Localization of Type IV Pilin Polymerization Proteins in *Clostridium perfringens*

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Abstract

Clostridium perfringens is a spore-forming anaerobic Gram-positive rod which has gliding motility through type IV Pili (TFP). Since the discovery of TFP in Gram-positive bacteria is relatively new, more studies are required to understand the mechanism and interaction of the proteins of this machinery. Moreover, the similarities between TFP and type 2 secretion system (T2SS) suggest that *C. perfringens* has also a T2SS.

We studied the localization of TFP ATPases, PilB1, PilB2 and PilT in *Bacillus subtilis* to compare the localization in an organism other than *C. perfringens* and which lacks any known genes similar to TFP. Unlike the case in *C. perfringens,* PilB1 in *B. subtilis* localized to the poles in the absence of PilT, with some central foci at the future division sites. Colocalization of PilB1 was also studied with PilT and the results suggested that PilB1 needs PilT to migrate from the poles to the center. Localization of PilB2 in *B. subtilis*, was similar to the results in *C. perfringens* and to the localization of PilB1 in *B. subtilis*. We have not been able to co-express PilB2 with PilT yet. Succeeding in this study will help us better understand the interactions between PilB proteins and PilT.

In another project, we studied a von Willebrand factor Type A-Domain Containing protein (vWA) which is secreted from *C. perfringens* strain 13. We overexpressed and purified

this protein and tested the effects on mammalian cells. We found that the vWA is probably not a toxin but since it seems to bind to macrophage membranes, we propose that the vWA could be part of a toxin complex, probably the subunit of the complex that binds to the host cells.

Dedications

To Mohammad,

My best friend and my husband for his continuous love and encouragement even through the most difficult times.

To my parents,

For their patience and never letting distance be a hurdle in being with me.

And to my little sister, Zahra,

For her faith that has always motivated me towards being a better person.

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Life Science I is not just a building but a community where assistance and support is always given to the members. I was privileged to be part of this family and I would like to thank all these great people whom I came to know during the past two years, particularly the Popham lab members, Casey Bernhards for her help with *B. subtilis* transformation and Sean Mury for his patience with my endless questions on fluorescence microscopy.

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CHAPTER ONE

Introduction and Literature Review

General characteristics of *Clostridium perfringens*

The genus *Clostridium* consists of Gram-positive spore-forming bacilli that grow anaerobically, unable of dissimilatory sulfate reduction (1, 2). Although clostridia cannot grow aerobically, various degrees of oxygen tolerance have been observed. Most clostridia prefer neutral conditions and the optimum pH is in the range of 6.5-7. Metabolic diversity is huge among clostridia as they can use many types of organic molecules (2). *Clostridium perfringens* is the most abundant pathogen in nature and can be isolated from different environments such as soil, sewage and the intestines of humans and animals as part of the normal flora (4). This bacterium has a short generation time under optimum conditions (8-10 minutes) (5) and is auxotrophic for many amino acids including arginine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine. Therefore, it obtains the required nutrients by production of toxins and enzymes which degrade host cells (6). The chromosome has a low G + C content of ~28% (6, 7).

C. perfringens pathogenicity

C. perfringens is the causative agent of a number of enterotoxic and histotoxic infections in humans (2). About fifteen different toxins have been identified in *C. perfringens* and based on the toxins, these species are divided in to five major types, A-E (7, 8) (Table 1.1(9)). These five types denote the presence or absence of plasmids which carry beta, epsilon or iota toxin genes.

Table 1.1) Diseases associated with different types of *C. perfringens*.

Type	Disease	Toxin	Host
A	Food poisoning Gas gangrene (Clostridial myonecrosis) Antibiotic associated diarrhea	CPE Major: alpha, theta Minor: mu, alpha-clostripain, collagenase, hemolysin, caseinase CPE	Humans and animals
B	Enteric diseases in animals	beta	Animals
C	Necrotizing enteritis in humans and animals (Pig Bel)	beta	Humans and animals
D	Enteric diseases in animals	epsilon, iota	Animals
E	Enteric diseases in animals	epsilon, iota	Animals

Gas gangrene (clostridial myonecrosis)

Gas gangrene, which is characterized by production of toxins, gas and severe tissue damage, is caused by the entry of Type A *C. perfringens* vegetative cells or spores in wounds either from trauma or surgery. The lack of blood supply due to the destruction of blood vessels in deep wounds, injuries inflicted during surgical procedures or previous clostridial infection provide the bacteria with anaerobic conditions and facilitate disease (8, 10). Myonecrosis along with necrotizing fasciitis, cutaneous necrosis and changes in skin color are observed as the infection spreads. *C. perfringens* escapes the phagosomes of macrophages in the early stages of the infection, survives in the cytoplasm and stays there until the conditions become anaerobic, when they multiply and spread the infection (4). Clostridial myonecrosis requires immediate treatment by antibiotics and often amputation of damaged tissue, otherwise it will progress rapidly and lead to systemic shock and death (4).

The main toxin involved is α-toxin, a zinc-metalloenzyme which is activated by calcium (11) and has phospholipase C and sphingomyelinase activity. The cytotoxicity, necrosis and hemolysis effect of this bacterium is attributed to this toxin which is encoded by the *plc* gene. Another toxin that is involved in these infections is θ-toxin (perfringolysin O),which causes necrosis and depletion of polymorphonuclear cells (PMNS) along with α-toxin by causing the PMNs to adhere to the vascular epithelium. The θ-toxin is a member of the pore-forming, cholesterol-binding cytolytic toxins which act against cholesterol-containing mammalian membranes [2]. Other toxins include μ-toxin (hyaluronidase) which degrades hyaluronic acid in the connective tissue and κ-toxin (collagenase) (1, 8).

Food poisoning

Food poisoning is also attributed to Type A *C. perfringens* and it is currently the third most identified foodborne disease in the United States (2). Food poisoning is caused by consumption of food (typically beef and poultry) that is contaminated by Type A *C. perfringens.* In the intestines, the vegetative cells multiply and produce an enterotoxin (CPE) before sporulation. The enterotoxin is stored in the cytoplasm of the mother cell and is released along with the mature endospore when the cell lyses (2). CPE, which is coded by the *cpe* gene, is a 35 kDa heat-labile, single polypeptide protein, which leads to the secretion of fluids, electrolyte loss and diarrhea (8, 12). The enterotoxin has cytotoxic effects and damages the intestinal epithelial cells at the tips of the villi. This disease is mostly self-limiting and in healthy adults the issue is resolved in one or two days (8). CPE also causes antibiotic–associated diarrhea, mostly affecting children and the elderly in hospital settings (1).

Enteritis Necroticans (Pig Bel)

C. perfringens Type C is responsible for Pig bel which is caused by consumption of high amounts of undercooked meat protein, after a low protein diet. An extracellular heat-sensitive toxin, β-toxin which is normally inactivated by trypsin in the gastrointestinal track is involved in this disease. In people with a low protein diet, the production of pancreatic enzymes including trypsin is reduced so the toxin is not inactivated. Similar symptoms have been reported sporadically and epidemically in Papua New Guinea during pig festivities in a population in which sweet potato, a source of trypsin inhibitors, is the staple food (13). β-toxin has a necrotizing effect on the intestinal villi, causing hemorrhagic necrosis which can be lethal (1, 8).

Enteric diseases of domestic animals

A number of enteric diseases in domestic animals can be caused by *C. perfringens* Types D and E, such as necrotic enteritis, enterotoxaemia and dysentery. The ε-toxin produced by these types is lethal and necrotizing, leading to pulpy kidney disease. Another toxin involved is ι-toxin which is mostly associated with Type E *C. perfringens* (1, 8).

Type IV pili (TFP) in *C. perfringens*

Type IV pili are surface filaments that are important virulence factors in many Gram-negative pathogens including *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Neisseria gonorrhea*. They are made of many pilin proteins that build a thin fiber that can be several microns long. They make a very strong structure that can withstand high stress forces (14). Although commonly found in Gram-negative bacteria, TFP were recently discovered in Gram-positive bacteria, including *C. perfringens* (15). Type IV pili are produced by assembly of pilin from the subunits in the membrane. First they are in the form of a prepilin which carries a signal sequence and is later cleaved with a signal peptidase. There are four pilin proteins in *C. perfringens* strain 13, PilA1, PilA2, PilA3 and PilA4. They are small proteins with a hydrophobic N-terminal region except for a glutamate at residue five [3]. The proposed model for pilin assembly is the polymerization of pilins by the energy provided from an assembly ATPase. TFP in various species are involved in many functions including attachment to host cells, DNA uptake and twitching motility (16, 17).

There are two subclasses of TFP, Type IVa and Type IVb. The TFP found in *C. perfringens* have the characteristics of type IVa. Both pilin types have an alpha helix at the N-terminal and a globular C-terminal but Type IVa possesses shorter leader sequences and different

N-methylated residues at the site of processing by the PilD prepilin peptidases (14). Fig. 1.1 shows the proposed assembly system of TFP in *C. perfringens.*

Main components of TFP assembly in *C. perfringens* [3]:

Prepilin peptidase (PilD). PilD is the signal peptidase that has two conserved aspartate residues required for peptidase activity and cleavage of the signal peptide of prepilins.

Assembly ATPases (PilB). *C. perfringens* has two assembly ATPases, PilB1 and PilB2. The designation of "1" and "2" is because the homologs were discovered on both primary and secondary operons. *pilB2* gene is in the primary operon with other main TFP assembly components and *pilB1* gene is on a secondary operon, encoded along with a pilin and a membrane core protein (Fig. 1).

Membrane core protein (PilC). There are two copies of *pilC* genes on the *C. perfringens* chromosome, labeled *pilC1* and *pilC2* because they are adjacent to *pilB1* and *pilB2* coding regions, respectively. It is suggested that each assembly ATPase works with its own core protein during the extension. The core protein is modeled to act like a piston, pushing the pilus out of the membrane by the energy provided from the assembly ATPase. The pilus is pushed far enough so that a new pilin can move to the gap from the membrane.

Retraction ATPase (PilT). PilT is involved in disassembly of the pilus and pulls back the pilins to the membrane. *pilT* mutants in *C. perfringens* cannot make pili and the reason could be related to the regulatory pathways for pilus assembly in these bacteria that requires expression of all the proteins in the machinery.

In summary, PilD is a signal peptidase that cleaves the signal sequence of PilA monomers which are assembled by PilB, an ATPase that seems to work with a membrane core protein to extend the pilus (18). The structure is pulled back to the membrane by the energy provided from PilT and PilA subunits are recycled back to the membrane. (19). Both PilB and PilT are hexamers, belonging to the Secretion Superfamily ATPases which are involved in Type 2 and Type 4 Secretion Systems (20).

