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Minimal Requirements for Assembly of a Stable Inner Membrane Platform for *Vibrio cholerae* Pilus Biogenesis

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Abstract

V. cholerae is a gram-negative bacterium that colonizes the human intestine via toxin co-regulated pilus (TCP) and can cause diarrhea with fluid losses severe enough to be fatal. Without TCP, *V. cholerae* cannot attach to the intestine, secrete toxins, or cause GI disease. At least 10 proteins encoded by genes within the *tcp* operon of *V. cholerae* are needed to assemble TCP. Four of these proteins -- TcpD, TcpE, TcpR, & TcpT -- localize to, or peripherally associate with, the inner membrane (IM). Previous experiments support the hypothesis that not all four proteins are needed to form a stable IM platform, believed to serve as an anchor & scaffold for outward pilus assembly. Instead, as seen within *E. coli*'s bundle-forming pilus (BFP) biogenesis apparatus, interactions among three proteins are predicted to be sufficient to form a stable IM platform in *V. cholerae*. To test this hypothesis, plasmids were genetically engineered to carry combinations of *tcp* genes and induced to co-express the four IM proteins required for TCP assembly in all groups of two and three in *E. coli*. Protein immunoblots on cell lysates were analyzed to help determine which proteins may form a stable IM platform, most likely by stabilizing the TcpR-TcpT interaction. Although not conclusive, results suggest that both TcpD and TcpE stabilize the TcpR-TcpT interaction. Therefore, as predicted, three proteins required for TCP biogenesis appear to form a stable IM platform – just as seen within *E.coli*'s BFP biogenesis machinery. Future pull-down and yeast two-hybrid studies can confirm or refute these implied protein interactions. Overall, understanding the structure of proteins needed for TCP biogenesis may provide clues as to how TCP forms & functions to cause cholera. Discovering how TCP forms may also help identify methods of disrupting pilus assembly, as well as novel targets for antibiotic

development & drug therapy. Finally, the mechanism of TCP biogenesis may serve as a useful model for studying protein secretion and DNA uptake in bacteria since the proteins involved in these processes share extensive sequence, structural, and functional homology.

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Introduction

Vibrio cholerae is a gram-negative bacterium that causes the gastrointestinal disease cholera characterized by diarrhea with fluid losses severe enough to be fatal. *V. cholerae* is transmitted to humans through contaminated food or drinking water and colonizes the human small intestine via TCP (Kaper *et al*., 1995). TCP is a type IV pilus (Tfp) composed of a single pilin protein, TcpA, polymerized into long fibers (Strom & Lory, 1993). Without TCP, *V. cholerae* cannot attach to intestinal epithelial cells, effectively secrete cholera toxin, or cause gastrointestinal disease (Kirn *et al*., 2003). At least ten proteins encoded by genes within the *tcp* operon of *V. cholerae* (depicted in **Figure 2**) are needed to make TCP. Four of these proteins -- TcpD, TcpE, TcpR, and TcpT -- localize to, or peripherally associate with, the inner membrane (IM). As shown in **Figure 1**, the current working model for the TCP biogenesis apparatus consists of 11 proteins encoded by genes within the *tcp* operon (Tripathi & Taylor, 2005 poster). *V. cholerae* mutants with single deletions of any gene within the *tcp* operon, except *tcpF*, fail to make TCP. Therefore, at least 10 known proteins are required for TCP biogenesis – four of which are found within or attached to the IM. Little is understood, however, about how the proteins involved in TCP biogenesis interact to assemble functional pili. Within the IM, for instance, TcpR and TcpT are the only proteins confirmed to interact through metal affinity pull-down and bacterial two-hybrid experiments (Tripathi & Taylor, 2007). Previous experiments support the hypothesis that not all four -- but combinations of three – IM proteins involved in TCP biogenesis are needed to form a stable IM platform, likely by stabilizing the TcpR-TcpT interaction as illustrated in **Figure 5** (Tripathi & Taylor, 2005). An IM platform within the TCP biogenesis

apparatus is believed to serve as an anchor and scaffold for outward pilus assembly, which involves: 1) pilus assembly in the periplasm and (2) transfer of the growing pilus to the cell surface (Wolfgang *et al*., 2000).

Background

Functions of TCP

V. cholerae requires two virulence factors to cause disease: cholera toxin and TCP (Waldor & Makalanos, 1996). First, cholera toxin is a potent enterotoxin largely responsible for the symptoms of cholera. Cholera toxin causes 'rice water' stool by disrupting electrolyte balance in the small intestine such that epithelial cells expel water and ions (Spangler, 1992; Kaper *et al*., 1995). Second, TCP is a Type IV pilus expressed on the surface of *V. cholerae* which is believed to function in: (1) adhesion to the human small intestine, (2) bacterial interactions mediating colonization, (3) DNA uptake via phage transduction and natural transformation, and (4) extracellular protein secretion.

TCP is believed to allow *V. cholerae* to adhere to epithelial cells of the small intestine where the bacteria can colonize, proliferate, and cause GI disease (Taylor et al., 1987). No epithelial cell receptor for TCP, however, has been identified.

Pilus-pilus contacts among *V. cholerae* mediate microcolony formation which promotes colonization of the small intestine (Kirn *et al*., 2000). TCP-deficient *V. cholerae* -- carrying mutations in the gene encoding the structural pilin TcpA -- are unable to colonize the human intestine (Herrington *et al*., 1988). Microcolonies facilitate colonization by increasing intestinal concentrations of bacteria, likely enhancing their proliferation, growth, persistence, and potentially dissemination to surrounding tissues (Kirn *et al*., 2000). Association into microcolonies may also help bacteria hide from antimicrobial host defenses like antibodies and the complement system (Kirn *et al*., 2000). Pilus-pilus interactions may also be responsible for the autoagglutination or clumping of *V. cholerae* observed in liquid cultures (Taylor et al., 1987).

TCP serves as a receptor for the bacteriophage CTXΦ which carries the structural genes for cholera toxin (Waldor & Makalanos, 1996; Wolfgang *et al*., 2000). Transduction of CTXΦ via TCP infects new virulent strains of *V. cholerae* (Waldor & Makalanos, 1996). *V. cholerae* must colonize the human intestine via TCP before it can secrete cholera toxin, a potent enterotoxin, via a T2S pathway called the extracellular protein secretion (EPS) system (Kirn *et al*., 2003). Cholera toxin acts by elevating cyclic AMP levels in the epithelial cells of the small intestine, thereby disrupting ion transport and electrolyte balance (Kaper *et al*., 1995; Kirn *et al*., 2003).

Regarding extracellular protein secretion, the TCP biogenesis apparatus mediates the extracellular secretion of the soluble colonization factor TcpF. In a two step process, (1) the EPS T2S pathway transports TcpF to the periplasm, then (2) the TCP biogenesis apparatus transports TcpF across the OM (Bose & Taylor, 2005). Mutant *V. cholerae* strains carrying deletions in *tcpF* (Δ*tcpF*) still assemble TCP, but have a remarkably reduced ability to colonize the mouse small intestine (Kirn *et al*., 2003).

Assembly of Type IV Pili

Expressed by gram-negative bacteria, type IV pili are related by their: (1) structural pilins which share extensive sequence homology (especially within their N-terminal ends) and contain a leader peptide that is cleaved prior to mature processing and (2) well-conserved biogenesis machineries (Strom & Lory, 1993; Wolfgang *et al*., 2000). Little is understood about how proteins within the biogenesis machineries of type IV pili function, interact, and ultimately work together to assemble functional pili. The functions of three proteins – TcpJ, TcpT, and TcpC -- involved in TCP biogenesis,

however, have begun to emerge and are providing insight into the mechanics behind this complex process (Bose & Taylor, 2005).

Type IV pilus biogenesis can be divided into two main steps: (1) pilus assembly in the periplasm and (2) extrusion of the growing pilus through the OM to the cell surface (Wolfgang *et al*., 2000). Regarding pilus assembly, TcpJ is a type IV prepilin peptidase which cleaves the 25 amino-acid N-terminal leader peptides from TcpA precursors to produce mature pilins that are polymerized into a fiber in the periplasm (LaPointe & Taylor, 2000). Removal of this leader peptide by TcpJ is required for stable pilin expression, polymerization, and translocation across the IM (Strom & Lory, 1993). In addition, TcpT is an ATPase tethered to the IM which is believed to generate all the energy needed for TCP biogenesis (Tripathi & Taylor, 2007).

Evidence suggests that type IV pili are extruded through the OM as intact fibers (Wolfgang *et al*., 2000). Translocation across the OM is believed to occur through a large secretion pore formed by a homomultimeric ring of secretin proteins (Wolfgang *et al*., 2000). In *V. cholerae*, 12 subunits of the lipoprotein TcpC are believed to form the OM secretion channel (Parsot *et al.*, 1991; Bose & Taylor, 2005). Evidence that type IV pili are translocated through the OM as intact fibers comes from *N. gonorrhoeae* mutants lacking the secretin pore-forming protein which express intact pili within the cell (Wolfgang *et al*., 2000). This suggests that pilus assembly occurs prior to and independently of pilus translocation to the cell surface. Furthermore, the diameter of protein transport channels formed by secretins in the OM of gram-negative bacteria like *P. aeruginosa* are large enough to accommodate the 80 Å diameter of functional TCP (Bitter, 2003; Taylor *et al*., 2004). Studies have proposed the secretin protein channel is

gated to retain the structural integrity of the OM as a cell barrier (Wolfgang *et al*., 2000). Within *V. cholerae*'s TCP biogenesis apparatus, TcpQ – proven to directly interact with TcpC using metal affinity pull-down experiments – has been proposed to facilitate the opening and closing of the gated OM pore (Bose & Taylor, 2005).

