystal structure shown in Figure 3-1. Residues 25-518 were expressed recombinantly for crystallization. (C) Alignment of the CofB β-repeats 1 and 2.
The WCC and Sup fractions were blotted with antibody against an N-terminal CofB peptide (residue 61-74) to determine CofB expression levels and cellular localization. CofB was not detected in ETEC 31-10 or MC4100-pcof WCC using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) but a faint band at ~57 kDa, corresponding to CofB, was observed for ETEC 31-10 and MC4100-pcof when the more sensitive Femto substrate was used, consistent with CofB being expressed at very low levels in the wild type strains (Figure 3-3A top panel). The CofB band is absent in MC4100pcofΔcofB but present in the MC4100-pcofΔcofB+pcofB WCC without rhamnose, confirming that CofB is produced under these conditions at a very low level that is nonetheless sufficient for pilus assembly. No CofB was detected in the culture supernatant using the N-terminal antibody against CofB peptide 61-74 (Figure 3-3B, Sup, middle panel), but it is detected in MC-4100pcofBΔcofB with an antibody against a C-terminal peptide, 403-419, using the Femto detection kit with a 60-second exposure (Figure 3-3B, Sup, lower panel), suggesting that CofB incorporates into pili but at very low levels.

Significant proteolysis of CofB was observed in the WCC when the minor pilin was expressed at high levels (Figure 3-3B, upper panel). These are N-terminal fragments as they are detected by antibodies specific for an N-terminal peptide. The most abundant fragment has a mass of ~29 kDa, which corresponds to a CofB fragment spanning residues 1 to ~260. This fragment is also present in ETEC 31-10 WCC.

It was previously shown that the CFA/III assembly apparatus secretes the soluble protein CofJ (Yuen et al. 2013). We used our heterologous expression system to determine if the minor pilin CofB is required for CofJ secretion. CofJ is expressed and secreted at lower levels in the MC4100-pcof strain than that of ETEC 31-10 (Figure 2C). Despite this, a faint CofJ band is detected in the Sup fraction for MC4100-pcofΔcofA, suggesting that some of this protein is secreted in a CFA/III independent manner. Surprisingly, deletion of cofB results in increased CofJ in the WCC fraction. This cannot be explained by accumulation of CofJ in the periplasm due to an inability to assemble pili, because an increase in total CofJ is not observed in the ΔcofA strain WCC, which is also unable to assemble pili. The higher levels of CofJ in the WCC fraction of the ΔcofB strain correspond with higher levels in the Sup fraction. Similar results are seen when
pcob is added to this strain without induction, despite CofB being made at low levels in this condition. Since we see similar levels of CofJ in the WCC and Sup fractions in the absence and presence of CofB and in the absence and presence of CFA/III assembly, we infer that CofJ is being released from the cells in a pilus-independent manner that is proportional to the amount of CofJ produced. This may be an artifact of the heterologous expression system we are using. Nonetheless, the results demonstrate a direct role for CofB in CFA/III assembly.
Figure 3-3  Immunoblots of ETEC CFA/III proteins in whole cell culture and supernatant fractions.
The indicated strains were grown on CFA plates, harvested and resuspended, and aliquots of this whole cell culture fraction (WCC) and the supernatant (Sup) fraction, by removal of cells by centrifugation and filtration, were analyzed by SDS-PAGE and immunoblotting. The WCC fraction represents the total protein and the Sup fraction contains CFA/III pili that have been shed from the cells and CofJ that is secreted by the CFA/III apparatus. Blots were probed with (A) anti-CofA, (B) anti-CofB (peptide 61-74), two upper panels, and anti-CofB (peptide 403-419), lower panel (C) anti-CofJ antibodies. Expression of CofB from pcofB and CofB259 from pcofB259 was induced with 0, 0.001 or 0.1% rhamnose as indicated. Molecular mass of markers are indicated on the left. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.