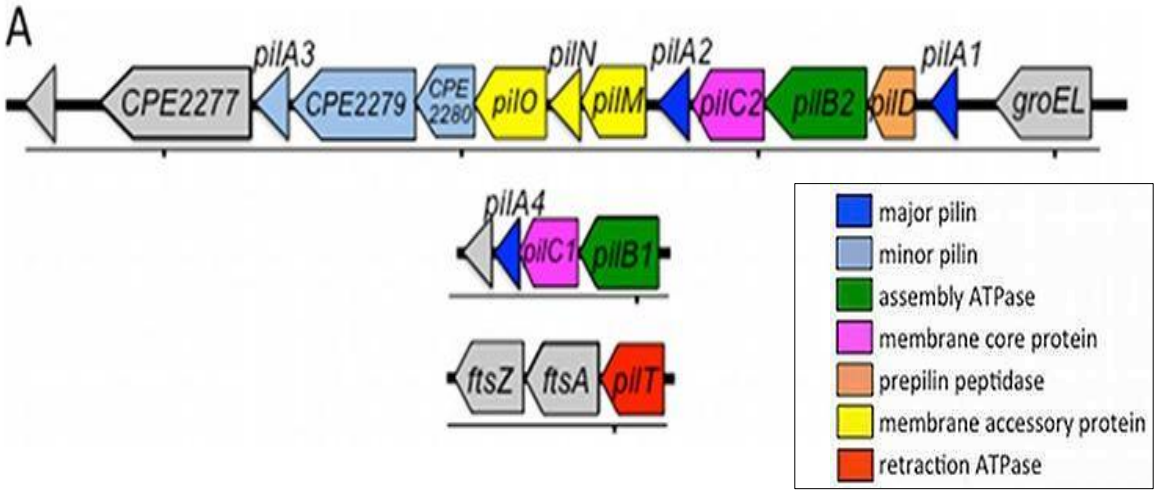

Figure 1.1) The primary TFP operon (top) and the secondary gene clusters in *C. perfringens* strain 13 (3). The box denotes proteins encoded in each gene, designated by color codes.

Type 2 Secretion System:

Type 2 secretion systems (T2SS) are a general secretory pathway which facilitates transportation of large folded proteins across the outer membrane in Gram-negative bacteria and plays a very important role in virulence. T2SS are evolutionarily related to TFP with similarities in structure and function (21, 22) (Fig. 1.2). Many components of the two systems share sequence similarities and they have many features in common. T2SS require proteins very similar to TFP pilins which can form structures called pseudopili (23-25).

T2SS are used for protein secretion in many Gram-negative bacteria including *Vibrio cholerae, E. coli, Klebsiella oxytoca, Yersinia enterocolitica and Legionella pneumophila*. They span from the inner membrane to the outer membrane and about 12-15 proteins are involved in the apparatus (22, 26). During secretion through T2SS, the substrate protein is first expressed with an N-terminal signal sequence which targets the protein for translocation across the inner membrane via Sec or Tat machinery. After the signal sequence is cleaved, the protein is released from the cytoplasmic membrane and temporarily remains in the periplasmic compartment before being translocated by outer-membrane complex. (21, 26).

There are four major subassemblies in T2SS (21, 26):

1. **The pseudopilus.**

   This structure is in the periplasm and includes five different pseudopilins, with several copies of a major pseudopilin. The N-terminal sequence has homology to Type IV pilins and the structure was named “the pseudopilus” for this reason. The signal sequence of the major pseudopilin is recognized and the pseudopilus is targeted for the insertion into the inner membrane. Then the N-terminal peptide is cleaved by a prepilin peptidase and may

temporarily remain in the inner membrane. Recent studies suggest that some pseudopilins form a helical fiber that spans the cell wall to the cell surface and can be involved in protein secretion.

2. **The outer-membrane complex.**

   The major protein of the outer-membrane complex is secretin, a multimeric protein. It is suggested that the outer-membrane complex may be the link between the inner-membrane and the outer-membrane and that it transduces energy between these two membranes. It may also have a role in pseudopilus assembly and activity.

3. **The inner–membrane platform.**

   The inner-membrane platform has several copies of four core membrane proteins and is closely associated with the secretion ATPase. T2SS mechanism of action centers around the inner–membrane protein as the complex is in contact with the outer membrane complex in the periplasm, the secretion ATPase in the cytoplasm and the major pseudopilin. It is suggested that the inner–membrane platform could be involved in extension of the pseudopilus which sends proteins out through the outer-membrane channel like a piston.

4. **The secretion ATPase.**

   The energy for secretion is provided by this single ATPase. The secretion ATPases have Walker Box motifs that contribute to the secretion and ATPase activity. Although the protein has been purified in monomer form, site-directed mutagenesis studies suggest that it is functional in the hexameric form (21).

The similarities that exist between Gram-positive TFP and Gram-negative T2SS suggest that a similar secretion system may be present in Gram-positive bacteria, particularly considering the fact that all the human pathogens in this group produce secretory toxins.

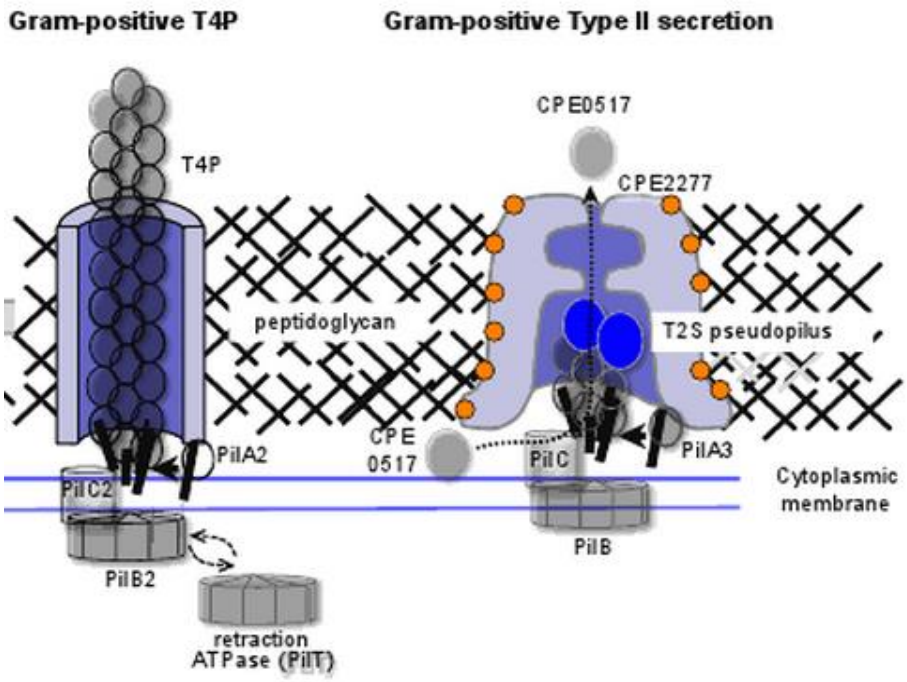

Figure 1.2) The proposed model for TFP and T2SS in Gram-positive bacteria. Due to similarities of T2SS (right) to TFP (left), we believe a Type 2 secretion system may be present in Gram-positive bacteria, *C. perfringens* in particular. From reference (3).

***Bacillus subtilis*:**

The genus *Bacillus* consists of Gram-positive rod-shaped, endospore-forming microorganisms that are aerobic or facultatively anaerobic. They are mostly motile through peritrichous flagella and produce catalase. *B. subtilis* produces one endospore per cell. Spores are highly resistant to unfavorable conditions such as heat, radiation, disinfectants and desiccation. Colony morphology is often in irregular shape with a range of consistency. *B. subtilis* is mostly isolated from soil and contamination is a problem in operating rooms, surgical dressings and pharmaceutical products (27, 28).

B. subtilis is naturally competent which permits the uptake of exogenous DNA; this physiological property is an important genetic tool (29). After *E. coli*, it is the best studied organism among prokaryotes and is used as a model for studying other Gram-positive bacteria because of easy genetic manipulation which make it a better choice over Gram-negative *E. coli* (30). It is a fast growing microorganism and multiplies rapidly in inexpensive media (31, 32). *B. subtilis* lacks a TFP system and belongs to the same group of bacteria as *C. perfringens*, that is Gram-positive bacteria so they have similar cell wall structure. Both species have the same type of *minCD* and *divIVA* genes (42) that are responsible for controlling the localization of cell division machinery, therefore it is suggested that both *B. subtilis* and *C. perfringens* use the same method for positioning cell division machinery (9). There are also different *B. subtilis* strains with mutations in cell division machinery available that make this species a valuable study model.

CHAPTER TWO

Localization of Type IV Pilin Polymerization Proteins in

Clostridium perfringens

Abstract

Clostridium perfringens is an anaerobic, spore-forming, Gram-positive bacterium which causes diseases such as gas gangrene and food poisoning. Recently, Type IV pili (TFP), which function by ATPase-based polymerization of pilin proteins, were discovered in *C. perfringens* which contribute to its gliding motility. TFP assembly is composed of different protein components but the mechanism of action and the structure in Gram-positive bacteria is not yet understood. In this study, we were interested in understanding the localization of the ATPase motor proteins, PilB (PilB1 and PilB2) and PilT- involved in pilus extension and retraction, respectively- during cell division. These proteins were tagged with green fluorescent protein variants, YFP (yellow fluorescent protein) and CFP (cyan fluorescent protein) and studied using fluorescence microscopy. The results suggested that PilT localizes independent of other TFP ATPases, whereas PilB1 requires PilT to localize and PilB2 does not (9). To have a better understanding of the localization of these proteins, they needed to be studied in a microorganism that lacks a TFP apparatus, therefore another Gram-positive bacterium, *Bacillus subtilis* was selected. It was transformed with the genes encoding the same fluorescent-tagged proteins. These clones were studied by fluorescence microscopy and the quantitative and qualitative data from PilT and PilB2 clones demonstrate localization similar to *C. perfringens* but PilB1 proteins seem capable of localization in the absence of PilT. How the localization of pilin proteins occurs and understanding the localization order are currently being studied.

Introduction

Clostridium perfringens is a Gram-positive anaerobic rod-shaped bacterium which is the most abundant pathogen in nature (1, 4). It can cause various diseases such as gas gangrene and food poisoning in humans and animals. *C. perfringens* is isolated from different environments such as soil, sewage and intestines of humans and animals as part of the normal flora (4, 15).

Type IV Pili (TFP) have been found in many Gram-negative pathogens including *Vibrio cholerae, Pseudomonas aeruginosa, Escherichia coli* and *Neisseria gonorrhea*

15). This machinery assembles the pilus by polymerizing pilin proteins through the energy provided by the PilB family of ATPases. These pili are involved in foreign DNA uptake for competence, motility and attachment to host cells (15, 16, 33). Recently, TFP were found in a number of Gram-positive bacteria including *C. perfringens* and it was suggested that the gliding motility of this bacterium depends on TFP (15).