Regarding the functional significance of IM proteins within *V. cholerae*'s TCP biogenesis apparatus, the IM platform may serve as an anchor, scaffold, and energy supplier for pilus assembly and localization (Py *et al.*, 2001). The IM proteins may help to anchor TCP in the IM (Py *et al.*, 2001). The IM platform also believed to serve as a scaffold on which other proteins of the TCP biogenesis apparatus can assemble outwardly to span the periplasm and outer membrane (Py *et al.*, 2001). In addition, the IM platform is important for generating energy to build TCP via the ATPase activity of TcpT (Tripathi & Taylor, 2007).

IM Protein Interactions within *E. coli*'s BFP Biogenesis Apparatus: A Model System

Enteropathogenic *E. coli*'s bundle-forming pilus (BFP) and *V. cholerae*'s TCP are well studied type IV pili. Since the biogenesis machinery of type IV pili is well conserved among gram-negative bacteria, what is known about the BFP biogenesis apparatus can be used to make predictions about protein interactions within the inner membrane platform of *V. cholerae*'s TCP biogenesis apparatus. **Figure 3** depicts the predicted structures of IM proteins comprising *V. cholerae*'s TCP biogenesis apparatus and *E. coli*'s BFP biogenesis apparatus based on biochemical evidence (Tripathi & Taylor, 2005 poster; Crowther *et al*., 2004). The TCP biogenesis apparatus consists of four IM proteins, while the BFP biogenesis apparatus consists of three (Crowther *et al*., 2004). *E. coli*'s BfpC, BfpE, and BfpT proteins resemble *V. cholerae*'s TcpR, TcpE, and

TcpT proteins, respectively (Tripathi & Taylor, 2007). To standardize the notation when comparing these biogenesis machineries, proteins from the BFP biogenesis apparatus of *E. coli* will be referred to as homologs of TcpR, TcpE, and TcpT.

Using a yeast two-hybrid system, the following IM proteins of the BFP biogenesis apparatus were found to interact: TcpT-TcpR, TcpT-TcpE, and TcpR-TcpE homologs (Crowther *et al*., 2004). To examine the stability of the IM proteins required for BFP biogenesis alone versus together, combinations of purified proteins were subjected to digestion by proteases. Individually, the TcpR and TcpT homologs were relatively resistant to degradation by protease. The susceptibility of the TcpR and TcpT homologs to protease digestion increased drastically, however, in the presence of one another (Crowther *et al*., 2004). Among IM proteins required for BFP biogenesis in *E. coli*, there appears to be a trend that each protein (homologous to TcpR, TcpE, and TcpT) is stable alone, any pair (homologous to TcpR-TcpT, TcpR-TcpE, and TcpT-TcpE) is unstable, and all three IM proteins (homologous to TcpR-TcpE-TcpT) are stable together (Crowther *et al*., 2004). This pattern of differential stability suggests that binary protein interactions induce conformational changes in the proteins rendering them more sensitive to protease digestion. The addition of a third protein, however, stabilizes the complex and protects the proteins from protease degradation (Crowther *et al*., 2004). Due to extensive homology, there is reason to believe this pattern of IM protein stability observed within the BFP biogenesis apparatus may also exist within the TCP biogenesis apparatus of *V. cholerae*.

IM Protein Interactions within *V. cholerae*'s TCP Biogenesis Apparatus

TcpR-TcpT Only Known IM Protein Interaction within TCP Biogenesis Apparatus

Localization, bacterial two-hybrid, and metal affinity pull-down studies have demonstrated that TcpR and TcpT interact (Tripathi & Taylor, 2007). Evidence includes the observation that TcpT properly localizes to the IM in a wildtype (O395) strain of *V. cholerae*, but mislocalizes to the cytoplasm when expressed in a *tcpR* deletion (Δ*tcpR*) strain of *V. cholerae* (Tripathi & Taylor, 2007). Complementation of the Δ*tcpR* strain of *V. cholerae* with a plasmid expressing TcpR rescued the wildtype phenotype -- as evidenced by the production of functional TCP able to autoagglutinate and secrete TcpF, as well as TcpT properly localized to the IM (Tripathi & Taylor, 2007). As in the wildtype strain of *V. cholerae*, TcpT remained properly localized to the IM when expressed in Δ*tcpD* and Δ*tcpE* strains of *V. cholerae*. These localization studies suggest that TcpR and TcpT interact, and that TcpR – but not TcpD or TcpE -- is needed to tether TcpT to the IM (Tripathi & Taylor, 2007). Without TcpR, TcpT mislocalizes to the cytoplasm.

TcpR-TcpT Interaction Requires Additional Proteins to Become Stable

After TcpR and TcpT were discovered to interact, the following question arose: is TcpR sufficient to tether to TcpT to the membrane? In other words, is the TcpR-TcpT interaction stable alone-- or are additional proteins required for its stabilization? To test this experimentally, TcpR was fused with the protein PhoA (the carboxyl-terminal portion of alkaline phosphatase) to ensure that TcpR properly localized to the IM. When TcpR-PhoA and TcpT were co-expressed in a Δ*tcpP* strain of *V. cholerae* (in which the

tcp operon is turned off), TcpT was found to degrade (Tripathi & Taylor, 2005 poster). That is, TcpT could not be detected in the cytoplasm or IM cellular fractions using protein immunoblotting with anti-TcpT antibody (Tripathi & Taylor, 2007). In contrast, when expressed alone in a Δ*tcpP* strain of *V. cholerae*, TcpT was found to be stable, but mainly mislocalized to the cytoplasmic fraction when detected via protein immunoblotting (Tripathi & Taylor, 2005 poster). This change in the stability of TcpT when expressed alone versus co-expressed with TcpR-PhoA suggests that, upon interaction, TcpR-PhoA may induce a change in the shape of TcpT which makes TcpT more unstable and susceptible to digestion by proteases. Complementation of the Δ*tcpR* strain of *V. cholerae* with a plasmid expressing TcpR-PhoA rescued proper localization of TcpT to the IM – suggesting that TcpT degradation is not a result of TcpR's fusion with PhoA (Tripathi & Taylor, 2007). Overall, the TcpR-TcpT interaction is not stable alone. Additional proteins are needed to stabilize the TcpR-TcpT interaction and to protect TcpT from degrading -- potentially by physically covering sites on TcpT where proteases can attack or preventing a conformational change that destablilizes TcpT.

TcpD and TcpE Appear to Perform Redundant Function in Stabilizing TcpR-TcpT

Another experiment providing clues as to the IM structure of proteins within the TCP biogenesis apparatus demonstrated that TcpT remains stable in all *V. cholerae* mutants carrying single deletions of a gene within the *tcp* operon (Tripathi & Taylor, 2007). That is TcpT can be detected via protein immunoblotting of cell lysates from all *V. cholerae* strains carrying single deletions of *tcp* genes. TcpT is detected in the IM fraction of all the single deletion mutants, except the TcpR deletion (ΔTcpR) strain in which TcpT is detected in the cytoplasm (Tripathi & Taylor, 2007). This data suggest

that more than one protein required for TCP biogensis is stabilizing the TcpR-TcpT interaction. Since TcpD and TcpE are the only other IM proteins involved in assembling TCP, they are likely candidates for performing a redundant function in stabilizing the TcpR-TcpT interaction. In other words, TcpD could be stabilizing the TcpR-TcpT interaction in a Δ*tcpE* strain of *V. cholerae*, while TcpE is stabilizing this interaction in a Δ*tcpD* strain. So, a third protein – namely TcpD alone or TcpE alone -- may be sufficient for stabilizing the TcpR-TcpT interaction. These two models of stable IM platforms within the TCP biogenesis apparatus -- TcpD-TcpR-TcpT and TcpE-TcpR-TcpT -- are illustrated in **Figure 5**. Of note, these models are consistent with evidence from *E. coli*'s BFP biogenesis apparatus that three proteins are sufficient to form a stable IM platform.

Similarities between Tfp Biogenesis Machinery & T2S & DNA Uptake Systems

Proteins involved in assembling TCP and other type IV pili share considerable structural and functional homology with proteins involved in type II protein secretion (T2S) and DNA uptake in gram-negative bacteria (Strom & Lory, 1993; Kirn *et al*., 2003). In some gram-negative bacteria, the same proteins function in more than one of these related processes. The extensive similarities among type IV pili biogenesis, T2S, and DNA uptake systems seem to suggest a shared evolutionary history and shared functional mechanisms. This seems likely because proteins involved in TCP biogenesis have been implicated in both the extracellular protein secretion of colonization factor TcpF and the uptake of bacteriophage CTXΦ DNA carrying the genes for cholera toxin (Kirn *et al*., 2003). Overall, the mechanism of Type IV pilus biogenesis may serve as a useful model for studying processes that facilitate the transport of macromolecules across cell membranes.

Tfp Biogenesis & T2S Machineries

The T2S pathway refers to the process by which proteins are transported across the outer membrane of gram-negative bacteria (Sandkvist, 2001). T2S is a two-step process in which: (1) proteins bearing a signal peptide are transported across the IM via the Sec pathway then (2) signal peptides are removed and proteins are translocated from the periplasm across the OM (Sandkvist, 2001). Several proteins involved in TCP biogenesis share structural and functional homology with proteins involved in the extracellular protein secretion (EPS) system, a T2S pathway of *V. cholerae* (Kirn *et al*., 2003). Four similarities between proteins involved in the EPS system of *V. cholerae* and proteins encoded by the *tcp* operon include: (1) "pseudoplin" proteins EpsG, H, I & J of the T2S system resemble the pilin protein TcpA comprising TCP; (2) EpsE of the T2S system resembles TcpT of the TCP biogenesis apparatus because both are ATPases that peripherally associate with the IM; (3) VcpD of the T2S system resembles TcpJ of the TCP biogenesis apparatus because both are type IV prepilin peptidases that remove the signal sequences of type IV prepilins to produce mature pilins, like TcpA, that can be secreted outside the cell; and (4) EpsD of the T2S system resembles TcpC of the TCP biogenesis apparatus because both are believed to be secretins that form a multimeric ring in the OM that serves as a gated channel for extracellular protein secretion (Kirn *et al*., 2003).