3.3.3. The ETEC minor pilin LngB is required for Longus pilus assembly and pilus-mediated autoagglutination

To examine the role of the ETEC minor pilins in a native T4b system, we turned to ETEC strain E9034A, which produces the Longus T4b pilus (Giron, Levine, and Kaper 1994). We deleted the lngB gene from ETEC E9034A using the λRED recombinase system (Datsenko and Wanner 2000). As with the MC4100-pcofΔcofB strain, pilus assembly as assessed by immunoblotting the supernatant fraction (Figure 3-4) and Longus-mediated autoagglutination were abrogated (Figure 3-5) in the ETEC E9034A ΔlngB strain, comparable to that of a ΔlngA mutant, and assembly and function were rescued when LngB was expressed ectopically (Figure 3-4A). LngB expression from plngB in ETEC E9034A ΔlngB does not appear to be as high as CofB expression from pcofB in MC4100-pcofΔcofB, requiring a higher level of induction to restore wild type Longus levels and wild type autoagglutination (0.001-0.1% rhamnose) (Figure 3-5). We were unable to detect LngB in the pilus fraction even when LngB was overexpressed and the ultra-sensitive Femto detection substrate was used to develop the immunoblot (Figure 3-4B, Sup, lower panel). Given that our results with the CFA/III pilus system showed that pilus assembly is most efficient when only low levels of CofB were produced, we tested whether increasing the major pilin, LngA, might disrupt pilus assembly by altering the major:minor pilin stoichiometry. However, we found that overexpressing the major pilin, LngA, in ETEC E9034A ΔlngA had no effect on pilus assembly, likely because the excess LngA was not processed by the prepilin peptidase (Figure 3-4A, upper panel). These results parallel those shown for the MC4100 heterologous CFA/III expression system and confirm that the ETEC minor pilins are necessary for T4b pilus assembly.
Figure 3-4  The ETEC minor pilin LngB is required for Longus pilus assembly.

The indicated strains were grown in TB. Whole cell culture (WCC) and culture supernatant (Sup), in which the cells are vortexed to shear the pili off then removed by centrifugation, were analyzed by SDS-PAGE and immunoblotting. The WCC fraction represents the total protein and the Sup fraction contains Longus pili that have been shed from the cells. Blots were probed with (A) anti-CofA and (B) anti-CofB antibodies, which cross-react with LngA and LngB, respectively. Expression of LngB from plngB was induced with 0, 0.001 or 0.1% rhamnose as indicated. Molecular mass of markers are indicated on the left. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.
ETEC E9034A autoagglutinates

Autoagglutination of ETEC E9034A strains. Cell cultures are shown after 4 hours of growth on rotating shaker sitting upright as described in materials and methods section, followed by 10 minutes of stationary incubation at room temperature. Similar to *V. cholerae* cells (Figure 2-3B), ETEC E9034A can form macroscopic aggregates that fall to the bottom of the tube leaving the supernatant almost clear. Rescue of the ∆lngA and ∆lngB strain by complementation of plngA and plngB, respectively, is most dramatic at the highest expression level. Pilin expression in cells transformed with constructs is induced with rhamnose (w/v). Red arrows point to bacterial aggregates formed.
3.3.4. The C-terminal domain is required for pilus assembly

Since CofB has such discrete and well-defined domains, we tested the requirement for the extended C-terminal region in initiating pilus assembly. We generated a CofB variant truncated at residue 259. This site was chosen because residues 260 and beyond do not interact with the pilin domain. Furthermore, a proteolytic fragment corresponding to this region is produced when CofB is overexpressed and appears to be stable (Figure 3-3B, upper panel). Unlike full length CofB, CofB259 is unable to rescue pilus assembly in the MC4100-pcofΔcofB strain when expressed ectopically, regardless that this variant appears to be stably expressed at all expression levels (Figure 3-3A). These results demonstrate the requirement for the CofB C-terminal region for initiating pilus assembly. Interestingly CofB259 is detected in the Sup fraction at the highest level of expression, which may represent CofB259 filaments. As with overexpression of full length CofB, CofJ is seen in the culture supernatant in the absence of CFA/III pili. CofB259 is also present in the Sup at the highest level of expression, suggesting that it might form its own filaments like TcpB.