Main components of TFP assembly in *C. perfringens* strain 13 are PilD, a signal peptidase which cleaves the signal sequence of pilin monomers (PilA1 – PilA4), PilB has two homologues, PilB1 and PilB2 which act as the assembly ATPases and work with a membrane core protein, PilC (which also has two homologues, PilC1 and PilC2) to push the pilus out of the cell with a piston-like mechanism. The pilus is pulled back into the membrane by the energy provided from the retraction ATPase, PilT (3).

In the studies by Andrea Hartman (9), the role of PilT and its interactions with the PilB homologues was studied and it seems that PilT works with only one of the PilBs. The movement of the ATPases from the poles to the septa was also studied, as the pili are at the poles of *C. perfringens* and we want to know how TFP apparatus localizes to the septum during cell growth

and division. It was discovered that PilT can localize independently of either PilB protein, but PilB1 requires PilT for localization while PilB2 could localize independently of PilT. Localization of PilT in *Bacillus subtilis* was also studied to see how this protein acts in a cell that lacks any TFP machinery and results similar to those obtained in *C. perfringens* were observed.

In this study, we wanted to determine the localization of PilB1, PilB2 and PilT individually and together in *B. subtilis* as a model organism with similarity to *C. perfringens* as they are both Gram-positive and possess similar cell wall structure. Moreover, *B. subtilis* lacks any known genes with similarity to TFP and easy genetic manipulation make it a good study model. By using fluorescently-tagged proteins and pDR111 (34), an integration plasmid which also carries an IPTG-inducible *hyperspac* promoter.

Materials and methods

Construction of the fusion genes:

The fusion genes and plasmids used in this study were constructed by Andrea Hartman (9) as described (See also Table 2.1 for a list of plasmids and primers in that study).

Transformation of *B. subtilis*:

B. subtilis strain PS832 was selected for transformation with the fusion genes. A lawn was prepared on Luria-Bertani (LB) agar and grown overnight at 25^0 C. In the morning, the competent cells were prepared (protocol from Popham Lab and (35)) and mixed with the fusion plasmid to take up the foreign DNA. After 30 minutes of incubation at 37^oC, 200 µl of each culture was plated on LB supplemented with 100 µg/mL of spectinomycin and incubated overnight at 37^oC. Homologous recombination should occur at the *amyE* locus which knocks out this gene. Therefore, the colonies were screened for loss of amylase production by patching them on starch plates and exposing them to iodine crystals. The colonies that did not show any clear zone of starch degradation were selected for microscopy.

Culture preparation for microscopy:

The clones were grown as lawns on Brain Heart Infusion (Difco) containing 5 mM $MgSO_4$ and $spec^{100}$ (BHI-$MgSO_4$) overnight at 25^0 C. The lawns were scraped off in the morning and resuspended in BHI-$MgSO_4$ broth with a turbidity of OD_{600} = 0.02 and were incubated (shaking, 37^oC) until the OD reached about 0.4. Then the cultures were induced with 0.5 mM IPTG, incubated for 30 minutes and concentrated to half their volume by centrifugation, decanting and re-suspending the pellet. One µl of the culture was transferred onto 2% agarose pads fixed on microscope slides as described (36). The pads contain BHI-$MgSO_4$ (37) and 0.5 mM IPTG. The

slides were incubated another 30 minute and then the pads were covered by coverslip and sealed by nail polish to perform fluorescence microscopy with Olympus IX71 microscope (1000X magnification) using Applied Precision SoftWorx program.

Data analysis and quantification:

The microscopy results were analyzed by human visualization and counting. Whenever a polar localization is mentioned, it refers to 20% of cell area from each end. Central localization refers to 20% of cell area in the middle. All the analysis (visual and computational) was performed only on the cells expressing the fluorescent proteins. MATLAB based software, MicrobeTracker and SpotFinderZ (38) were used for generating the histograms. MicrobeTracker finds the cells through image contrast with the background and outlines each cell and the fluorescent spots based on the defined parameters with the threshold minimum level set at 0.02. SpotFinderZ scans each image data file generated by MicrobeTracker and produces a histogram of all the cells based on pixel distribution from one pole to the other. The final result cannot be quantified and only displays the distribution of the fluorescent proteins in the cells.

Protein Assays

The production of the fusion proteins in *B. subtilis* was studied by western blotting. Two mL of each culture was taken an hour after induction and the pellets were obtained by centrifugation (11,000 rpm, 20 min.). The pellets were sonicated and centrifuged again (10,000 rpm, 15 min., 4^{o}C), and the supernatants were collected to be boiled in 1X SDS sample buffer and run on a 10% polyacrylamide gel (Amiresco® NextGel). Western blots were performed using antibodies against each tagged ATPase (the primary antibodies were diluted to 10^{-3} and the secondary antibody was diluted to 10^{-4} and were developed by chemiluminescence (Thermo Scientific).

Table 2.1) List of strains, plasmids and primers used in this study.

Strain	Description	Reference/Source
E. coli strain DH10B	*F-mcrA D(mrrhsdRMSmcrBC) F80d lacZ DM15 lacX74 deoR recA1araD139 D (ara, leu)7697 galU galK rpsL endA1 nupG*	
B. subtilis strain PS832		Popham Lab
Plasmids		
CT(BS)	pGEM-T Easy with *cfp-pilT* for transformation of *B. subtilis* (SalI-HindIII)	(9)
CTpDR111	pDR111 with the *cfp-pilT* fusion gene	This study
CTYB1pDR111	pDR111 with the *yfp-pilB1* and *cfp-pilT*	This study
CTYB2pDR111	pDR111 with the *yfp-pilB2* and *cfp-pilT*	This study
pAH8	pGEM-T Easy with *yfp-pilB1* (SalI-SphI)	(9)
pAH9	pGEM-T Easy with *yfp-pilB2* (SalI-SphI)	(9)
pCTYB2	pGEM-T Easy with *yfp-pilB2* for making CTYB2 (NheI-SphI)	This study
pDR111	Suicide plasmid for *B. subtilis*, ampicillin and spectinomycin resistance	K. Ramamurthy
pGEM-T Easy	Ampicillin resistance	Promega
YB1pDR111	pDR111 with the *yfp-pilB1* fusion gene	(9)
YB2pDR111	pDR111 with the *yfp-pilB2* fusion gene	This study

YFPpDR111	pDR111 with the *yfp* gene	(9)
Primers	**Sequence (5' to 3')**	
OSN10	GCT AGC TAA ATA ACA AAA AGG AGA AGG CAT ATT GTC AAA AGG AC	This study
OAH193	GCA TGC TTAC ATA TCA TAA GTT ATA TTT AAC	by Andrea Hartman

Results

Localization of TFP ATPases. Both PilB1 and PilB2 were tagged with YFP (yellow fluorescent protein) and the fusion genes were introduced to *B. subtilis* using pDR111, an *amyE* integration plasmid with *hyperspac* promoter which is inducible with IPTG. The localization results of PilB1 in *B. subtilis* shows that this protein is at either pole or both poles most of the time. Of all the counted spots, 80% had polar localization, with only 10% appearing in the center and 10% in between the poles and the center of the cell (Fig. 2.1A). A histogram of localization in PilB1 shows the distribution of proteins from pole to pole (Fig. 2.1 B). The first three bars demonstrate the poles on either side and the 4 bars in the middle represent the center of the cells. All the histograms are produced based on pixels distribution. This result is different from what was observed in *C. perfringens* (9) as PilB1 was unable to localize to the poles in a PilT mutant and appeared as faint foci all through the cell. The majority of PilB2 spots also appeared at the poles with 87% polar localization. Central localization was observed in 9% of spots and 4% appeared in between the poles and the center of the cell (Fig 2.A). Localization of PilB2 in *B. subtilis* is similar to what was observed in *C. perfringens* (9). This protein could localize to the poles in both wild type and the PilT mutant, suggesting that PilB2 does not require PilT for localization. Both PilB1 and PilB2 are at the poles most of the time and the central foci seem to be future division sites in the cells.

Colocalization of PilB1 and PilT. In order to see how PilB1 localizes in the presence of PilT, the localization of these two proteins together was studied in *B. subtilis* transformed with CTYB1pDR111 plasmid. In the images (Fig. 2.3), PilT appears blue and was observed at the poles 76% of the time and in the center 11% of the time. PilB1 (orange) has polar localization 88% of the time and central localization 7 % of the time. Of all the colocalized spots of PilB1

and PilT (white), 92% demonstrated polar localization and 5% of the spots were in the center which appears to be future division sites. About 6% of cells contained only PilB1 and 1% had only PilT present. Of all the CT proteins that localized to a non-polar position, 32% of them were associated with PilB1 (data not shown.)