Further evidence suggests that the processes for assembling type IV pili and secreting proteins via the type II system operate through similar mechanisms. In some bacterial species, the same proteins function in both T2S (or secretion pathways closely resembling T2S) and type IV pilus biogenesis. Regarding *V. cholerae*, the TCP

biogenesis apparatus facilitates TcpF secretion by helping the soluble colonization factor transit the OM (Kirn *et al*., 2003). That is, TcpF secretion appears to occur via the TCP biogenesis apparatus in a process closely resembling T2S (Kirn *et al*., 2003). The secretion pathway for TcpF appears to at least partially overlap with that for TcpA because over-expression of TcpF results in reduced TCP assembly, presumably due to reduced TcpA secretion (Kirn *et al*., 2003). Yet another link between these systems is that proteins from the T2S system of some gram-negative bacteria, like *Pseudomonas aeruginosa*, have been shown to form pili when over-expressed (Durand *et al*., 2003). One proposed function of the pilus formed by T2S machinery is to serve as a piston, extending and retracting to force proteins across the OM through a secretion channel (Crowther *et al*., 2004; Sandkvist, 2001).

Tfp Biogenesis & DNA Uptake Machineries

DNA uptake in bacteria functions: (1) to promote genetic adaptability and diversity by acquiring DNA that potentially confers some benefit for survival -- such as antibiotic resistance or virulence factors, (2) to repair damaged DNA by using DNA acquired from closely related bacteria as a template for synthesis, and (3) to use DNA as a source of carbon, nitrogen, and phosphorous (Dubnau, 1999; Chen & Dubnau, 2004). In *V. cholerae*, TCP directly functions in DNA uptake as a receptor for the transduction of CTXΦ – a filamentous bacteriophage whose DNA encodes the genes for cholera toxin (Waldor & Makalanos, 1996). After infection via TCP, the CTXΦ genome can integrate into the bacterial chromosome or replicate independently as a plasmid -- giving rise to new virulent strains of *V. cholerae* by (Waldor & Makalanos, 1996). In naturally competent gram-negative bacteria, extensive similarities exist between cellular

machinery involved in DNA uptake during transformation and the biogenesis machinery of type IV pili (Chen & Dubnau, 2004). DNA uptake systems in gram-negative bacteria also form competence pseudopili that resemble that type IV pili regarding their major structural pilins, prepilin peptidases, ATPases associated with IM, and OM secretins (Chen & Dubnau, 2004). A correlation exists between the production of competence pseudopili and ability of gram-negative bacteria to acquire DNA via natural transformation (Chen & Dubnau, 2004).

Among gram-negative bacteria, proteins involved in DNA uptake via natural transformation resemble proteins involved in Type IV pilus biogenesis (Chen & Dubnau, 2004). In general, natural transformation in gram-negative bacteria can be divided into three steps: (1) binding of DNA to the cell surface, (2) DNA uptake into the periplasm through an OM pore made of secretins, and (3) transport of one DNA strand across the IM while the other strand is degraded (Dubnau, 1999). DNA uptake specifically refers to the transition of DNA from a DNase-sensitive to a DNase-protected state and is generally accomplished in gram-negative bacteria by passage through the OM (Chen & Dubnau, 2004). Another link between these systems is that proteins involved in DNA uptake are believed to form a pilus-like structure (Dubnau, 1999). Still further support these processes operate via similar mechanisms emerges from the discovery that several proteins required for type IV pilus biogenesis -- including IM peptidases and OM secretins – are also required for natural competence in some gram-negative bacteria like *N. gonorrhoeae* (Dubnau, 1999; Wolfgang *et al.*, 1998; Crowther *et al.*, 2004). For instance, the structural pilin comprising the type IV pilus of *N. gonorrhoeae* is required for the binding of DNA to the cell surface in the first step of natural transformation

(Dubnau, 1999; Chen & Dubnau, 2004). Overall, discovering how proteins interact within *V. cholerae*'s TCP biogenesis machinery has promising implications for understanding diverse cellular processes.

Methods

Engineering Plasmids pAJ1-pAJ5

To investigate IM protein interactions within *V. cholerae*'s TCP biogenesis apparatus, five plasmids -- pAJ1-pAJ5 -- were engineered to carry various combinations of *tcp* genes (shown in **Figure 6**). Plasmids expressing *tcpE* alone and *tcpR* alone were previously engineered. The cloning strategy was devised to use the fewest restriction enzymes needed to build compatible plasmids for co-expression of *V. cholerae*'s IM Tcp proteins in all combinations of two and three in *E. coli* (see **Figures 7 & 8**, respectively). The cloning strategy was also designed to express all combinations of Tcp proteins in *E. coli* by transformation with just two plasmids.

To construct the plasmids pAJ1-pAJ5, *V. cholerae* genomic DNA was extracted and purified using Gentra Systems' PureGene Purification System. IM *tcp* genes were amplified from *V. cholerae*'s genome by PCR. PCR primers were designed to contain the restriction sites shown flanking the *tcp* genes in **Figure 6**. By utilizing primer pairs with different restriction sites, PCR-amplified *tcp* genes were more likely to ligate with vectors in the proper orientation. Amplified *tcp* genes were purified with Qiagen's Qiaquick PCR Purification Kit (according to the manufacturer's "microcentrifuge" protocol). In addition, the following plasmid vectors were extracted and purified from bacteria using Qiagen's Miniprep Plasmid Extraction Kit (according to the manufacturer's "miniprep" protocol): (1) empty pBAD33 and (2) pBAD33 carrying *tcpE* fused to a 6-histidine tag (or pEF) from *E. coli* and (3) pBADTOPO carrying *tcpR* fused to a 6-histidine tag from *V. cholerae*. The purity and amount of extracted plasmid DNA

were measured using the NanoDrop ND-1000 Spectrophotometer at an absorbance of 260nm. Of note, all the plasmids contained arabinose-inducible promoters.

The extracted plasmids (vectors) and PCR-amplified *tcp* genes (inserts) were all digested with two restriction enzymes as outlined in **Figure 6**. Double-digested vectors and inserts were separated by electrophoresis on a 1% agarose gel, visualized with ethidium bromide, gel-extracted, and purified using Qiagen's Gel Extraction and Purification Kit (according to the manufacturer's "microcentrifuge" protocol). Surprisingly, DNA bands in the gel's pAJ2 - pAJ5 lanes revealed that the Nhe restriction site was not unique to the multiple cloning site (MCS) of pBAD33 vectors (see **Figures 1 & 2, Appendix A**). Rather, two distinct Nhe restriction sites exist as depicted in the accurate pBAD33 vector map in **Figure 3** in **Appendix A**. To work around this obstacle without re-designing the entire cloning strategy, plasmids were digested with the restriction enzyme Nhe alone for 10, 30, 60, and 180 minutes. After ten minutes, Nhe was found to cut some pBAD33 vector only once. At longer times, Nhe completely digested the plasmids by cutting at both restriction sites (see **Figure 3** in **Appendix A**). So, only the band representing "partially digested plasmids – those cut once at the Nhe site within the MCS -- were gel-extracted, digested with a second restriction enzyme, and used for genetic engineering.

Vectors and inserts digested with the same restriction enzymes were joined with DNA ligase to form pAJ1-pAJ5 (shown in **Figure 6**). The NanoDrop ND-1000 Spectrophotometer and quantitative DNA gels helped determine the relative amounts of vector and insert needed to combine to make pAJ1-pAJ5. The pAJ1-pAJ5 ligations were spot dialyzed against dH_20 for 30 minutes to remove excess salts. Electrocompetent *E.*

coli were electroporated with each pAJ1-pAJ5 ligation at 1.7 kV during a one second pulse. Of note, electocompetent *E. coli* were prepared by growing cells to an OD 600 of 0.3-0.7, then repeatedly centrifuging and re-suspending the cells in cold sterile water. Vials of electroporated cells were taped to a rotating test tube rack and incubated for 45 minutes at 37°C to allow sufficient time for gene expression of critical proteins, including those conferring antibiotic resistance. For example, the pBAD33 and pEF vectors (pAJ1-pAJ5) carry chloramphenicol resistance, while pBADTOPO (p*tcpR*) contains the ampicillin resistance gene. To select *E. coli* transformed with the pAJ1-pAJ5 ligations, electroporated cells were spread on antibiotic plates containing 100 μg/mL ampicillin or 15 μg/mL chloramphenicol.

Using "Colony PCR" to Select *E. coli* Transformed with pAJ1-pAJ5

Since plasmid vectors carry the antibiotic resistance genes, bacteria transformed with empty plasmids are able to grow on the antibiotic selection plates. "Colony PCR," therefore, was used to screen for "positive" colonies – colonies transformed with plasmids carrying the properly inserted *tcp* gene(s) of interest (see **Figures 4-7** in **Appendix A**). From the antibiotic selection plates for *E. coli* transformed with the pAJ1-pAJ5 ligations, cells from 18 colonies were dilution-streaked on new antibiotic plates (3 plates divided into sixths) and incubated at 37°C overnight. Isolated colonies from 12 of the 18 dilution-streaked parent colonies were removed from each pAJ1-pAJ5 ligation plate, suspended in 25uL dH_20, and pre-heated to 95°C using a PCR machine. For each pAJ1-pAJ5 transformation, PCR was performed using: cells from the 12 selected colonies, primers specific to the *tcp* gene(s) on pAJ1-pAJ5, Taq polymerase, 10X pfu buffer, dNTPs, and dH_20. PCR products were run on a 1% agarose gel to detect

amplified *tcp* genes and reveal "positive" colonies transformed with pAJ1-pAJ5. *Tcp* genes amplified from the wildtype *V. cholerae* genome were used as positive controls.