3.3.5. CofB Glu5 is not needed for pilus assembly

Most minor (pseudo)pilins has a glutamate at position 5, a conserved residue in the major pilins that is critical for efficient pilus assembly (Aas et al. 2007; Horiuchi and Komano 1998; Li, Egelman, and Craig 2012; Pasloske and Paranchych 1988; Strom and Lory 1991). CofB has this Glu5, but it also shares similarities with pilins classified in the GspK family, which are larger than their major pilin counterparts and lack this conserved Glu5. In the ETEC T2S system, the pseudopilin GspK is thought to form the tip of the pseudopilus (Korotkov and Hol 2008), and as the first subunit in the filament it would not require Glu5 as there is no existing charge to neutralize. Our data suggests that CofB initiates pilus assembly, suggesting it is the primary subunit at the tip of a CFA/III pilus. Therefore, we tested this implication by generating several CofB Glu5 variants by substitution into pcofB and testing for the ability of these mutants to restore CFA/III assembly in MC4100-pcofΔcofB. As with the MC4100-pcofΔcofB+pcofB strain, pilus assembly was restored for each of the CofB Glu5 variant with and without rhamnose induction (Figure 3-6A). CofB is detected in the WCC of all CofB Glu5 variants, even
without rhamnose, confirming that the minor pilin is produced at a level adequate for pilus assembly (Figure 3-6B). These results show that the conserved Glu5 is not required to initiate pilus assembly, and concur with our hypothesis that the minor pilin is likely the first subunit added to the pilus.

Figure 3-6  CofB Glu5 variants can assemble CFA/III pili

The indicated strains were grown on CFA plates, harvested and resuspended, and aliquots of this whole cell culture fraction (WCC) and the supernatant (Sup) fraction, obtained by removal of cells by centrifugation and filtration, were analyzed by SDS-PAGE and immunoblotting. The WCC fraction represents total protein and the Sup fraction contains CFA/III pili that have been shed from the cells. Blots were probed with (A) anti-CofA and (B) anti-CofB antibodies. Protein levels can be compared across each panel which represents a single blot; while vertical lines delineate strains.
3.4. Discussion

The CofB minor pilin is like no other pilin or minor pilin structure published to date. A 2 Å resolution CofB crystal structure has been determined by our research associate, S. Kolappan (Figure 3-1) (S. Kolappan et al, submitted). While it contains a canonical pilin domain, the extended flexible nature of the C-terminal region suggests an ability for the structure to adapt to conformations. CofB most closely resembles the ETEC minor pseudopilin GspK, as both proteins have a pilin domain linked to a larger globular domain and are considerably larger than their respective major (pseudo)pilins (GspK has 316 amino acids whereas GspG has 138 amino acids). However, the globular domain of GspK though bulky is compact. Its extra α-helical region is not at the C-terminus but instead inserted between two of the framework β-strands of the pilin domain and interacts intimately with the globular domain; this forms an outsized and rigid globular domain in GspK (Korotkov and Hol 2008). In contrast, CofB is segmented, with a discrete globular domain that resembles the one found in the major pilin CofA and two discrete extended C-terminal domains that connect to the pilin domain via flexible linkers. Whereas GspK and other members of this family work in concert and in complex with several other minor pseudopilins to prime pseudopilus assembly, CofB itself appears sufficient for this task. Deletion of cofB results in loss of pilus formation and replacement with cofB, expressed ectopically, restores pilus assembly.