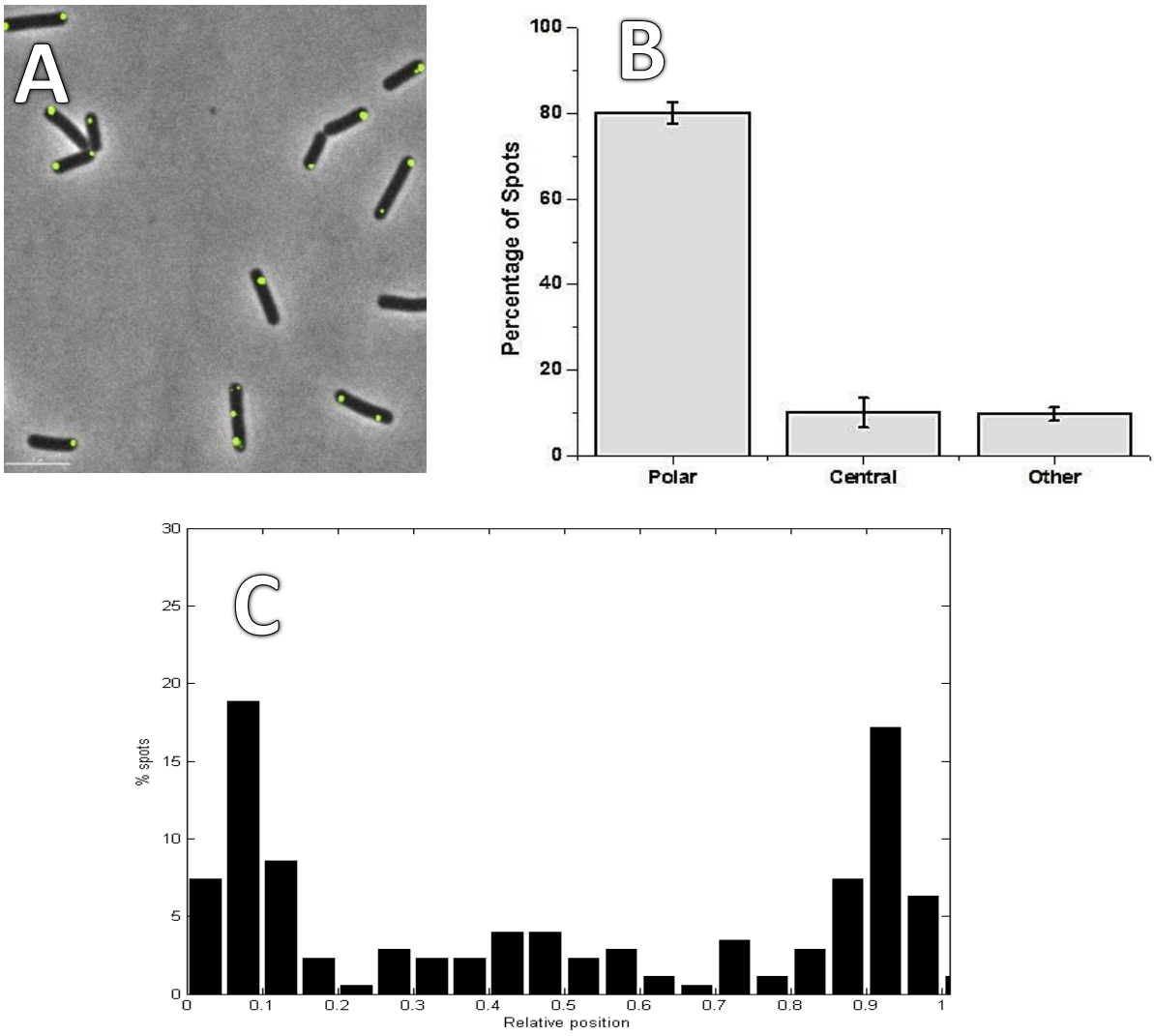

Figure 2.1) Localization of PilB1 in *B. subtilis*. A) Fluorescence microscopy image of YFP-PilB1. YFP appear green in this image (scale: 5 μm). B) Graph of PilB1 localization. Eighty percent of spots had polar localization, 10% showed central localization and the rest appeared between the poles and the center of the cell. Triplicate samples were studied by counting 50 cells each. C) Histogram demonstrating relative position of spots in the cells.

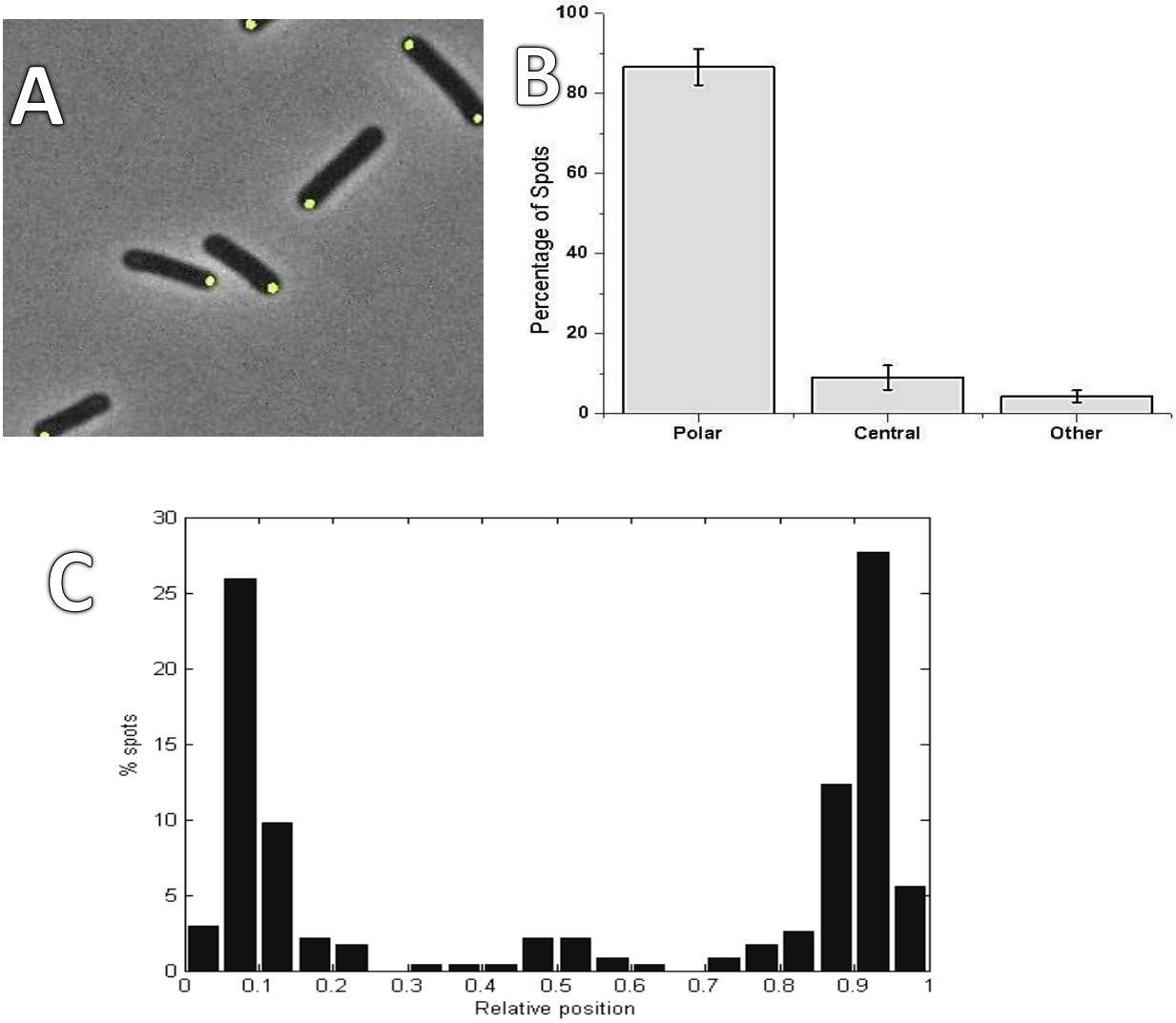

Figure 2.2) Localization of PilB2 in *B. subtilis*. A) Fluorescence microscopy image of YFP-PilB2. YFP appears green in this image (scale: 5 μm). B) Graph of PilB2 localization. Eighty seven percent of spots had polar localization, 9% showed central localization and the rest appeared between the poles and the center of the cell. Triplicate samples were studied by counting 50 cells each. C) Histogram demonstrating relative position of spots in the cells.

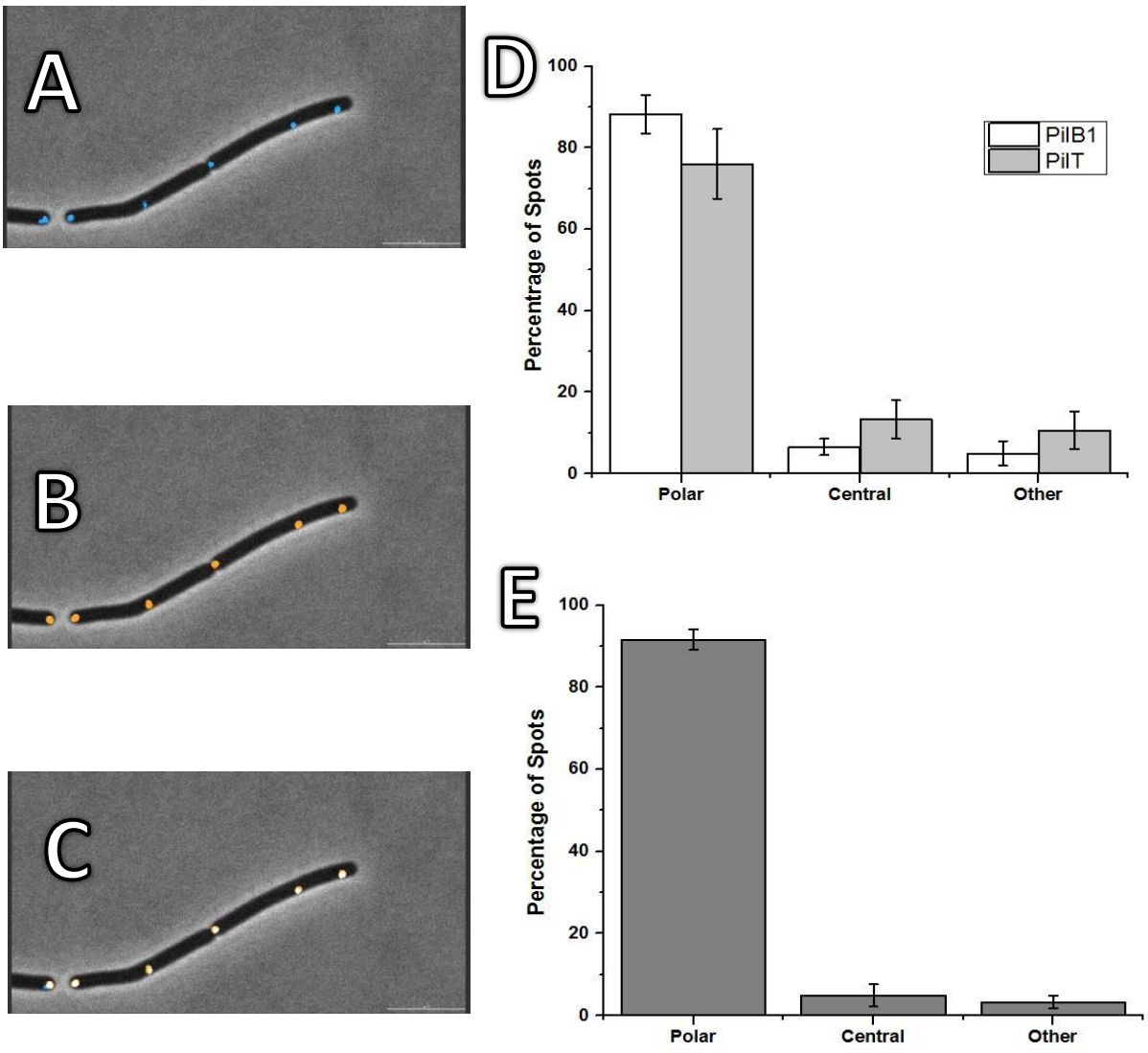

Figure 2.3) Localization of PilB1 and PilT and their colocalization in *B. subtilis*. A) Fluorescence microscopy of CFP-PilT which appears as blue spots. PilT localized to the poles 76% of the time and had central localization 11% of the time. B) Fluorescence microscopy of YFP-PilB1 which appears as orange spots. PilB1 localized to the poles 88% of the time and had central localization 7% of the time (*P* value< 0.0001). A graph (D) was produced to compare the localization of these two proteins (n=50). Fluorescence microscopy of CFP-PilT with YFP-PilB1 colocalization which appears as white spots where both proteins are present together. Colocalized proteins appeared at the poles 92% of the time and in the center 5% of the time (*P* value< 0.0001) – See the graph (E). Triplicate samples were studied by counting 50 cells each.