Testing Ability of Engineered Plasmids to Rescue WT Phenotype

When feasible, complementation tests helped reveal whether plasmids expressed the *tcp* genes they were engineered to carry. *V. cholerae* strains with single deletions (Δ) of *tcp* genes were electroporated with plasmids engineered to carry these *tcp* genes. For example, the *V. cholerae* deletion strains Δ*tcpD*, Δ*tcpR*, and Δ*tcpT* were electroporated with pAJ1 (p*tcpD*), p*tcpR*, and pAJ5 (p*tcpT*), respectively, at 2.5 kV during a one second pulse. Electroporated cells were incubated at 30°C for 45 minutes and transformed *V. cholerae* cells were selected on antibiotic-containing agar plates. Liquid cultures of "positive" colonies transformed with pAJ1, p*tcpR*, and pAJ5 were grown overnight at 30°C and examined for their ability to "complement" or "rescue" the wildtype *V. cholerae* phenotype. Wildtype rescue was qualitatively measured by the ability of cells to auto-agglutinate. So, rescue was visually confirmed by clumping of cells along the test tube walls of liquid cultures.

Building *E. coli* Strains for Co-Expression of *V. cholerae*'s IM Tcp Proteins

To build *E. coli* strains co-expressing *V. cholerae*'s IM Tcp proteins in all pairs and groups of three, pAJ1-pAJ5 were extracted and purified from their respective "positive" colonies using the "Qiagen Miniprep Plasmid Extraction Kit." Extracted plasmids were spot dialyzed against dH_20 for 30 minutes. Electrocompetent *E. coli* were electroporated with the pairs of plasmids (or "co-expression vectors") outlined in **Figures 7 & 8**. Electroporated cells were incubated at 37°C for 45 minutes to allow sufficient

time for the transcription and translation of critical genes, including those conferring antibiotic resistance. To select only *E. coli* transformed with the appropriate co-expression vectors, cells were spread on agar plates containing both chloramphenicol and ampicillin. Various volumes of electroporated cells were plated, then incubated at 37°C overnight to find a concentration at which single, isolated colonies grew.

Of note, due to time pressures, the *tcp* genes carried by pAJ1-pAJ5 were sequenced through Dartmouth's cycle sequencing service only after the *E. coli* co-expression strains carrying these plasmids were built and used in experiments. However, *E. coli* strains expressing TcpR alone and TcpE alone were previously built and frozen in glycerol stocks. Sequencing experiments confirmed these strains contained plasmids carrying the wildtype genes.

Immunoblotting to Predict Interactions Among IM Proteins within *V. cholerae*'s TCP Biogenesis Apparatus

SDS-PAGE protein immunoblotting was used to detect when proteins were stably expressed in *E. coli*. To make samples for the protein gel, liquid cultures of the *E. coli* "co-expression strains" -- strains genetically engineered to co-express IM TCP proteins in groups of two or three – and of the relevant controls were prepared. Portions of isolated *E. coli* colonies transformed with each pair of co-expression vectors were removed from the antibiotic selection plates and mixed with 5mL LB broth containing 5μL chloramphenicol and 5μL ampicillin. Varying concentrations (0%, 0.02%, and/or 0.1%) of arabinose were added to the liquid cultures of each co-expression strain. This helped determine at what concentration arabinose increased protein expression from the plasmids' arabinose-inducible promoters. Liquid *E. coli* cultures were incubated on a

rotating test tube rack at 37°C overnight, while *V. cholerae* cultures were incubated at 30°C overnight. For each co-expression strain, 500µL of liquid culture was centrifuged at 14,000 rpm for 2 minutes and cell pellets were re-suspended in 200µL 2X SDS protein sample buffer (composed of 0.5mL 1% bromophenol blue, 1mL beta-mercaptoethanol, 6mL 10% SDS, 2.5mL 4X upper buffer [6.06 gm Tris base and 4mL 10% SDS with pH adjusted to 6.8 and volume adjusted to 100mL with dH_20], 4mL glycerol, and 6mL dH_20). Samples were boiled for 10 minutes and 30µL of each sample was loaded on a 12.5% acrylamide gel. Gel electrophoresis was used to separate proteins from the cell lysates of each co-expression strain. Proteins were transferred to a nitrocellulose membrane in 1X transfer buffer (composed of 100mL 10X transfer buffer [30.3 gm 0.25M Tris base and 0.144 gm glycine with volume adjusted to 1L with dH_20], 200mL MeOH, and 700mL dH_20 with volume adjusted to 1L) at 200V for one hour. Qiagen's anti-His antibody was used to detect TcpE and TcpR since they are fused to a 6-Histidine tag. Anti-TcpT and anti-TcpD polyclonal antibodies directly recognized these proteins. Overall, protein immunoblots were used to predict interactions among IM proteins within the TCP biogenesis appartus.

Results

Based on previous experimental results, this paper makes the following predictions about the stability of IM proteins required for TCP biogenesis when expressed in *E. coli*: (1) all proteins, except TcpR, will be stable alone, (2) any pair of proteins (TcpR-TcpT, TcpR-TcpE, TcpT-TcpE, TcpR-TcpD, TcpE-TcpD, TcpT-TcpD) will not be stable, and (3) two triplets of proteins (TcpD-TcpR-TcpT and TcpE-TcpR-TcpT) will be stably expressed. These predictions closely reflect the known IM protein interactions within *E. coli*'s BFP biogenesis machinery and account for preliminary experimental results in *V. cholerae*.

Sequencing Results for *tcp* Genes on pAJ1-pAJ5

Due to time constraints, *E. coli* strains containing the co-expression vectors in **Figures 7 & 8** were built before the *tcp* genes on the plasmids were sequenced. Eventually, however, the sequencing results (summarized in **Table 1**) helped to explain some unexpected findings and unveiled mutants that are valuable for future studies. Results concluded the *tcpD* and *tcpT* sequences of pAJ2, as well as the *tcpE* and *tcpT* sequences of pAJ4 were all correct. PAJ1 and pAJ5, however, were found to carry mutations useful to study. For instance, the mutated *tcpD* gene on pAJ1 results in a175 amino acid long truncated TcpD that is missing 103 amino acids from its C-terminus. Although this truncated protein contains the TcpD antibody binding region, it cannot be detected on a protein immunoblot. Moreover, the mutated *tcpT* gene on pAJ5 changes the fourth cysteine in TcpT's tetra-cysteine bridge to serine. This mutation is especially valuable to investigate further because the tetra-cysteine bridge is believed to be critical

for TcpT to function properly as an ATPase. Lastly, the sequencing reaction for *tcpD* and *tcpE* on pAJ3 was not successful and must be repeated.

Protein Stability Analysis via Immunoblotting to Predict IM Protein Interactions Within TCP Biogenesis Apparatus

Only protein immunoblots were used to detect stable TCP proteins because Coomassie-staining techniques were not specific enough to detect the desired proteins among total cell proteins. The top of **Figure 10** shows an anti-His protein immunoblot to detect TcpR which carries a 6X-His tag. The positive control for TcpR is the dark band in the ΔR + R lane which proves the plasmid carrying *tcpR* complements the ΔR deletion strain of V. cholerae. TcpR can be seen in the pAJ2 + TcpR lane in which the three proteins TcpD, TcpT, and TcpR -- predicted to form a stable IM platform – are expressed. Detection of an at least partially stabilized TcpR in the presence of TcpD and TcpT is one major novel finding of this paper. The anti-His protein immunoblot does not suggest, however, how the stability of TcpR in the presence of TcpD and TcpT compares with the stability of TcpR in wildtype *V. cholerae* because the wildtype strain was not run as a control. Surprisingly, TcpR appears very faintly in the pAJ1 + TcpR lane in which TcpD and TcpR are expressed. This is unexpected because previous experiments have consistently shown that when wildtype TcpD and TcpR are co-expressed, TcpR cannot be detected on a protein immunoblot. This apparent discrepancy is resolved by sequencing results showing the TcpD expressed by pAJ1 is a largely truncated protein. Apparently, this truncated TcpD is in a conformation, different from the wildtype TcpD, that partially stabilizes TcpR. So, some region within the first 175 amino acids of TcpD is important in stabilizing TcpR.

The bottom of **Figure 10** is an anti-TcpD protein immunoblot of the same membrane as above after stripping and re-probing with antibody. Although there is no positive control, TcpD is represented by the lowest band on the membrane. As shown, TcpD is stable in the pAJ2 and pAJ2 + R lanes when induced with 0.02% and 0.1% arabinose. The arabinose concentration of 0.02% is more effective in enhancing the expression of TcpD from the pBAD33 vector than 0.1% since the band is darker at this lower concentration. Interestingly, TcpD does not appear in the pAJ1 or pAJ1 + R lanes. Sequencing results again explain the unexpected finding that even though it still contains the TcpD antibody-binding region, the largely truncated TcpD cannot be detected on a protein immunoblot. This observation again suggests the truncated protein is in a conformation different from that of wildtype TcpD -- which blocks or hides the site where TcpD antibody binds.