The structure of CofB, its function in initiating pilus assembly, and its similarity to GspK minor pseudopilin together imply that it is located at the tip of the CFA/III pilus. Our immunoblots show that CofB is present in the pilus fraction at very low levels, consistent with it being a tip-associated pilin. CFA/III pili are several microns in length. Based on the TEM image reconstruction of the close related V. cholerae TCP, which has an axial rise per subunit of 8.4 Å (Li, Egelman, and Craig 2012), a 5 μm long CFA/III pilus is comprised of ~6000 CofA subunits. Thus, a single CofB molecule for every 6000 CofA molecules will be difficult to detect. Although we have demonstrated by immunoblot that CofB is found in low levels in the pilus fraction, we have been unable to demonstrate CofB localization at the pilus tip using immunogold TEM. Perhaps anti-CofB antibody, raised against a peptide, is not capable of binding to the folded protein in the context of the pilus filament.
CFA/III pili are not assembled when full length CofB is substituted with the CofB259 construct that has the pilin domain but lacks the C-terminal region, despite this protein being expressed stably in the cell. We propose that the minor pilin CofB is the first pilin subunit in a new pilus and that it recruits the first CofA subunit via interactions between its C-terminal region and the CofA globular domain (Figure 3-7). The position of the C-terminal region as shown in the crystal structure, together with the bulky αβ-loop, would prohibit insertion of CofB into a growing pilus filament, but would it fit well at the tip of the pilus. Importantly, the C-terminal region would lie across the top of the filament. When modeled as a rigid body, the C-terminal domain of CofB would protrude from the filament and clash with the secretin channel as the pilus grows across the outer membrane. However, the longer links between the β-repeat domain and the C-terminal domain could allow the C-terminal domain to tuck in to the end of the pilus without extending the diameter of the pilus. This mechanism would allow the pilus to grow extracellularly, whereas the rigid and bulky GspK T2S minor pilin would block pseudopilus passage through the secretin, relegating it to the periplasm (Korotkov and Hol 2008; Reichow et al. 2011; Reichow et al. 2010; Yanez et al. 2008a, 2008b).

It is not clear why the ETEC, and likely *V. cholerae*, T4b pili utilize this simple mechanism with a single minor pilin to initiate pilus assembly whereas T2S and T4a pili require multiple minor pilins. This may be related to the requirement to balance assembly with disassembly. In the case of the T2S systems, the pseudopilus must rapidly assemble and disassemble to act as a piston to extrude substrate across the outer membrane. In the case of T4a pili (and EPEC T4b pili) assembly must work against retraction mediated by the retraction ATPase. Both systems evolved from the primitive ETEC and *V. cholerae* T4P pilus systems to refine secretion or more complex pilus functions like twitching motility and host cell signaling.
Figure 3-7  Model of CofB incorporation into CFA/III pilus

Proposed model for CofB-mediated initiation of CFA/III assembly. The minor pilin (red) is the first subunit in pilus assembly, an incoming major pilin (blue) is stabilized by the β-repeats (yellow) and β-sandwich C-terminal domain (green). As more pilins are incorporated, the minor pilin exits the cell as a tip-associated component. Figure adapted from Lisa Craig, Simon Fraser University.
Chapter 4. General discussion and conclusion

The hallmark of diseases caused by enteric bacterial pathogens such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* is the acute watery diarrhea, which is caused by the release of bacterial enterotoxins that disrupt the water and electrolyte balance within the intestinal lumen (Kaper, Morris, and Levine 1995; Kaper, Nataro, and Mobley 2004; Nataro and Kaper 1998). In regions endemic to diarrheal pathogens, this strategy allows the bacteria to disseminate and persist easily within the population, especially in areas where clean water and sanitation infrastructures are not readily available (Kosek, Bern, and Guerrant 2003). For the bacteria to reach its toxin-releasing stage, it must first successfully colonize the host intestinal epithelium. In many bacterial pathogens, this critical task is mediated by Type IV pili, which are long and thin filaments that are expressed on the bacterial cell surface. *V. cholerae* and ETEC produce the Type IVb class pili, which are unique in their simplistic genetic organization (Giron, Levine, and Kaper 1994; Taniguchi et al. 2001; Taylor et al. 1987). All the proteins needed for assembly are encoded within a single operon. We have proposed that many of the T4b pilus functions requires a dynamic filament that can extend out and retract back into the cell. However, T4b pilus systems lack the retraction ATPase commonly found in the other more complex T4a systems.