Localization of proteins during growth. Time-lapse movies were created to study localization as the cells were growing and dividing. Using the Applied Precision SoftWorx program and at a rate of one frame per 30 seconds, an hour long movie was made of each fusion protein culture. The location and migration of fluorescent proteins in 50 cells were tracked and recorded and the results are as follows:

- In fusions of PilB1 and PilB2, similar localization pattern were observed. The spots remained in their initial location (mostly polar) but as the cells grew, new spots emerged in the center where the cell was about to divide, with polar localization in the new daughter cells (movie 1 and movie 2). Both proteins were stationary throughout the movie and no migration was observed. This is different from what has been seen in *B. subtilis* and *C. perfringens* when PilT was studied. PilT moves through the cell from one end to the other or in a small area in both *B. subtilis* and *C. perfringens.*

- In CFP-PilT+YFP-PilB1 clones that both fusion proteins were present, colocalization was observed with 67% of the spots at the poles all the time and 8% in the center. Approximately, 25% of the spots moved from the pole to the center.

Seventy eight percent of all the PilB1 proteins had polar localization and remained at the pole throughout the movie and the rest of them moved from the pole to the center. This could be due to the presence of PilT but because of the weak CFP signal and fast photo-bleaching of this fluorescent protein, we are not able to see the CFP.

About 64% of all PilT proteins moved from sub-polar position to the center, 9% moved from the pole to the center, 18% were at the center throughout the movie and 9% were at the poles all the time (movie 3).

Protein expression. The expression of fusion proteins was also demonstrated by western blotting, using chemiluminescence technique (Fig. 2.4). The molecular weight of the ATPases are as follows: PilB1 is 53 kDa, PilB2 is 63 kDa and PilT is 39 kDa in monomeric form and the fluorescent proteins are about 27 kDa. The sizes of all the fusion proteins were correct, although some proteins also appeared without the fluorescent tag and some degradation was observed. This was more dominant in the case of PilB1 and PilB2. We expected to see a band at 70 kDa for CTYB2 but it seems the CFP protein is not expressed. This result confirms the fluorescence microscopy results of CTYB2pDR111 as PilB2 was observed but PilT could not be seen. We still do not know why cloning YB2 downstream of the CT interferes with the expression of the CFP protein in CTYB2, while PilT and YFP-PilB2 are expressed in those clones.

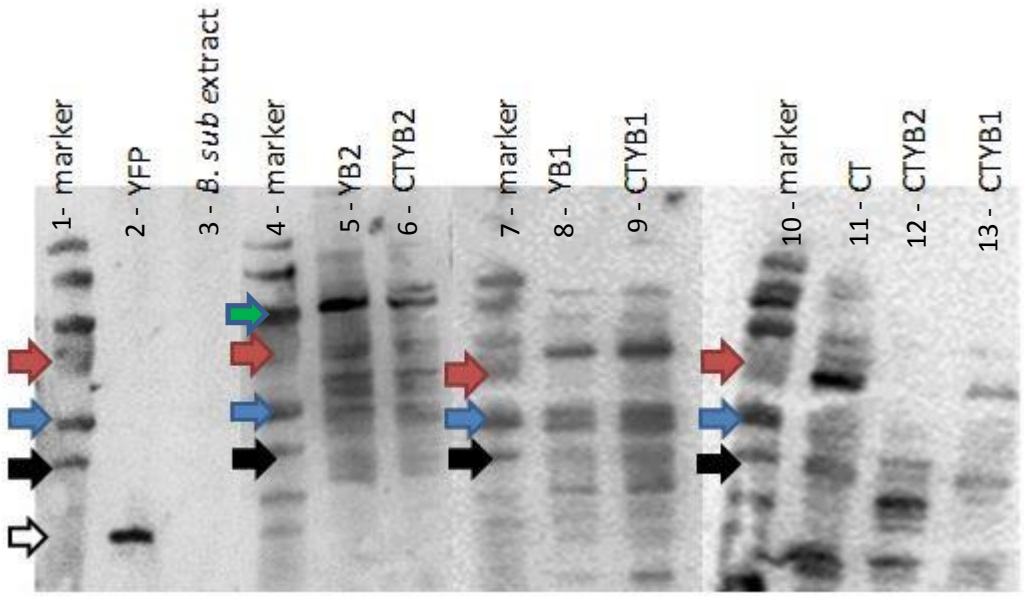

Figure 2.4) Western blot results of the fusion proteins expression in *B. subtilis* strain PS832 using GFP, PilB2, PilB1 and PilT antibodies, respectively.

Lane 2 contains the positive control for fluorescent protein expression, YFP and a 29 kDa can be seen (white arrow). Lane 3 is the negative control, containing *B. subtilis* cells extract. GFP antibody was used on both samples.

5) YB2 6) CTYB2 (with PilB2 antibodies). A band above the 100 kDa maker (green arrow) is visible which denotes YFP-PilB2 expression.

8) YB1 9) CTYB1 (with PilB1 antibodies). A band above the 72 kDa maker (red arrow) is visible which denotes YFP-PilB1 expression. There is a band present at 55 kDa marker (blue arrow) which denotes expression of PilB1 protein not tagged with YFP.

11) CT 12) CTYB2 13)CTYB (with PilT antibodies). Red arrows denote the 72kDa marker and we can see expression of fusion proteins CT and CTYB1 fusion proteins but no expression of CTYB2 fusion protein. A band is visible below lane 12 at 40 kDa marker (black arrow) which denotes expression of PilT protein.

Some protein degradation is observed in most samples.

Discussion

The localization results in *C. perfringens* suggested that PilB1 needs PilT for localization, unlike PilB2 which could localize to the poles in a *pilT* mutant. When these proteins were studied in *B. subtilis*, although we expected to see similar requirements since *B. subtilis* does not possess any TFP machinery proteins, PilB1 could localize to the poles similar to what was observed in wild type *C. perfringens* (see table 2.2 for a comparison of all localization results in *C. perfringens* and *B. subtilis*). Localization of PilB1 in *B. subtilis* can be attributed to specific proteins that are present in either *C. perfringens* or *B. subtilis* that affect PilB1 localization in these bacteria. Another possibility is that there are some artifacts present that make PilB1 proteins aggregate at the poles in these bacteria but we also saw new proteins emerge in the center of the cells as they were growing and expressing PilB1, before the bacteria divide, therefore the effect of absence or presence of other proteins on PilB1 is more likely. These results could also indicate that PilT is not highly necessary for localization of PilB1.

PilB2 localization is independent of PilT. The majority of the proteins appear at the poles and as the cells grow, new fluorescent foci appear in the center where the cells are about to divide. This was observed in the *C. perfringens* wild type and *pilT* mutant, as well as in *B. subtilis*. Since PilB has two homologues, it appears that PilT interacts with PilB1 but this does not explain PilB2-independent localization manner because in *C. perfringens* PilB2 colocalized with PilB1.

In colocalization of PilB1 and PilT, about 25% of colocalized proteins moved from the poles to the center where the cells were about to divide. We never observed any movement in PilB1 proteins when PilT was not present but when colocalized, it seems PilT localizes PilB1 proteins to the center as it moves along the cell. PilT motion has been observed in both *C.*

perfringens and *B. subtilis*, and it seems PilT is required for migration of PilB1 from the poles to the center of the cell. Moreover, the *pilT* gene is located in the same operon as cell division genes, *ftsA* and *ftsZ* in *C. perfringens* strain 13 and it may have a role related to cell division; probably a link between TFP assembly and division. However, cell division in a *pilT* mutant of strain 13 was not affected (9). When localization of PilT was studied in *B. subtilis* strain AH93 (9) that contains MciZ, a peptide that blocks the formation and function of FtsZ (43), PilT could not localized when MciZ was previously induced, suggesting that PilT requires division machinery for localization although cell division proteins are not dependent on PilT.

We still need to learn the effect of PilT when coexpressed with PilB2 in *B. subtilis* to have a better understanding of the roles of these proteins. But the CTYB2 microscopy has been unsuccessful because PilT could not be seen. Western blotting with anti-pilT antibodies revealed that CFP is not expressed in these clones. We will try constructing the plasmid again. Moreover, using a different cloning technique or changing the vector may help us study coexpression of PilB2 and PilT in *B. subtilis.*

Table 2.2) Comparison of localization results in *C. perfringens* and *B. subtilis*.

Fusion protein	*C. perfringens*			*B. subtilis*		
	polar	central	other	Polar	central	other
YFP (control)	Diffused through the cell			Diffused through the cell		
PilT in WT	70%	10%	20%	76%	16%	8%
PilB1 in WT	73%	10%	17%	80%	10%	10%
PilB2 in WT	73%	15%	12%	87%	9%	4%
PilT and PilB1 colocalization in WT	70%	13%	17%	92%	5%	3%
PilT and PilB2 colocalization in WT	70%	13%	17%	No data yet		
PilB1 in *ΔpilT*	Faint foci, unable to localize to poles			N/A	N/A	N/A
PilB2 in *ΔpilT*	73%	15%	12%	N/A	N/A	N/A
PilT localization in *ΔpilB1B2*	70%	10%	20%	N/A	N/A	N/A

CHAPTER THREE

Secretion and Activity of a von Willebrand Factor Type A-Domain Containing Protein in *Clostridium perfringens*

Abstract

Clostridium perfringens is an anaerobic Gram-positive bacterium which causes gas gangrene and food poisoning. Although commonly found in Gram-negative bacteria, Type IV pili (TFP) were discovered in *C. perfringens* and contribute to its gliding motility. TFP are assembled by ATPase-based polymerization of pilin proteins and are important virulence factors. Due to the similarities between TFP and Type 2 Secretion System (T2SS), we believe *C. perfringens* actually utilizes a T2SS for protein secretion. In previous studies, the secretome of a *pilA3* deletion mutant was missing the product of the gene *CPE0517*, a 71 kD protein characterized as a von Willebrand factor type A domain-containing (vWA) protein. We hypothesize that vWA protein is secreted by a T2SS and our goal is to understand the secretion and activity of this protein in *C. perfringens* and its role in pathogenesis. We have been able to purify the vWA protein by designing an overexpression system and using nickel affinity chromatography. The purified protein was tested on mouse myoblasts for toxicity but no effect was observed. We have also performed binding assays using fluorescently-labeled vWA to study its ability to bind to eukaryotic cells and preliminary results suggest that it does. An in-frame deletion mutant of *CPE0517* in strain HN13 was also constructed to study the phenotypes such as secretome and adherence. We believe that vWA is secreted by *C. perfringens* and that it could be part of a toxin complex where it is the subunit that binds to host cells.

Introduction

Clostridium perfringens is a Gram-positive rod-shaped anaerobic bacterium (1, 2) with a low G + C content and relatively short generation time (8-10 minutes) (5-7). It is the most abundant pathogen in nature causing various diseases such as gas gangrene and food poisoning. It is found in different environments such as soil, sewage and intestines of humans and animals as part of the normal flora (4).