The anti-His protein immunoblot in **Figure 11** should visualize TcpR and TcpE which both carry identifying 6X-His tags. All of the plasmids used were induced with the ideal arabinose concentration of 0.02%. For some unknown reason, the wildtype *V. cholerae* ("0395") strain failed as a positive control because TcpR and TcpE cannot be seen in this lane. While the dark band in the ΔR + R lane can serve as a positive control for TcpR, no positive control exists for TcpE. The lanes for pEF, pAJ4, and pAJ5 – all of which carry TcpE – do not share a unique band. Therefore, only the stability of TcpR can be addressed on this membrane. TcpR is seen in the pAJ4 + TcpR lane in which the three proteins TcpE, TcpT, and TcpR – also predicted to form a stable IM platform – are expressed. Detection of an at least partially stabilized TcpR in the presence of TcpE and TcpT is the other major novel finding of this paper. Surprisingly, TcpR appears even

more stable in the pAJ5 + R lane in which only TcpT and TcpR are expressed. This observation contradicts previous experimental results that demonstrate when wildtype TcpT and TcpR are co-expressed, neither can be detected on a protein immunoblot. However, the apparent discrepancy can be resolved by sequencing results showing the TcpT expressed by pAJ5 has a mutation within its tetra-cysteine bridge, believed to be critical for proper ATPase functioning. This suggests that the mutated TcpT is in a conformation, distinct from wildtype TcpT, that is able to stabilize TcpR. Clearly, TcpT's tetra-cysteine bridge must be very important since the mutated TcpT differed from wildtype by only a single amino acid. Of note, TcpR can no longer be detected in the pAJ2 + R lane as it was in the top of **Figure 10**. This is believed to have occurred because a 'negative' pAJ2 colony was accidentally used – that is, a colony which took up the plasmid, but without the correctly inserted *tcpD* and *tcpT* genes.

Figure 12 is an anti-TcpT blot. As in **Figure 10**, all of the represented plasmids were induced with the ideal arabinose concentration of 0.02%. Moreover, the wildtype *V. cholerae* (0395) strain again failed as a positive control because TcpT cannot be detected in this lane. Based on band position, however, TcpT is almost certainly represented by the highest band on the membrane. So, TcpT is detectable in the pAJ4 and pAJ4 + R lanes. Interestingly, TcpT appears even more stable in the pAJ5 and pAJ5 + R lanes. As previously mentioned, this observation conflicts with past data showing that wildtype TcpT is not stable when expressed with wildtype TcpR and that TcpT degrades in the presence of TcpR-PhoA. This conflict is also resolved by acknowledging the TcpT expressed by pAJ5 is mutated within its tetra-cysteine bridge. Apparently, the mutated TcpT's single amino acid change prevents wildtype TcpR from inducing a

conformational change in this protein which, in turn, renders it more susceptible to digestion by proteases.

Overall, these experimental results paint a partial, yet very intricate picture. This paper's novel findings suggest that TcpD and TcpT together, as well as TcpE and TcpT together can at least partially stabilize TcpR. Results would likely show that TcpD and TcpE can each stabilize the TcpR-TcpT interaction if the membranes in **Figures 10** and **11** were probed with ant-TcpT antibody to confirm the presence of TcpT along with TcpR. So, the data seems to suggest that TcpD-TcpR-TcpT and TcpE-TcpR-TcpT can -- in fact -- form stable IM platforms, although no combination of all three proteins was successfully detected on protein immunoblots presented in this paper. With TcpD-TcpR-TcpT (pAJ2 + R) co-expression, TcpT was not detected because the membrane was not re-stripped and probed with anti-TcpT antibody. This could, however, easily be done. With TcpE-TcpR-TcpT (pAJ4 + R) co-expression, TcpE could not be detected for some reason. So, further experiments with reliable controls are critical to prove that TcpD-TcpR-TcpT and TcpE-TcpR-TcpT are stably expressed as triplets and indeed represent the smallest combinations of proteins required to form a stable IM platform within the TCP biogenesis apparatus.

Discussion

Interpreting Experimental Results

While many research efforts are aimed at studying the diverse functions of TCP and other type IV pili, little is understood about how they are assembled. The goal of this project was to help elucidate the structure of proteins required for TCP biogenesis – specifically, which proteins can form a stable IM platform. Based on the methods used, experimental results did not offer direct biochemical evidence of protein interactions—which could be obtained through pull-down, co-immunoprecipitation, bacterial two-hybrid, or cross-linking studies. Instead, this project used protein immunoblotting to analyze protein stability and help determine which IM proteins required for TCP biogenesis are likely to physically interact. The integrity of this analysis rests on the belief that patterns of protein stability are useful predictors of protein-protein interactions. That is, analysis of experimental results relies on the following premises: (1) the TCP biogenesis apparatus assembles in a proteolytic environment and (2) when co-expressed, the proper arrangement of proteins within the TCP biogenesis apparatus will form a stable complex, protected from protease degradation. Consider the following example: when expressed in *E. coli*, TcpT is stable alone, unstable in the presence of TcpR, and appears to be stable in the presence of both TcpR and TcpE. This differential stability, as detected via protein immunoblotting, suggests that TcpT and TcpR interact, but TcpR induces a conformational change in TcpT that renders it susceptible to protease degradation. So, the TcpT-TcpR interaction is not stable in isolation. Furthermore, the data suggest that TcpE can stabilize the TcpT-TcpR interaction and that TcpE-TcpT-TcpR form a stable IM platform within *V. cholerae*'s TCP biogenesis apparatus. The

validity of this type of analysis -- based on detecting patterns of protein stability on immunoblots-- has been confirmed by experiments conducted with *E. coli*'s BFP biogenesis machinery and *V. cholerae*'s EPS T2S system which have accurately predicted protein-protein interactions (Ramer *et al*., 2002).

Future Directions & Research Implications

Many steps of the experimental design must be repeated to provide further support that TcpE-TcpR-TcpT and TcpD-TcpR-TcpT are the smallest combinations of protein needed to form a stable IM platform within the TCP biogenesis apparatus. First, *E. coli* co-expression strains must be re-built to carry plasmids with correctly sequenced *tcp* genes that express wildtype proteins and complement their respective *tcp* deletion strains of *V. cholerae*. Then, wildtype TcpD, TcpE, TcpR, and TcpT must be co-expressed in all combinations of two and three in *E. coli*. Protein immunoblotting of cell lysates can be used to detect which IM Tcp proteins are stably expressed *E. coli*. Collectively, protein immunoblots will test the stability of each IM Tcp protein in the presence of all possible combinations of the remaining three IM proteins required for TCP biogenesis. To support the notion that three proteins are indeed the *minimal* requirements for stable platform assembly, evidence must show that no pair of IM Tcp proteins is stable together. In addition, subcellular fractionation studies can confirm that stable IM Tcp proteins detected via immunoblotting are indeed assembling within and localizing to the IM – as opposed to the cytoplasm, periplasm, or OM.

Furthermore, TcpD, TcpE, TcpR, and TcpT can be co-expressed in all pairs and groups of three in a Δ*tcpP* strain of *V. cholerae*. The *tcp* operon is essentially turned off in the Δ*tcpP* strain of *V. cholerae* due to the absence of TcpP, a positive regulator of the

tcp operon. This experiment will reveal whether the results obtained in *E. coli* can be mimicked in *V. cholerae*, the natural cellular environment for assembly of the TCP biogenesis apparatus. Lastly, as previously mentioned, any protein-protein interactions predicted by stability patterns on protein immunoblots must ultimately be confirmed by experiments proving direct biochemical interaction, such as bacterial two-hybrid and metal-affinity pull-down studies.

Overall, understanding the intricate structure of proteins needed for TCP biogenesis may provide clues as to how TCP forms & functions to cause cholera. Discovering how TCP forms may lead to breakthrough methods for disrupting pilus assembly. That is, each discovery of a novel protein-protein interaction required for TCP assembly represents a potential target for antibiotic development or drug therapy. One therapeutic intervention that seems especially promising is a drug that could prevent both the secretion of virulence factors and colonization by pathogenic bacteria by simultaneously interfering with type IV pilus biogenesis and type II secretion pathways. This type of inhibitor could prevent infections by a wide range of pathogenic bacteria and provide an alternative to antibiotic use. Finally, the mechanism of TCP biogenesis may serve as a useful model for studying type II protein secretion and DNA uptake in bacteria since the proteins involved in these processes share extensive sequence, structural, and functional homology.