One of my thesis objectives is to elucidate the molecular mechanism that allows T4b systems to retract their pilus without a retraction ATPase. With the help of Dr. Biais, we demonstrated that *V. cholerae* TCP are indeed retractile. We showed the single minor pilins expressed in the TCP, CFA/III, and Longus systems play an important role in regulating pilus dynamics and functions. The T4b minor pilins share N-terminal homology with the major pilins but they possess a bulkier C-terminal globular domain. We established in *V. cholerae* that TcpB is indeed the minor pilin of TCP and its expression levels in the cell need to be very low for optimal functions. Furthermore, we provided a precise quantification of the major:minor pilin stoichiometric ratio, which is 800 major pilin for each minor pilin. This balance between TcpA and TcpB is only
indicative of the relative total protein levels in the cell as the ratio was determined from analysis of the whole cell lysate, the ratio of major:minor pilin within a pilus filament is likely much higher as the minor pilin is only surface-displayed as a tip-associated component of the filament.

Through immunodetection and immunogold electron microscopy we showed that minor pilins have a functional role in initiating pilus assembly and is found incorporated, likely as a tip associated protein, in surface-displayed pili. There is a clear structural delineation between the canonical N-terminal pilin domain and the more variable and bulkier C-terminal domain. The crystal structure of the CFA/III minor pilin CofB, determined by S. Kolappan, suggests that the C-terminal domain has a large degree of flexibility in its structure. We found that the C-terminal domain is necessary for minor pilins to initiate pilus assembly, both in the TCP and CFA/III systems. Based on the sequence homology between CofB and the Longus minor pilin, LngB, we contend that LngB will also require its C-terminal domain for pilus biogenesis. We propose that the single minor pilin found in the T4b pilus systems is the first subunit in an assembling pilus and its main function is to stabilize the subsequent major pilin subunit that docks into the assembly apparatus (Figure 2-12 top panel and Figure 3-7). In our model, the minor pilin exits the cell as a tip-associated component but it remains unclear whether T4b minor pilins can mediate adhesion to other cells or the host epithelium like their T4a counterparts. In the more complex P. aeruginosa system, the minor pilins are also incorporated into the pilus filament and have an indirect role in adhesion through their association with the adherence protein PilY1 (Giltner, Habash, and Burrows 2010; Nguyen et al. 2015). Another T4a minor pilin, N. meningitidis PilX, is found to incorporate into the filament and directly modulates bacterial aggregation and host adherence (Bernard et al. 2014; Brissac et al. 2012; Helaine et al. 2005; Helaine et al. 2007; Imhaus and Dumenil 2014; Kuchma, Griffin, and O’Toole 2012), though one study did find PilX could modulates its function from within the periplasm (Imhaus and Dumenil 2014).

We have also established that the conserved Glu5 residue found in T4b minor pilins is critical to inducing pilus retraction and pilus-mediated functions. This invariant
Glu5 is also found in the major (pseudo)pilins in all pilus systems and we have hypothesized this residue provides charge complementarity to stabilize pilin:pilin interactions as the positively charged methylated N1+ of the terminal subunit is positioned in close proximity to this negatively charged residue on an incoming pilin subunit during assembly (Figure 1-6B) (Craig 2009; Craig, Pique, and Tainer 2004). The charge interaction is likely transient, but sufficient, to hold the pilin until the pilus apparatus extrudes the pilin and the filament a short vertical upward to allow the next pilin subunit to dock. Since the T4b minor pilins also have an invariant Glu5 in the N-terminal polymerization domain, we hypothesized this residue allows the minor pilin to interact with the assembling filament and this incorporation is the mechanism that initiates pilus retraction. We predicted the absence or presence of Glu5 does not affect the minor pilin’s ability to prime pilus assembly as there is no existing N1+ charge to neutralize. Indeed, when we substituted Glu5 with another amino acid in the V. cholerae minor pilin TcpB, we found pilus assembly is restored but pilus mediated functions that require pilus retraction were impaired. We assert that the minor pilin must be able to polymerize with TCP in order to exert its function and we have proposed a model (Figure 2-12) in which incorporation at the base of the filament stalls assembly and prevent further addition of pilin subunits. This leads to a spontaneous disassembly process and the pilins are free to diffuse back into the inner membrane, leading to processive retraction of the filament.