C. perfringens has Type IV Pili dependent gliding motility (15). TFP are very important virulence factors in many Gram-negative pathogens including *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Neisseria gonorrhea* (14) .

Type IV pili (TFP) are produced by ATPase-based polymerization of pilin protein and they are involved in the attachment to host cells, DNA uptake, and twitching motility (14). Type 2 Secretion Systems (T2SS) are evolutionarily related to TFP with similarities in structure and function and are used for protein secretion in many Gram-negative bacteria (21, 22). We believe *C. perfringens* also has a T2SS because it has two TFP systems and genetic evidence suggests one of these may actually be a T2SS (3). Secretion through this system involves protein expression with a signal sequence which is transported either via Sec or Tat machinery, followed by the removal of the signal sequence, and translocation of the folded protein (21, 26).

During a study on pilin genes in the Melville lab (9), it was observed that a *pilA3* mutant lacked a protein in the secretome which was identified as the product of gene *CPE0517*, a 71 kDa protein known as a von Willebrand type A-domain containing protein. The vWA domain has 30% similar sequence identity with von Willebrand Type A domains found in both prokaryotes and eukaryotes. They are generally involved in cell adhesion and found in

extracellular matrices and integrin receptors (40). In *C. perfringens*, the vWA protein is encoded in an operon where the upstream genes products are SipW (a signal peptidase), camelysin, and a protein with unknown function (Fig. 3.1). We hypothesized that vWA is secreted by a T2SS and in this study we wanted to understand the secretion and activity of this protein in *C. perfringens* strain HN13.

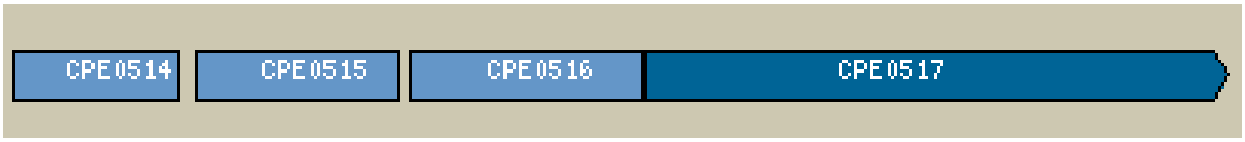

Figure 3.1) The vWA (*CPE0517*) operon in *C. perfringens* strain 13. *CPE0514* encodes SipW, *CPE0515* encodes camelysin and the product of *CPE0516* has unknown function.

Materials and Methods

Overexpression system for a vWA protein:

The *CPE0517* gene was amplified by primers OOG5 and OOG6, cloned into a pGEM®-T Easy vector (Promega) and electroporated into *E. coli* strain DH10B which was spread on Luria-Bertani (LB) containing 40 μg/ml X-gal and 100 μg/ml ampicillin. Two plasmids isolated were pJM3 and pJM4 (courtesy of Jordan Mancl). The white colonies were screened by restriction digestion using NcoI and XhoI (New England Biolabs®) and after gel electrophoresis (0.8% agarose in 1X TAE buffer), the inserts were excised, extracted by a QIAGEN QIAQUICK Gel Extraction Kit (QIAGEN) and ligated into pET28-A vector (EMD Millipore) which adds a histidine hexamer to the protein. The ligation products were first introduced into strain DH10B and were screened by restriction digestion again to identify one clone, pJM5. pJM5 was electroporated into *E. coli* strain BL21-CodonPlus®-RIL Competent Cells (Stratagene), a protein expression system for bacteria with low G-C content, and plasmid pJM8 was identified. The cells were grown overnight in LB broth containing 100 ug/ml kanamycin at 37^{o} C and subcultured the next day in to fresh LB Kan^{100} medium. Overexpression was induced at OD_{600} = 0.4 by addition of 0.1 mM IPTG and after six hours, the *E. coli* culture was centrifuged (10,000 rpm, 20'). Both the supernatant and the pellet were collected. The supernatant was precipitated using 2 M trichloroacetic acid (41). For loading buffer, 1X SDS was used and the precipitates were boiled for 20 minutes in it. Protein sample was loaded (10 μl) on a 10% polyacrylamide gel (Fisher BioReagents). Presence of vWA protein was demonstrated by Coomassie protein staining and InVision™ His-tag In-gel Stain, and the protein was found to be in the supernatant. (See Table 1 for a list of plasmids and primers).

Purification of the vWA protein:

For protein purification, a culture of *E. coli* strain BL21-CodonPlus®Ril with pJM8 was prepared as previously described but 1 liter was induced with IPTG (1mM). The supernatant was collected and precipitated by ammonium sulfate (3.9M) at 0^{o} C, and the pellet was resuspended in phosphate buffered saline (20 mM sodium phosphate, 0.5 M NaCl, pH=7.6) , dialyzed in Snakeskin tubing (Thermo Scientific) with a 3.5 kDa cut-off, and loaded onto a HisTrap FF 5 mL Nickel affinity column (GE Healthcare). First the column was equilibrated in a buffer (20 mM sodium phosphate, 0.5 M NaCl, 150 mM imidazole, pH=7.6), then the vWA was eluted from the column by stepwise addition of a gradient of imidazole (500 mM) in buffer. The presence of vWA protein fractions was confirmed by western blot using antibody against histidine hexamer and Coomassie staining, then it was concentrated by spin column (EMD) to 500 µl and the protein concentration was estimated using Pierce™ BCA Protein Assay kit. To purify the protein further for future experiments, size exclusion chromatography was performed using a HiPrep 26/60 Sephacryl S-200 HR column (GE Healthcare). The purified protein was then run on a 10% SDS-PAGE gel, and the gel was stained with Coomassie Blue, and a western blot was performed using vWA antibodies (final dilution: 10^{-3}) and ThermoScientific DyLight® 594 conjugated secondary antibody (final dilution: 10^{-4}). The result was detected using a Typhoon 9400 Variable Imager (GE Healthcare). The protein purity was estimated by ImageJ software.

Assessment of the vWA effect on eukaryotic cells:

To study the effect of the vWA on eukaryotic cells *in vitro*, the purified protein was tested on the murine myoblast cell line C2C12 for toxicity. The protein was diluted at different concentrations in PBS (10 µg/µl, 5 µg/µl, 2.5 µg/µl, 1.25 µg/µl, 0.5 µg/µl, 0.25 µg/µl, 0.1 µg/µl, 0.05 µg/µl,

0.001 μg/μl) and incubated with the cells for 24 hours at 37^{o}C, which were then observed by a Microscoptics IV900 series microscope.

Binding assay:

The vWA protein was labelled with a fluorescent dye using Alexa Fluor® 594 Protein Labeling Kit. Murine cells (myoblast strain C2C12 and macrophage strain J774) were exposed to different concentrations of this fluorescently labelled vWA (concentrations: 0.1 μg/ml, 1 μg/ml, 5 μg/ml, 40 μg/ml). Fluorescence microscopy was performed on an Olympus IX81 microscope.

Construction of a vWA mutant:

Over-lapping PCR on the *CPE0517* gene was performed to create the deletion. The primers for the first reactions were OOG1 and OOG2 at 5' end and OOG3 and OOG4 at 3' end. Over-lap extension PCR was performed by oOG1 and oOG4. The products were run on a 1.5% agarose gel and the 2 kb bands were excised and purified as previously described, ligated into pGEM®-T Easy vector, electroporated into *E. coli* DH10B and plated on LB Xgal^{40} amp^{100} agar. White colonies were screened by restriction digest (BamHI and SalI) and the insert DNA was ligated into the pCM-galK suicide plasmid and electroporated into DH10B strain and spread on LB chloramphenicol (20 μg/ml) plates. Once again the ligation products were screened by restriction digest. Using a Qiagen Midiprep kit, the plasmid was purified and electroporated in *C. perfringens* strain HN13 (pJM6) and the mutant strain was made based on a previously published protocol (42). The mutant clones were screened by PCR using primers OSN1 and OSN2, designed inside the ORF region, and flanking region primers, OSN3 and OSN4 and the mutant was isolated (pJM7).

A protein secretion assay was also performed on the mutants against the wild type. Both were grown inside a Coy anaerobic chamber in Brain Heart Infusion (BHI) broth overnight (37^{o}C), diluted to fresh broth in the morning (dilution factor: 10^{2}) and grown for 8 hours. Then the supernatant was collected by centrifuging the culture (15,000 x g, 2', 4^{o}C). The supernatant was filtered through a 0.2 μm filter (Fisherbrand) and was TCA precipitated (42). Ten microliter of samples were loaded and run on SDS-PAGE after being boiled in SDS sample buffer for 20 minutes. The gels were stained by SYPRO®-Ruby to visualize the bands. (See Table 1 for a list of plasmids and primers.).

Table 3.1) A list of plasmids and primers used in this study.

Plasmids	**Description**	**Reference/Source**
pJM1 pJM2	*CPE0517* over lapping PCR product in pGEM-T Easy with BamHI, SalI digestion sites/ amp^R	This study
pJM3 pJM4	*CPE0517* PCR product in pGEM-T Easy with NcoI and XhoI site/ amp^R	This study
pJM5	pJM4 in pET-28a, a protein expression vector, carrying a His Tag coding sequence/ kan^R	This study
pJM6	pJM1 in pCM-GalK suicide plasmid to create in-frame deletion mutants/ cam^R	This study
pJM7	pJM1 in pCM-GalK suicide plasmid to create in-frame deletion mutants/ cam^R after electroporation into *C. perfringens* str.13	This study
Primers	**Sequence** **(all sequences are 5' to 3')**	

OOG1 OOG2 OOG3 OOG4	GGATCCACTGCAAACTTATTAGAAAGTGTTAC CAAAAATAAAAAGTTTTCCATCTTAATTTATC TTCATATCTCTCCCCACCTAAC GTTAGGTGGGGAGAGATATGAAGAATTTAAA TTAAGATGGAAAACTTTTTATTTTTG GTCGACACATAAAATTACATATCGCCTATTC	This study
OOG5 OOG6	CCATGGAAGAATATAAGAAAATTTTTTGTG CTCGAGATTTAATTTTAATATACCAAAATC	This study
OSN1	GGATATTGGTAATGAAAGCCAAGG	This study
OSN2	CTTTTGTTTTCTGAACTTTCTGTAACC	This study

Results

The overexpression and purification of a vWA protein. The vWA protein overexpression was induced by IPTG in *E. coli* BL21 using pET-28a vector and it was shown to be secreted by the bacteria. The secreted protein was precipitated by ammonium sulfate and dialyzed using Snakeskin tubing to be purified by Nickel affinity –chromatography. The vWA protein was eluted by stepwise addition of 500 mM imidazole and the eluted fractions were loaded on an SDS-PAGE gel. The results were observed by Coomassie staining and Western blot using anti-histidine antibodies where a 70 kDa band appeared which corresponded with the size of this protein (Fig. 3.2A and 3.2B). The protein was concentrated using spin columns and the concentration was estimated by Pierce BCA Assay (24 μg/μl). The concentrated protein was purified further with size exclusion chromatography, fractions were separated on a gel stained with Coomassie, and western blot was performed using anti-vWA antibodies (Fig. 3.2C and 3.2D). The 71 kDa band purity (99% purity estimation) was quantified using ImageJ software.