Figures & Tables

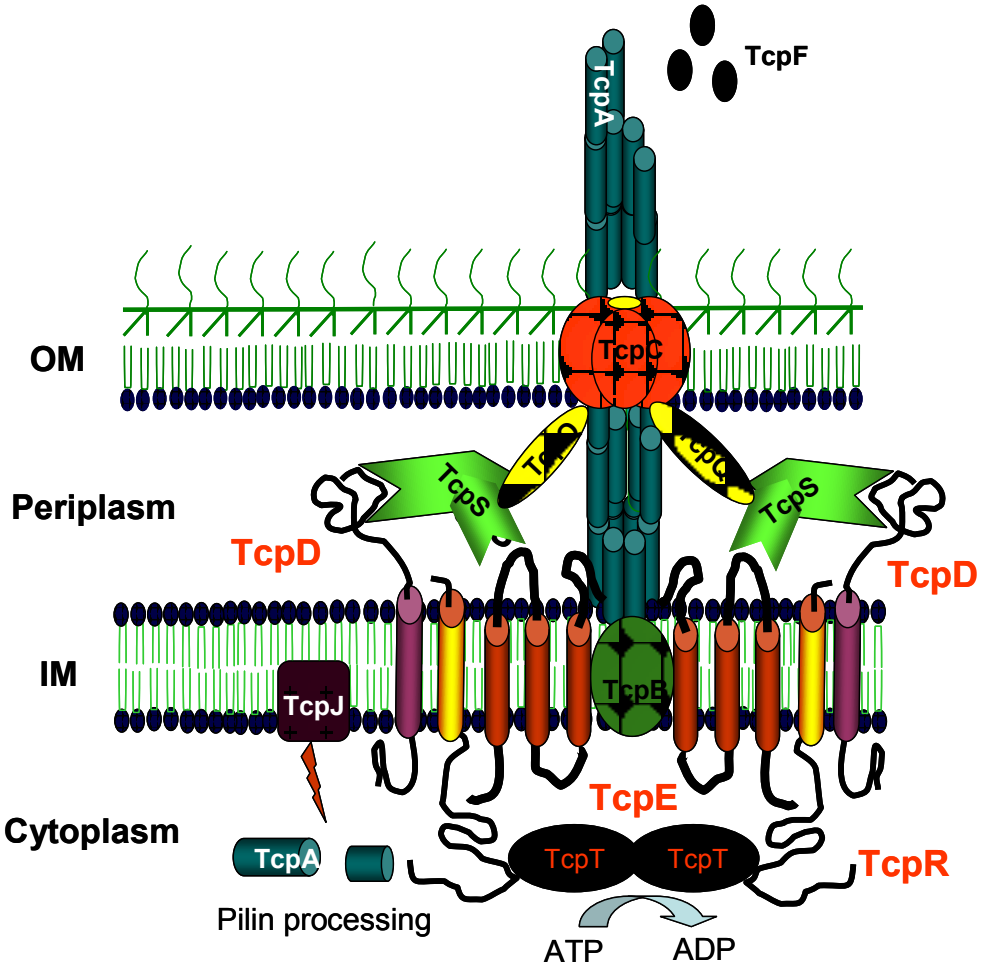

Fig. 1: Current working model for TCP biogenesis apparatus.

Fig. 2: The *tcp* operon; *V. cholerae* mutants with single deletions of any gene within the *tcp* operon, except tcpF, fail to make TCP.

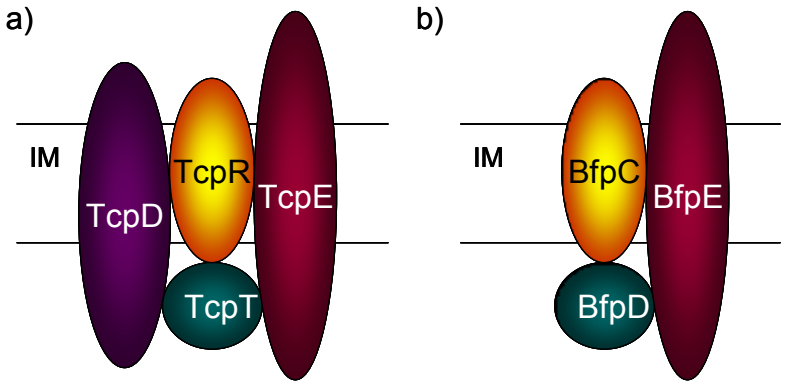

Fig. 3: Predicted IM protein structure of a) TCP in wildtype *V. cholerae* (0395) and b) BFP in wildtype *E. coli* (BL21).

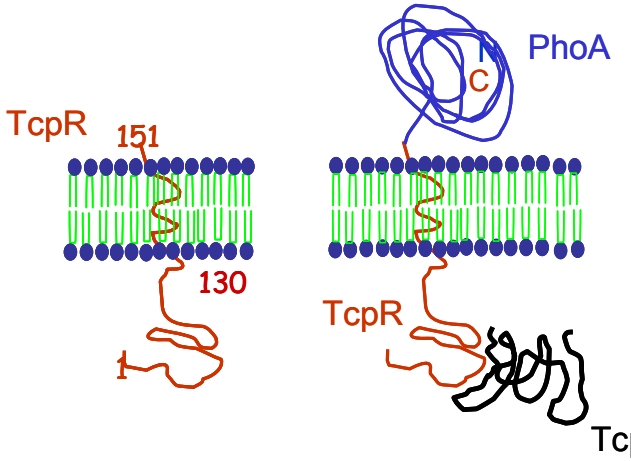

Fig. 4: TcpR is not stable alone, but becomes stable and properly localizes to the IM when fused to PhoA.

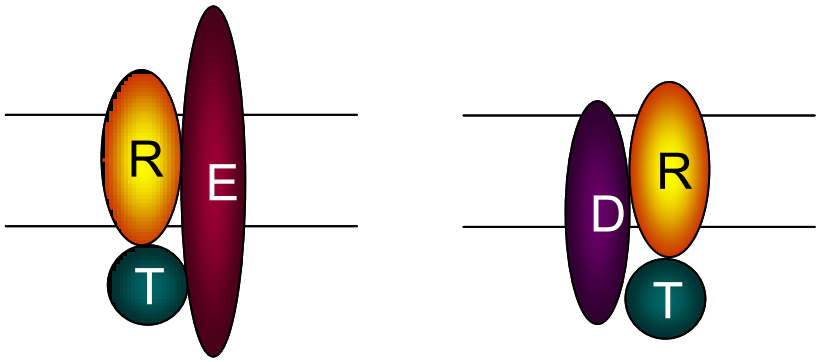

Fig. 5: Predicted models for stable IM platforms in *V. cholerae*.

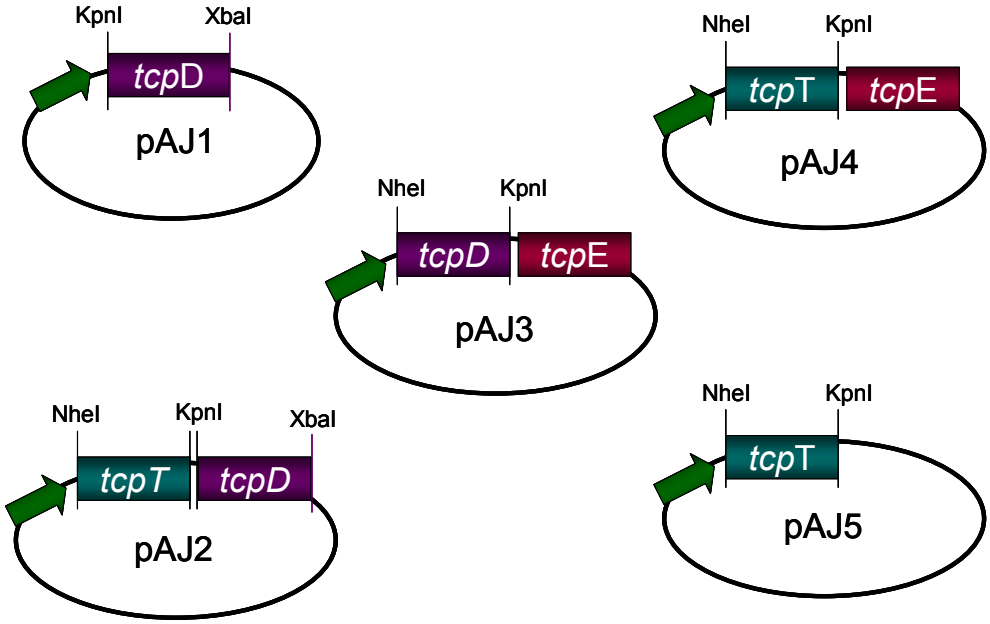

Fig. 6: Plasmids pAJ1-pAJ5 showing inserted *tcp* genes and restriction sites used for genetic engineering.

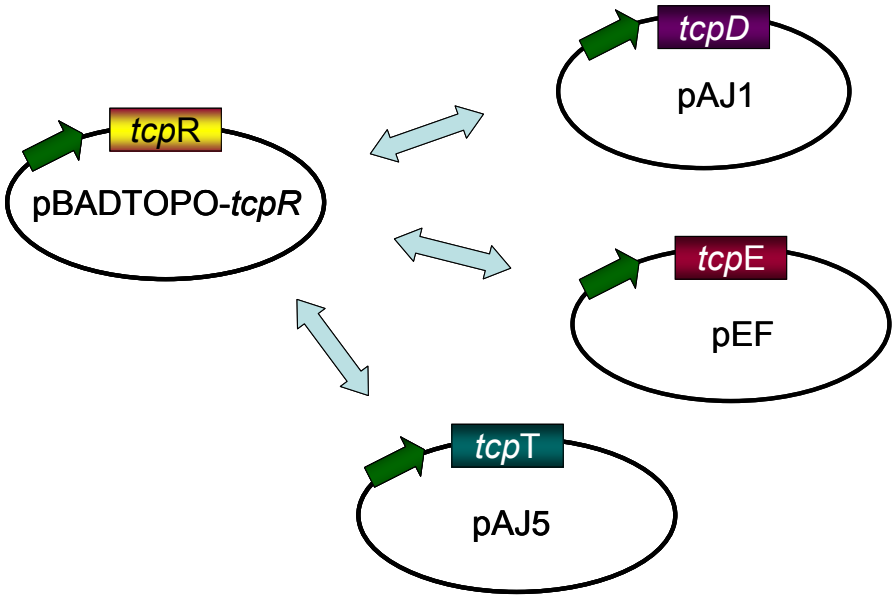

Fig. 7: Plasmid pairs used to co-express 2 *V. cholerae* IM Tcp proteins in *E. coli*.