We have additional results that strongly suggest the minor pilins can polymerize with TCP. When TcpB is expressed ectopically at high levels in V. cholerae, the minor pilin is exported into the pilus fraction in high levels. This export is significantly impeded in V. cholerae ΔtcpA, a major pilin deletion strain, suggesting that export is associated with pilus assembly. In TcpB overexpression conditions, immunogold EM show novel filaments that are largely comprised of TcpB. These results show that TcpB can form its own filaments but requires TcpA, suggesting that they can polymerize within one filament.

It remains unclear whether TcpB exerts its functional mechanism at the base of the filament or after its incorporation into a filament within the periplasmic space. In the latter scenario, I hypothesize that TcpB is incorporated within the extending pilus but is
not able to pass through the secretin channel because its larger C-terminal domain either sterically clashes with the secretin channel or interacts with another periplasmic components of the assembly apparatus, thus stalling pilus assembly and inducing spontaneous disassembly. This is similar to the model in T2S systems where the GspK family of proteins are thought to cap pseudopilus extension by creating a steric clash with the secretin (Korotkov, Sandkvist, and Hol 2012; Korotkov and Hol 2008). However, a steric clash between TcpB and the assembly apparatus is an unlikely mechanism to induce retraction as we have observed large and variable diameter in TcpB filaments that rely on the TCP assembly apparatus to be exported onto the cell surface. This suggests that the outer membrane secretin can allow passage of objects larger than the typical diameter of TCP. Furthermore, the CTXϕ bacteriophage has been shown to associate with the periplasmic protein TolA (Ford et al. 2012), which requires the pilus filament to retract into the periplasm and the secretin to expand and accommodate the larger bacteriophage. Further studies are needed to identify any potential binding partners of TcpB, as their localization might provide insight into where TcpB exerts its functional mechanism. A crystal structure of TcpB will also guide us in identifying surface residues on the globular domain that might interact with the major pilin TcpA or other proteins of the TCP assembly apparatus.

Our results suggest that the minor pilin has two roles in pilus dynamics and functions. The minor pilins initiates the pilus assembly process and are exported as tip-associated components of the pilus, they also play a second role by incorporating at the base of an assembling filament to induce pilus retraction – the invariant Glu5 is critical to this interaction – to modulate different cellular functions. Our findings suggest that the single minor pilin in the T4b systems can facilitate the same functions as multiple minor pilins found in the T4a and T2S systems. Indeed there are many similarities between these minor pilins. The T4b minor pilins most closely resemble the GspK super family of proteins, which are minor pseudopilins in the T2S systems that are also considerably larger than their major pilin counterparts. ETEC GspK has been crystallized in complex with two other minor pseudopilins GspI and GspJ and all three have the canonical Type IV pilin fold (Korotkov and Hol 2008). GspK is hypothesized to form the cap of the pseudopilus (Korotkov, Sandkvist, and Hol 2012), similar to the pilus biogenesis role we have observed for TcpB, CofB, and LngB. In the P. aeruginosa Xcp T2S system, when
the GspK-homolog is deleted, the pseudopilus can form long filaments when the major pseudopilin is overexpressed (Durand et al. 2005).