The effect of vWA on eukaryotic cells. The purified vWA was tested on mouse myoblast cell line (C2C12) to test it for toxicity at different concentrations. Microscopy results showed no morphological changes in the C2C12 cells which suggest that the vWA protein does not have a toxic effect.

vWA ability to bind to host cells. The purified protein was labeled by Alexa Fluor 594 protein labeling kit and murine cells (myoblast C2C12 and macrophages J774) were exposed to different concentrations of vWA. Microscopy results showed that the protein binds to macrophage membrane at 40 μg/ml concentration (Fig. 3.3).

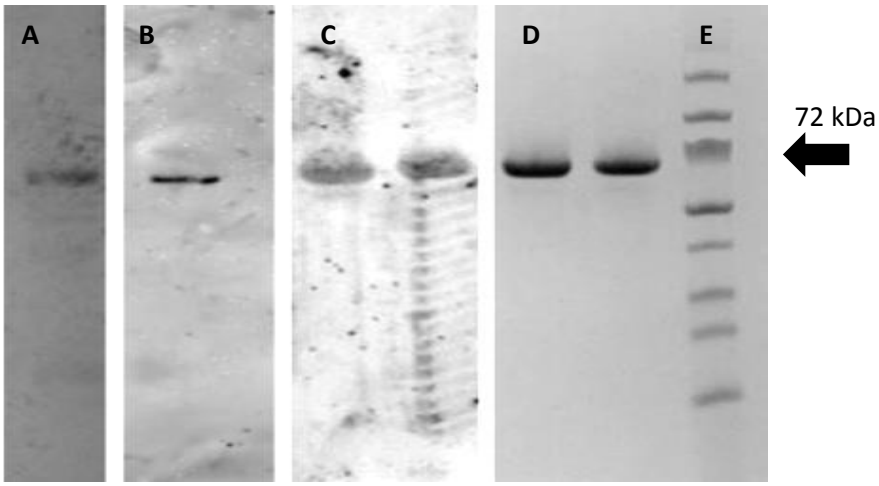

Figure 3.2) the purification of vWA protein by Nickel Affinity column and Size exclusion Chromatography. A) Coomassie Blue staining after SDS-PAGE of one collected fraction from Ni2+ affinity chromatography B) Western blot with histidine antibodies after Ni2+ affinity chromatography. C) Western blot of two collected fractions after size exclusion chromatography using vWA antibodies. D) Coomassie Blue staining after SDS-PAGE of one collected fraction from size exclusion chromatography. E) E-Z Run protein marker. Sample volume in each lane = 10 μl.

The ability of the vWA protein to form crystals. The purified protein was sent to Dr. Florian Schubot's lab for testing the vWA in crystal formation and the results suggest that this protein can form crystal structures. No further work was done on this.

Construction of a vWA mutant. To have a better understanding of the vWA protein function, we constructed an in-frame deletion mutant of this gene in *C. perfringens* strain HN13 by using pCM-GalK as the suicide plasmid (42). The mutants were confirmed by PCR screening. The mutants and the wild type were grown inside an anaerobic chamber in BHI overnight, subcultured to fresh media in the morning and grown for 3 hours. Then the supernatant was collected and TCA precipitated. Samples were run on SDS-PAGE and the gels were stained by Sypro®-ruby to visualize the bands. Comparison of the mutant secretome with the one of the wild type shows the 70 kDa band is missing in the mutant and confirms the protein is not secreted in those cells (Fig. 3.4).

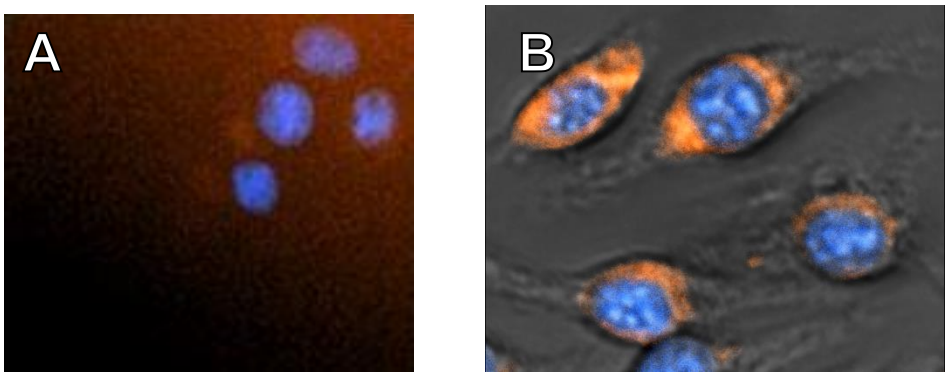

Figure 3.3) Fluorescently labeled vWA and murine macrophages (J774). Macrophage DNA appears blue by Hoechst stain and vWA appears red by Alexa Fluor dye. A) J774 control cells (not exposed to vWA protein). B) J774 cells exposed to 40 μg/ml of vWA. In this image, red spots bound to the cell membrane are the fluorescent vWA protein.

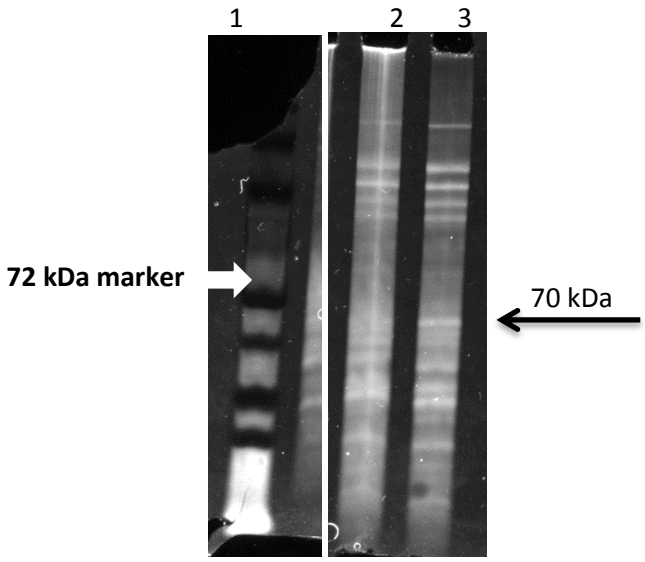

Figure 3.4) SDS-PAGE gel showing Sypro Ruby stained secretome profiles of the *ΔCPE0517* (lane 2) and WT strain (lane 3). The white arrow points to the 72 kDa band on the E-Z run marker. The image is courtesy of Dr. Melville.

Discussion

We were able to purify vWA, a 71 kDa protein with an unknown function, which is secreted by *C. perfringens*. We tested this protein on murine myoblast cell line but no morphological changes were observed in the cells so we concluded that vWA is likely not a toxin. This protein was also tested in a binding assay and murine myoblast and macrophages were exposed to fluorescently labelled vWA. Binding of the protein to the macrophage membrane was observed but the vWA did not show any attachment to the myoblast cells. Since *vWA* gene is downstream of an operon which includes *sipW* (a gene coding for a signal peptidase), a gene encoding camelysin and another gene with unknown function, respectively (Fig. 1), we hypothesize vWA can be part of a toxin complex with the other proteins. It is possible that SipW cleaves the signal sequence of each member of the complex. In order to test this hypothesis, more experiments are required. An immunoprecipitation assay needs to be performed to pull-down the Camelysin-CPE0516 product-vWA complex and test its toxicity. Moreover, the *CPE0515* and *CPE0516* gene products can be overexpressed and tested along with vWA for toxic effect on mammalian cells and also if this complex is capable of binding to such cells.

We also constructed an in-frame deletion mutant of *vWA* and compared the protein secretion to wild type *C. perfringens* secretome and confirmed the missing 71 kDa band in the mutant. The phenotype of this mutant needs to be understood by performing binding assays. Another experiment can be the construction of in-frame deletion mutants of the 3 genes upstream *cpe0517* and check their secretomes for vWA secretion. Additionally, we already know that *pilA3* mutant cannot secrete the vWA protein and we can analyze other Type IV Pili mutants for the secretion of this protein.

The next step would be purifying more vWA and define the protein structure by X-ray crystallography, especially since we have found that vWA is able to form crystal structures (F. Schubot, personal communication).

CHAPTER FOUR

Final Discussion

Clostridium perfringens is an anaerobic spore-forming Gram-positive bacterium which is motile because of Type IV Pili (TFP). The TFP are found abundantly in Gram-negative bacteria and they are involved in DNA uptake, attachment to host cells and twitching motility but recently TFP were found in Gram-positive bacteria including *C. perfringens.* The TFP assembly mechanism is based on polymerization of pilin proteins by the energy provided from assembly ATPases. The similarities that exist between TFP and Type 2 Secretion Systems (T2SS) may indicate that Gram-positive bacteria also have a T2SS for protein secretion. The significance of these findings lie in their function as virulence factors in these bacteria. Having a better understanding of both systems is important in discovering new treatment methods for bacterial infections.

In previous work in the Melville lab, localization of TFP ATPases was studied. There are two extension ATPases homologues, PilB1 and PilB2 and one retraction ATPase, PilT. Since the pili are at the poles of *C. perfringens*, it is assumed that the TFP apparatus is at the poles as well. The research goal was to understand how these ATPases localize to the septa during cell growth and in what order this localization happens. The results suggested that PilT is required for localization of PilB1 but not PilB2. Meanwhile, PilT could localize normally in the absence of PilBs.

In the next step, we studied the localization of these proteins in *Bacillus subtilis*, as it does not possess any genes similar to the TFP genes so we could study the localization in an organism free from the effect of TFP machinery. An integration plasmid with a *hyperspac* promoter was used for carrying the fusion genes and introducing them to *B. subtilis* and localization of fluorescent proteins were studied using fluorescence microscopy.

In *B. subtilis*, PilB1 could localize to the poles although PilT was not present. This could be due to presence of specific proteins in either *C. perfringens* or *B. subtilis* that affect PilB1 localization. Localization of PilB2 was similar to *C. perfringens* and did not require PilT. When PilB1 was co-expressed with PilT, movement of fluorescent proteins was observed in time-lapse movies. Majority of the PilB1 proteins that migrated from the poles to the septa were associated with PilT proteins and those that were singular, did not move, unlike singular PilT which moved in different motions through the cell. Therefore, it seems PilB1 needs PilT to localize from the poles to the septum of the dividing cells.