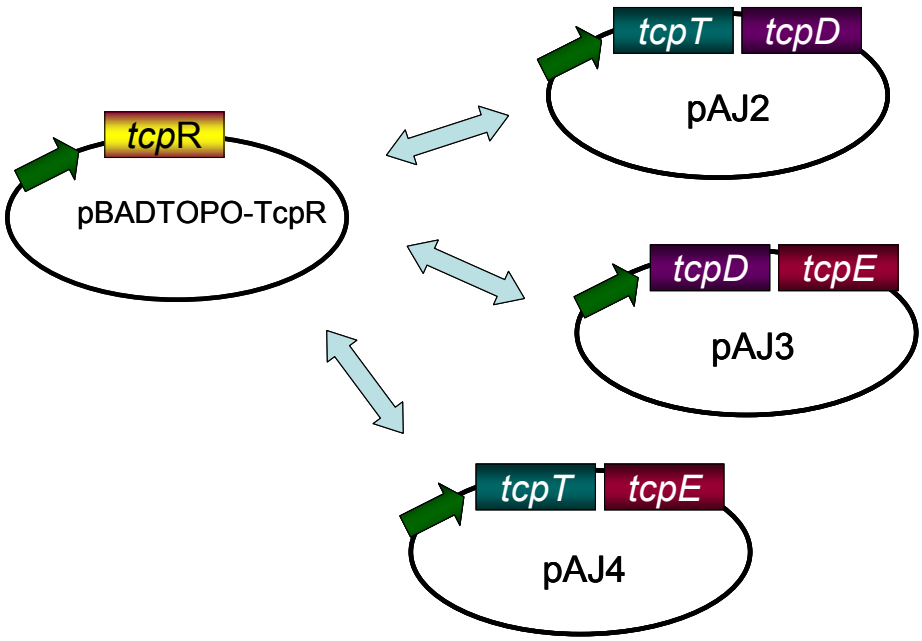

Fig. 8: Plasmids pairs used to co-express 3 *V. cholerae* IM Tcp proteins in *E. coli*.

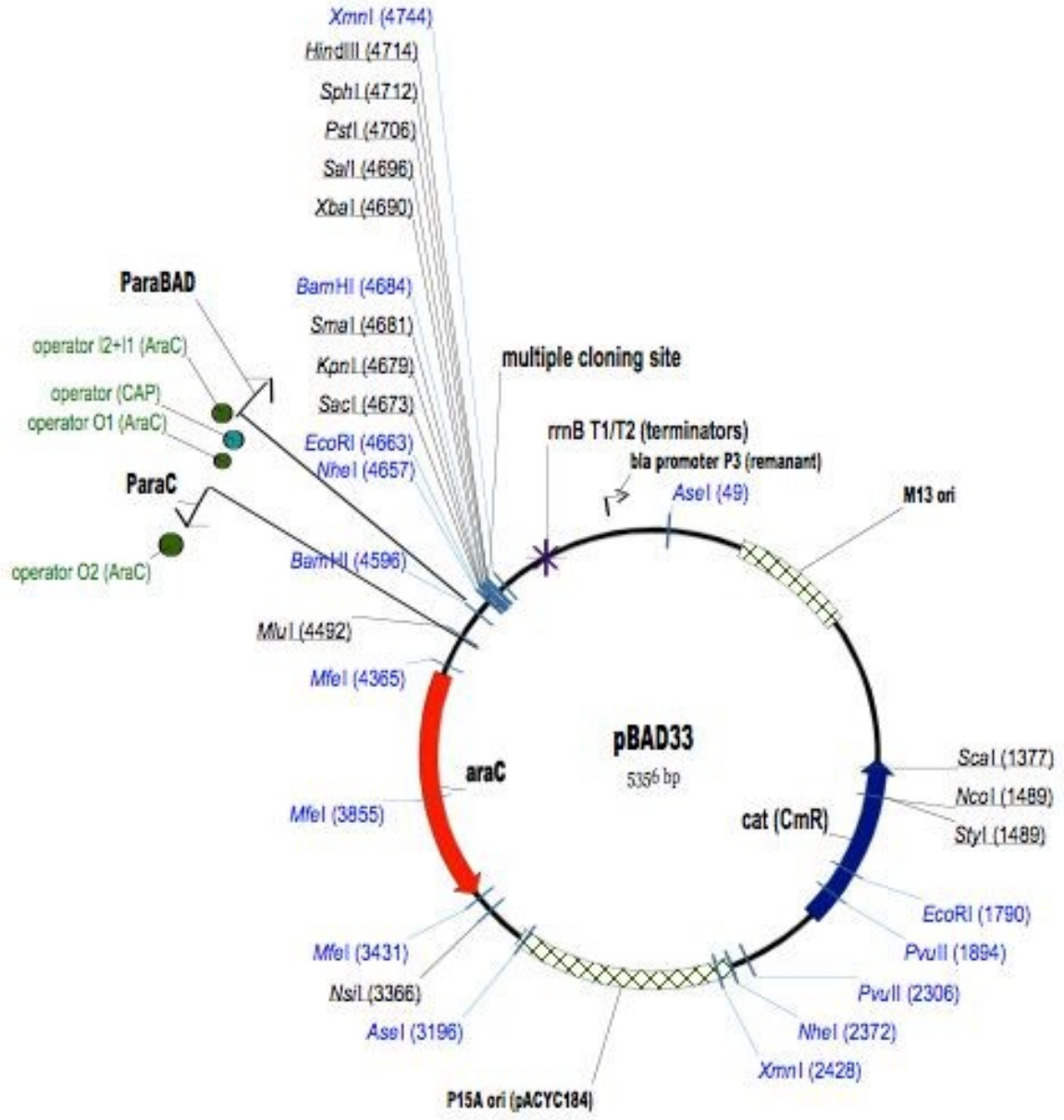

Figure 9: pBAD33 vector map displaying two Nhe restriction sites -- located at 2372 bases and within the MCS at 4657 bases.

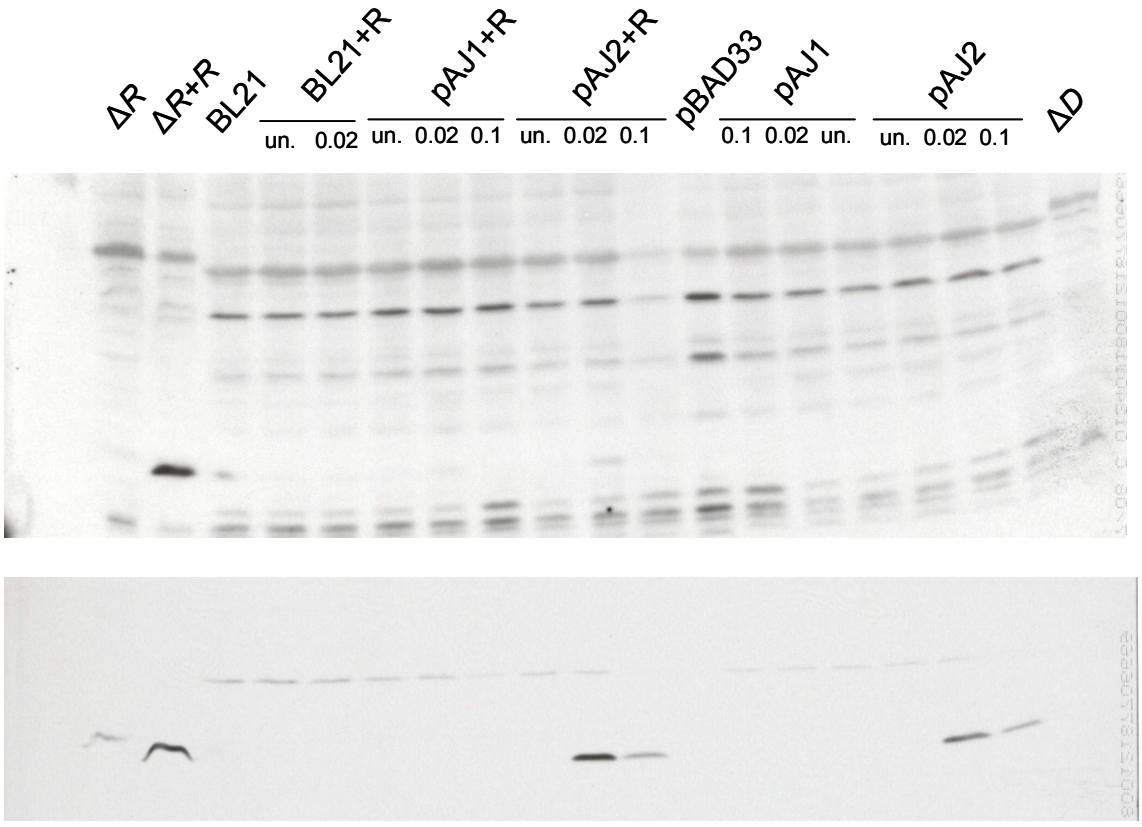

Fig. 19: ***Top*****: Anti-His blot to visualize TcpR;** ***Bottom*****: Anti-TcpD blot of same membrane after stripping. (Subscripts "un," "0.02," and "0.1" refer to 0%, 0.02%, and 0.1% concentrations of arabinose used to induce the engineered plasmids' arabinose-inducible promoters)**

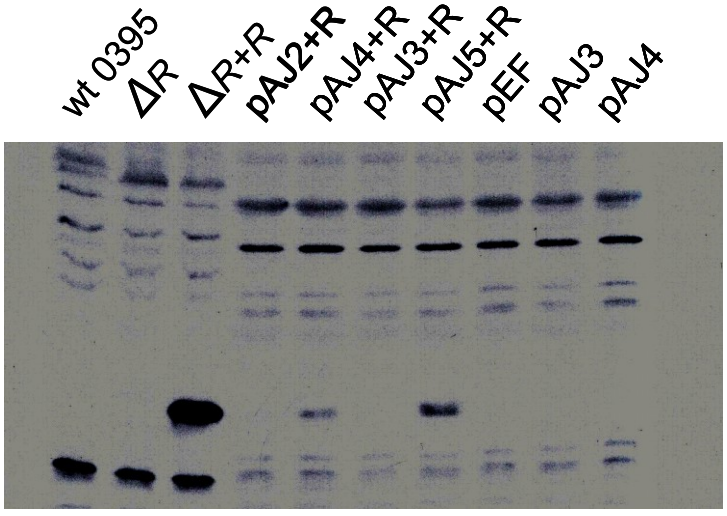

Fig. 20: Anti-His blot to visualize TcpR and TcpE.

