Type IV Pili-Dependent Secretion of Biofilm Matrix Material Proteins in *Clostridium perfringens*

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Abstract:

Clostridium perfringens is a Gram-positive bacterium that secretes a biofilm matrix material. The goal of these experiments was to identify pilin mutants that are needed for secretion of the biofilm matrix and develop a functional model for a type II secretion system (T2SS) in *C. perfringens.* Protein tagging, western blot, and slot blot experiments were done to quantify protein secretion. After performing experiments using a CPE0515-FLAG construct, it was concluded from immunoblot densitometry data that, except for the *pilA1* deletion mutant, none of the 18 tested pilin mutants had a statistically significant difference from the wild type (WT) with regard to protein secretion. From slot blot densitometry assays, it was concluded that the *pilA1* and *CPE2280* mutants showed statistically significant lower values than the WT but the *pilA2* and *CPE1841* mutants had values that were higher than the wild type. Testing the construct containing only *CPE0514* and *CPE0515-FLAG* showed that CPE0516 and CPE0517 are not needed for secretion of the protein CPE0515. HA-tagged CPE0516 qualitative immunoblots showed that, unlike CPE0515, oligomerization of CPE0516 is not occurring, and that this protein likely forms a heat stable dimer. Overall, the data did not allow us to construct a T2SS model, since there were not enough proteins revealed to be involved to create a complete Type II secretion system.

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General Audience Abstract

The methods by which *C. perfringens* can persist and survive in environmental conditions is something that would be useful to learn more about. One of the methods that many bacteria use to survive is by creating a biofilm matrix material, which provides protection for the bacteria from environmental stresses. In this study, the goal was to determine which specific proteins are needed for the secretion of the biofilm matrix material. Using molecular biology techniques, the proteins thought to be involved in biofilm formation quantified. The results showed that while two proteins ultimately appeared to be needed for secretion, there were not enough proteins involved to create a complete model for a functional secretion system in *C. perfringens*.

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Chapter 1: Literature Review

Clostridium perfringens Overview

Clostridium perfringens is an anerobic, spore forming, Gram-positive, rod-shaped bacterium (Yao & Annamaraju, 2021). Because it is an anaerobe, it is limited by the location and amount of oxygen it encounters in the host it is infecting. *C. perfringens* has many notable characteristics that allow it to be successful in invading host tissue and can cause numerous types of diseases. *C. perfringens*, like other types of *Clostridia,* secretes toxins that are designed to kill the host they infect, including both humans and animals (Rood *et al.,* 2018). *C. perfringens* also has virulence factors that are proposed to be used for secretion of protein toxins and overall pathogenesis (Geier *et al.*, 2021).

Diseases caused by *C. perfringens.* *C. perfringens* causes many different types of diseases, as shown in Table 1.1. In humans, toxinotype A causes gas gangrene, toxinotype C causes human enteritis necroticans, and toxinotype F causes food poisoning (Uzal *et al.*, 2018). Table 1.1 also summarizes animal diseases, where toxinotype B and D causes enterotoxemia of sheep, toxinotype C causes necrotic enteritis, toxinotype E causes enterotoxemia in calves and rabbits, and toxinotype G, and rarely toxinotype F, causes necrotic enteritis in birds.

Food poisoning (a form of gastroenteritis) caused by *C. perfringens* is common, and occurs when spores are found in food, often meat (Ghoneim & Hamza, 2017). The spores withstand the heat of cooking, and if the food is not stored properly the spores are able to germinate into vegetative cells and multiply in the food (Sanchez-Plata *et al.*, 2005). When ingested, the bacteria then sporulate and produce a toxin that causes intestinal inflammation (Kiu & Hall, 2018). The strain used in this study, HN13 (Nariya *et al.*, 2011), was constructed from *C. perfringens* strain 13, which primarily causes deep tissue infections known as gas gangrene (clinically referred to as myonecrosis), as well as food poisoning (Katayama *et al.,* 2009). Additionally, necrotic enteritis can be caused by toxinotype type C in humans and some animals, and type F in birds, however this is a rare small intestinal infection (Uzal *et al.*, 2014). Enterotoxemia is another kind of intestinal disease caused by *C. perfringens*, as observed in animals (Uzal & Songer, 2008). The diseases that *C. perfringens* causes have many different toxins associated with them, as summarized in Table 1.1.

Toxins utilized by *C. perfringens.* Toxins are one of many mechanisms that *C. perfringens* uses to effectively cause disease in its host (Kiu & Hall, 2018). There are seven toxinotypes of *C. perfringens* that can be grouped based on toxigenic type, including A, B, C, D, E, F, and G, as mentioned above. (Uzal *et al.*, 2018). The categorization of the toxinotypes primarily depends on the toxin profile (Table 1.1).