A recent study by the Francetic group examined these long pseudopili and the EM reconstruction of the pseudopilus show a continuously variable twist along the length of the filament (Nivaskumar et al. 2014). They proposed a rotary assembly model in which the GspIJK minor pilin complex initiates pseudopilus assembly through a spool-like mechanism. In this model, major pseudopilins are targeted to a complex formed by the minor pseudopilins and the inner membrane platform protein GspF. Upon ATP hydrolysis, the assembly ATPase drives rotation of the GspIJKF complex which is coupled to the major pseudopilin. This process extracts the major pseudopilin from the inner membrane into the pseudopilus and repositions the GspIJKF complex to allow the next pseudopilin subunit to dock. Rotation of the GspIJKF complex and the pseudopilus itself occurs upon the addition of each subsequent pseudopilin, this drives rotational twist into the growing pseudopilus. In contrast, our assembly model proposes a different mechanism in which the major pilins are added sequentially to the base of the filament from three different positions, each position representing a single start of a filament with a 3-start helical symmetry. Each ATP hydrolysis cycle would extrude the filament a short vertical distance but would not require components of the assembly apparatus or the pilus itself to rotate when incorporating a new pilin subunit. While the rotary mechanism proposed in the Francetic model is plausible for the shorter T2S pseudopili, which only span the periplasm, it remains unclear how the rotational forces can assemble substantially longer T4P filaments as each pilin added would require the rotational force to twist the entire filament into the correct orientation for the next subunit to incorporate. In addition, some T4P are involved in attachment to host cells, which would limit the degree of rotation within the filament, it is not clear how the rotational forces are resolved in this scenario when both ends of the pilus are anchored. Lastly, the Francetic model does not account for (pseudo)pilus disassembly or retraction. Based on their model, the GspIJKF complex must rotate in the opposite direction to allow for pilus disassembly. As T2S systems lack a retraction ATPase, how is the process regulated and driven without active energy input?
The minor pilins between T2S and T4P are interchangeable to a degree in priming (pseudo)pilus assembly (Cisneros, Pehau-Arnaudet, and Francetic 2012; Sauvonnet, Gounon, and Pugsley 2000) though (pseudo)pilus mediated functions are not recovered through heterologous assembly. While GspK-homologs work in conjunction with other pseudopilins to prime assembly, this task is accomplished by just one minor pilin in T4b systems. However, it remains unclear how pseudopili are disassembled or retracted as GspK-like proteins do not have an invariant Glu5 residue, suggesting that they are not likely to incorporate at the base of the pseudopilus to induce retraction. The large GspK-like proteins are hypothesized to form an oversize cap at the tip of the pseudopilus and its sheer size prevents passage through the outer membrane secretin channel, stalling assembly and immediately inducing retraction of the pseudopilus. While minor (pseudo)pilin mediated priming of pilus assembly appears to be universal between T4P and T2S systems, minor (pseudo)pilin mechanism for function appears to be different. In the N. meningitidis T4a system, it is unclear whether the functional mechanism for the minor pilins is based on being incorporated into surface-displayed pili (Bernard et al. 2014; Brissac et al. 2012) or from within the periplasm (Imhaus and Dumenil 2014). In contrast, the T4b minor pilins appear to exert their effects from within the cell. TcpB and CofB are only found in low levels within the pilus fraction and are likely exported only as a tip-associated component. We showed that minor pilin incorporation via Glu5, which can only happen within the periplasm, is critical to retractile functions.

In the simpler T4b systems, the minor pilins have a major role in pilus dynamics and functions. While one minor pilin can initiate pilus assembly, another minor pilin can subsequently incorporate into the assembling filament to induce retraction. The opposite nature of their two functions likely explain why they are expressed at such low levels as optimal pilus functions require this stoichiometric balance. There are many parallels between the T4b systems, the more complex T4a systems, and the related T2S systems. The T4b system is relatively simple, with all the proteins necessary for assembly encoded within a single gene cluster. This system has a single minor pilin and lacks a retraction ATPase. Perhaps the simpler T4b system is the most primitive system of the three, giving rise to the T2S and T4a systems (Figure 4-1). Both the T2S system and the unique T4b BFP system encode several minor pilins that might have arisen from
duplication of the minor pilin gene. The BFP system also possess a putative retraction ATPase in its operon (Brossay et al. 1994), suggesting this system could be the intermediate precursor to the more complicated T4a systems. The T4a system is the most complicated of the three, encoding multiple minor pilins and retraction ATPase(s) in multiple gene clusters. Understanding how a single minor pilin can balance both pilus biogenesis and retraction will provide insight into how multiple minor pilins facilitate the same tasks in the more complex (pseudo)pilus systems.

Figure 4-1  Evolutionary relationship between type IV pilus and type II secretion systems

Proposed evolutionary relationship between the type IVa, type IVb, and type II secretion systems. The simpler T4b pilus systems have all the proteins necessary for assembly encoded within a single gene cluster. The system is unique for its single minor pilin (red) and its lack of a retraction ATPase. The T2S and the EPEC BFP systems encode several minor pilins. The BFP system also has a putative retraction ATPase (purple). We propose that the more complex T4a systems, which have multiple minor pilins and retraction ATPases, like arise from the T4b systems.
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