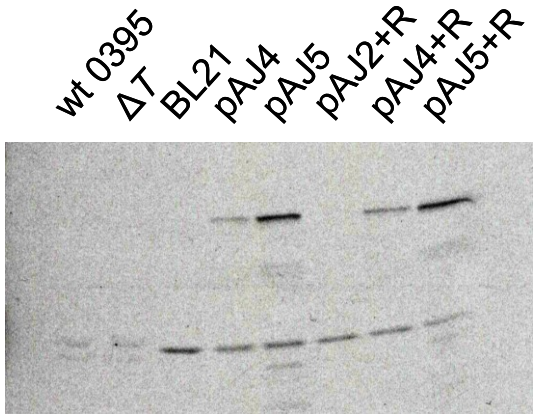

Fig. 21: Anti-TcpT blot.

Construct	Sequencing Results
pAJ1	A frame-shift mutation creates a 175 amino acid truncated TcpD (103 aa missing from C-terminus).
pAJ2	Both *tcpT* and *tcpD* sequences are correct.
pAJ3	Needs to be sequenced.
pAJ4	Both *tcpT* and *tcpE* sequences are correct.
pAJ5	A missense mutation in *tcpT* results in a change from cysteine to serine at amino acid 406.

Table 1: Summary of automated DNA sequencing results for pAJ1-pAJ5.

Appendix A

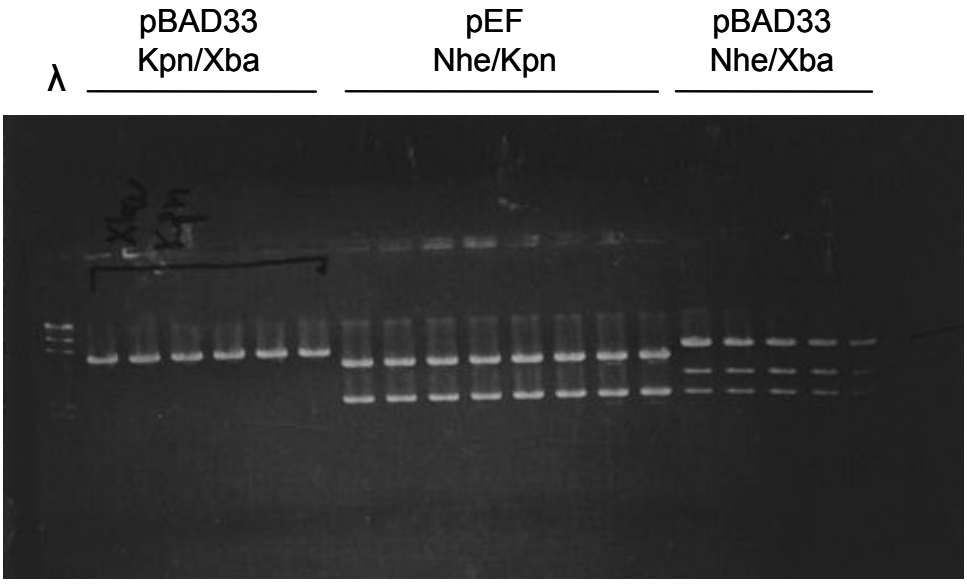

Fig. 1: DNA gel showing pEF digested with the restriction enzymes Nhe and Kpn, as well as pBAD33 digested with Nhe and Xba; reveals Nhe restriction site is not unique to MCS of these vectors.

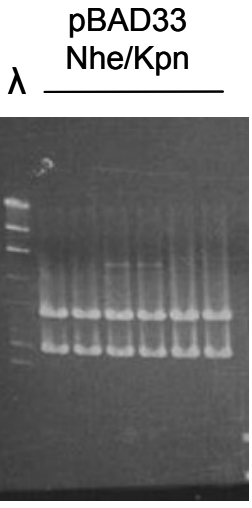

Fig. 2: DNA gel showing pBAD33 digested with Nhe and Kpn; confirms Nhe restriction site is not unique to the MCS.

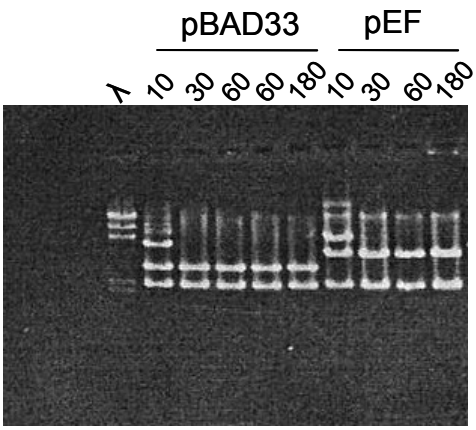

Fig. 3: DNA gel showing pBAD33 & pEF digested with Nhe alone for 10, 30, 60, & 180 minutes; 10 minutes is only time interval at which some vector is cut only once at the Nhe restriction site within the MCS.

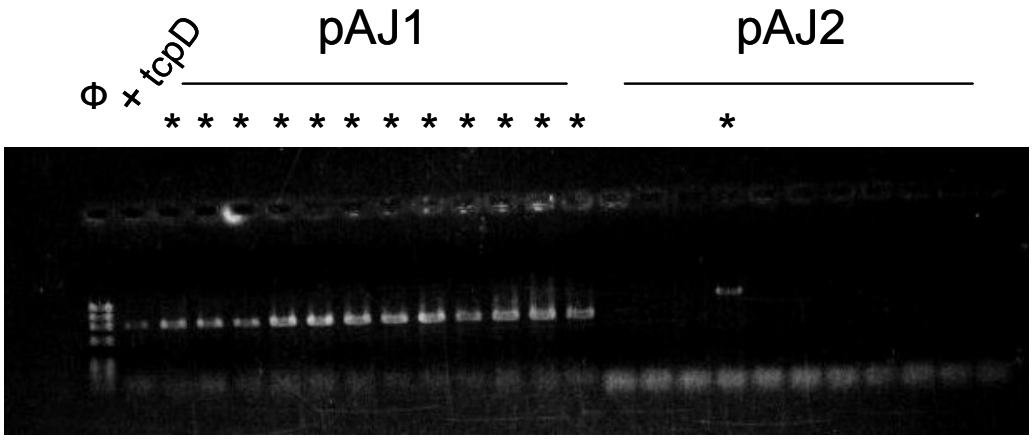

Fig. 4: Colony PCR results showing "positive" pAJ1 & pAJ2 colonies designated by asterisks above their lanes. Primers were designed to amplify the full-length *tcpD* gene from pAJ1, while the 3' end of *tcpT* through the 5' end of *tcpD* from pAJ2 (explaining higher band from positive pAJ2 colony versus positive *tcpD* control and positive pAJ1 colonies).

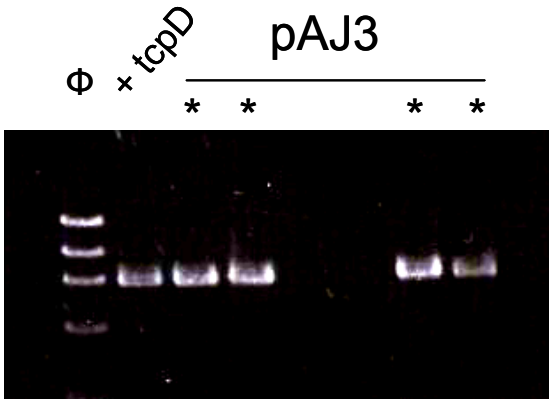

Fig. 5: Colony PCR results showing "positive" pAJ3 colonies designated by asterisks above their lanes. Primers were designed to amplify the full-length *tcpD* gene from pAJ3.

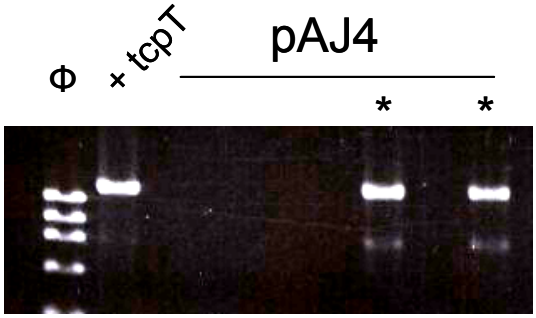

Fig. 6: Colony PCR results showing "positive" pAJ4 colonies designated by asterisks above their lanes. Primers were designed to amplify the full-length *tcpT* gene from pAJ4.

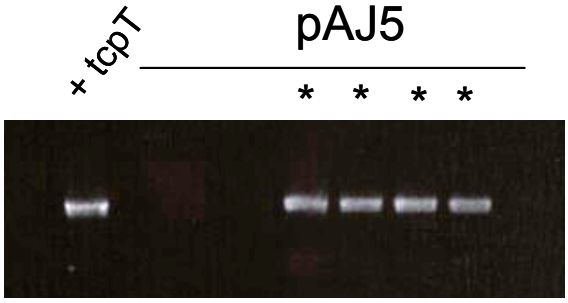

Fig. 7: Colony PCR results showing "positive" pAJ5 colonies designated by asterisks above their lanes. Primers were designed to amplify the full-length *tcpT* gene from pAJ5.

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