The critical role of PilT could be due to the location of the gene in *C. perfringens* strain 13 chromosome. This gene is in the same operon as *ftsA* and *ftsZ* genes and it may have a role as a linking protein between cell division proteins and TFP assembly. Moreover, the observed motion of PilT in the cells could also be due to its association with the division proteins.

In order to have a better understanding of the localization, we still need to study the localization of PilB2 when co-expressed with PilT in *B. subtilis* but the fluorescence microscopy results along with western blot suggest that the CFP portion is not expressed when YB2 is present. The issue seems to be from the CT plasmid and it should be resolved by repeating the cloning method.

In another project, secretion and activity of a vWA protein was studied in *C. perfringens.* This protein was identified when a 71 kDa band disappeared from the secretome of a *pilA2* mutant. An overexpression system was designed to produce the protein and using Nickel affinity chromatography and gel filtration techniques, we were able to purify the vWA protein. This

protein was tested on mammalian cells for toxic effects and also in binding assays. The results suggested that vWA is probably not a toxin but it can bind to macrophage membranes.

Since *vWA* coding sequence is in the same operon as a signal peptidase and camelysin, it could be part of a toxin complex that is secreted via a T2SS and vWA is the binding subunit of the complex. In order to better learn if such a complex exists, vWA can be overexpressed along with other proteins encoded on that operon and then test the effect of all these proteins together on host cells.

An in-frame deletion mutant of *vWA* was also constructed and the secretome was compared to wild type and the 71 kDa band was missing from the mutant. This mutant can be tested in binding assays to see if it is deficient in binding to mouse myoblasts (the C2C12 cells).

Our results suggest that vWA is capable of forming crystals, so it may be possible to determine the structure of this protein by using X-ray crystallography which would help us in predicting the protein function and also the interactions of the amino acids of the vWA.

The ultimate goal of studying TFP and T2SS and the components of these two systems may help in understanding the mechanism of some virulence factors and toxin secretion in *C. perfringes* which is an important pathogen for humans and other mammals, capable of producing a variety of toxins and causing a number of different diseases. The findings may help in developing prevention and treatment methods for Clostridial infections and other bacteria that use type IV pili and type 2 secretion systems.

References

1. **Rood JI, Cole ST.** 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiological reviews **55:**621-648.
2. **Bahl H, Durre, P.** 2001. Clostridia: Biotechnology and Medical Applications. Wiley.
3. **Melville S, Craig L.** 2013. Type IV pili in Gram-positive bacteria. Microbiol Mol Biol Rev **77:**323-341.
4. **Flores-Diaz M, Alape-Giron A.** 2003. Role of *Clostridium perfringens* phospholipase C in the pathogenesis of gas gangrene. Toxicon **42:**979-986.
5. **Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, Ogasawara N, Hattori M, Kuhara S, Hayashi H.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc Natl Acad Sci U S A **99:**996-1001.
6. **Sebald M, Costilow RN.** 1975. Minimal growth requirements for *Clostridium perfringens* and isolation of auxotrophic mutants. Applied microbiology **29:**1-6.
7. **Dürre P.** 2005. Handbook on Clostridia. Taylor & Francis, Boca Raton.
8. **Rood JI.** 1998. Virulence genes of *Clostridium perfringens*. Annu Rev Microbiol **52:**333-360.
9. **Hartman A.** 2012. Construction and Characterization of an Inducible Promoter and Type IV Pili Homologues in *Clostridium perfringens*. Master of Science. Virginia Polytechnic Institute and State University.
10. **Rood JI, McClane BA, Songer JG, Titball RW.** 1997. The Clostridia: molecular biology and pathogenesis. Academic Press.

11. **Moreau H, Pieroni G, Jolivet-Reynaud C, Alouf JE, Verger R.** 1988. A new kinetic approach for studying phospholipase C (*Clostridium perfringens* alpha toxin) activity on phospholipid monolayers. Biochemistry **27:**2319-2323.

12. **Novak JS, Juneja VK.** 2002. *Clostridium perfringens*: hazards in new generation foods. Innovative Food Science & Emerging Technologies **3:**127-132.

13. **Walket TGCMaPD.** 1991. The pigbel story of Papua New Guinea. Transactions of the Royal Society of Tropical Medicine and Hygiene (1991) 85, 119-122.

14. **Craig L, Pique ME, Tainer JA.** 2004. Type IV pilus structure and bacterial pathogenicity. Nature Reviews Microbiology **2:**363-378.

15. **Varga JJ, Nguyen V, O'Brien DK, Rodgers K, Walker RA, Melville SB.** 2006. Type IV pili-dependent gliding motility in the Gram-positive pathogen *Clostridium perfringens* and other Clostridia. Molecular microbiology **62:**680-694.

16. **Aas FE, Wolfgang M, Frye S, Dunham S, Løvold C, Koomey M.** 2002. Competence for natural transformation in Neisseria gonorrhoeae: components of DNA binding and uptake linked to type IV pilus expression. Molecular microbiology **46:**749-760.

17. **Craig L, Taylor RK, Pique ME, Adair BD, Arvai AS, Singh M, Lloyd SJ, Shin DS, Getzoff ED, Yeager M.** 2003. Type IV pilin structure and assembly: X-ray and EM analyses of Vibrio cholerae toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. Molecular cell **11:**1139-1150.

18. **Craig L, Volkmann N, Arvai AS, Pique ME, Yeager M, Egelman EH, Tainer JA.** 2006. Type IV pilus structure by cryo-electron microscopy and crystallography: implications for pilus assembly and functions. Molecular cell **23:**651-662.

19. **Nudleman E, Kaiser D.** 2004. Pulling together with type IV pili. Journal of molecular microbiology and biotechnology **7:**52-62.

20. **Jakovljevic V, Leonardy S, Hoppert M, Sogaard-Andersen L.** 2008. PilB and PilT are ATPases acting antagonistically in type IV pilus function in *Myxococcus xanthus*. J Bacteriol **190:**2411-2421.

21. **Korotkov KV, Sandkvist M, Hol WG.** 2012. The type II secretion system: biogenesis, molecular architecture and mechanism. Nat Rev Microbiol **10:**336-351.

22. **Sandkvist M.** 2001. Type II secretion and pathogenesis. Infection and immunity **69:**3523-3535.

23. **Bose N, Payne SM, Taylor RK**. Type 4 pilus biogenesis and type II-mediated protein secretion by *Vibrio cholerae* occur independently of the TonB-facilitated proton motive force. J Bacteriol. 2002;184:2305–2309.

24. **Kulkarni R, Dhakal BK, Slechta ES, Kurtz Z, Mulvey MA, et al**. (2009) Roles of Putative Type II Secretion and Type IV Pilus Systems in the Virulence of Uropathogenic Escherichia coli. PLoS ONE 4(3): e4752. doi:10.1371/journal.pone.0004752

25. **Vignon G, Kohler R, Larquet E, Giroux S, Prevost MC, Roux P, Pugsley AP.** 2003. Type IV-like pili formed by the type II secreton: specificity, composition, bundling, polar localization, and surface presentation of peptides. J Bacteriol **185:**3416-3428.

26. **Johnson TL, Abendroth J, Hol WG, Sandkvist M.** 2006. Type II secretion: from structure to function. FEMS Microbiol Lett **255:**175-186.

27. **Gillespie, S. H. and Hawkey, P. M.** (eds) (2006) Index, in Principles and Practice of Clinical Bacteriology, Second Edition, John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9780470017968.index

28. **Meyer, J. (1987), J. G. Holt** (Editor-In-Chief), Bergey's Manual of Systematic Bacteriology, Volume 2 (Editors: P. H. A. Sneath, N. S. Mair, H. E. Sharpe). XXIII + 630S., 262 Abb., 168 Tab. Baltimore-London-Los Angeles-Sydney 1986. Williams and Wilkins. $ 75.00. ISBN: 0-683-07893-3. J. Basic Microbiol., 27: 398. doi: 10.1002/jobm.3620270714

29. **Dubnau D.** 1991. Genetic competence in *Bacillus subtilis*. Microbiological reviews **55:**395.

30. **Baumann, P., L. Baumann, C.-Y. Lai, D. Rouhbakhsh, N. A. Moran, and M. A. Clark**. *Bacillus subtilis* and its closest relatives: from genes to cells. ASM Press, Washington, D.C. 5. 1995.

31. **Vavrova L, Muchova K, Barak I.** 2010. Comparison of different *Bacillus subtilis* expression systems. Res Microbiol **161:**791-797.

32. **Yansura, D.G., Henner, D.J.**, 1984. Use of the *Escherichia coli* lac represor and operator to control gene expression in *Bacillus subtilis*. Natl. Acad.Sci. USA. 81, 439e443

33. **Craig L TR, Pique ME, Adair BD, Arvai AS, Singh M, Lloyd SJ, Shin DS, Getzoff ED, Yeager M, Forest KT, Tainer JA. 20**03. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. Mol Cell 2003 May;11(5):1139-50.

34. **Britton R, Gonzalez-Pastor JE, Fawcett P, Monson R, Losick R, and Grossman AD.** 2002. Genome-Wide Analysis of the Stationary-Phase Sigma Factor (Sigma-H) Regulon of *Bacillus subtilis*. Journal of Bacteriology 184.17 (2002), pp. 4881–4890.

35. **C. Anagnostopoulos and J. Spizizen**. “Requirements for transformation in *Bacillus subtilis*”.In: Journal of Bacteriology 81 (1961), pp. 74–76.

36. **P. T. Tran, A. Paoletti, and F. Chang**. “Imaging green fluorescent protein fusions in living fission yeast cells”. In: Methods 33.3 (2004), pp. 220–5.

37. **Ballesteros-Plaza B, Holguera I, Scheffers D, Salas M, and Muñoz-Espín D .** Phage {phi}29 protein p1 promotes replication by associating with the FtsZ ring of the divisome in *Bacillus subtilis*. PNAS 2013 110: 12313-12318.

38. **E. C. Garner**. “MicrobeTracker: quantitative image analysis designed for the smallest organisms”.In: Mol Microbiol 80.3 (2011), pp. 577–579.

39. **Whittaker CA, Hynes RO.** 2002. Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Molecular biology of the cell 13:3369-3387.

40. **Nariya H, Miyata S, Suzuki M, Tamai E, Okabe A. 2011.** Development and application of a method for counterselectable in-frame deletion in *Clostridium perfringens*. Appl Environ Microbiol 77:1375-1382.

41. **clw**. 2001. TCA protein precipitation protocol. http://wwwitscaltechedu/~bjorker/Protocols/TCA_ppt_protocolpdf.

42. **I. Barak** et al. “Lipid spirals in *Bacillus subtilis* and their role in cell division”. In: Mol Microbiol 68.5 (2008), pp. 1315–27.

43. **A. A. Handler, J. E. Lim, and R. Losick**. “Peptide inhibitor of cytokinesis during sporulation in *Bacillus subtilis*”. In: Mol Microbiol 68.3 (2008), pp. 588–599.