The major toxins include alpha (lecithinase or PLC), beta, epsilon, and theta toxins (PFO), in addition to CPE (enterotoxin), NetB, and iota toxin, (Uzal *et al.*, 2018). Alpha toxin has phospholipase C

(PLC) and sphingomyelinase activity, and is hemolytic, cytolytic, necrotizing, and lethal (Saint-Joanis *et al.*, 1989). PLC acts by catalyzing the hydrolysis of a phosphodiester bond, yielding a water-insoluble 1,2 diacylglyceride and phosphorylcholine (Cocco *et al.*, 2015). PLC is the major virulence factor that allows *C. perfringens* to cause gas gangrene infections, shown using animal models (Flores-Diaz & Alape-Giron, 2003). Epsilon toxin is important for types B and D, and causes enterotoxemia in animals such as sheep, foals, and goats (Gil *et al.*, 2015). Theta toxin (PFO) assists PLC in its activity, but PFO itself is not necessary for a full gangrene infection (O'Brien & Melville, 2004). CPE is produced by *C. perfringens* cells that have high rates of spore formation (Lindsay, 1996). CPE can form pores in the membranes the host cells, and does so by binding to claudin receptors (Benz & Popoff, 2018). Claudins are the most crucial part of the tight junctions, and when CPE binds to claudins this causes a conformational change in the structure of the claudins (Benz & Popoff, 2018). This leads to a disruption in the host cell cytoplasmic membrane ultimately ending with apoptosis, making CPE cytotoxic (Chakrabarti *et al.*, 2003). NetB is another toxin that is pore-forming, and is the major toxin associated with type G toxinotypes (Uzal *et al.*, 2018). To summarize, *C. perfringens* produce a number of toxins, notably a pore forming toxin (PFO) and phospholipase (PLC), which are the two main toxins associated with gas gangrene infections, in addition to CPE, which is mainly associated with food poisoning (Rood, 1998).

The strain of *C. perfringens* that was used primarily in this study, strain HN13, falls under category type A and produces alpha toxin (PLC) in copious amounts (Rood, 1998). Type A strains also produce enterotoxin (CPE), which is the cause of acute food poisoning (Garcia *et al.*, 2014). While *C. perfringens* has a multitude of toxins to utilize for toxicity to host cells, it also has other virulence factors that are used for their successful survival (Geier *et al.*, 2021).

Other virulence factors associated with *C. perfringens.* There are virulence factors that many types of *Clostridia* can produce as well, including delta-toxin (Seike *et al.*, 2019) and neuraminidase (Fraser & Collee, 1975). In addition, kappa toxin (collagenase) and hyaluronidase are known to be secreted, however the role of these virulence factors in pathogenesis is not well understood (Fourie *et al.*, 2020). *C. perfringens* possesses quorum-sensing systems that serve to regulate virulence and toxin production (Yu *et al.*, 2017). One method utilized for adherence by *C. perfringens* involves a sortase-dependent pilus it can produce, which aids in successful pathogenesis (Lepp *et al.*, 2021). *C. perfringens* also has Type IV pili (T4P) (see next section), which are used for adherence and could function as a virulence factor (Soncini *et al.*, 2020).

Type IV Pili (T4P)

T4P structure and function introduction. An additional characteristic of *C. perfringens* that aids in survival of the bacteria are T4P (Soncini *et al.*, 2020). T4P are structures that have a variety of functions across the bacterial world, including motility, horizontal gene transfer, adherence to host cells, biofilm formation, and protein secretion (Piepenbrink & Sundberg, 2016). While T4P have been studied extensively in Gram-negative bacteria, there is still much to be discovered about T4P in Gram-positive bacteria (Melville & Craig, 2013).

Gliding motility in *C. perfringens*. *C. perfringens* lacks flagella for swimming motility but has a type of gliding motility, which allows them to propel themselves along surfaces at the air-agar interface (Melville & Craig, 2013). The bacteria can glide along the surface when the cells line up end to end, then use extension and division of each bacterium to push the filament forward (Varga *et al.*, 2006). The filament moves away from the colony, and there is a model that suggests T4P attach the filament to the surface, allowing the filament to move in a straight line (Liu *et al.*, 2014). In Gram-negative bacteria, T4P are required for twitching motility (Collins *et al.*, 2005). While twitching motility observed in Gram-negative bacteria involves single cells, gliding motility in *C. perfringens* is only observed in filaments of cells (Liu *et al.*, 2014).

When *C. perfringens* is grown on a surface (such as a plate) they elongate in shape, and display a distinctive type of social gliding motility on plates (Liu *et al.*, 2014). T4P are found on the outside of the cell when grown on a surface, but have not been observed in cells grown in liquid media (Soncini *et al.*, 2020). In addition, it has been shown that PilC (core protein) and PilT (retraction ATPase) are required for T4P activity in *C. perfringens* for gliding motility (Varga *et al.*, 2006). This was deduced since Δ*pilC* and Δ*pilT* mutants were observed to be nonmotile, indicating that these proteins are necessary for motility (Varga *et al.*, 2006).

Assembly of T4P in Gram-negative bacteria. In comprising the T4P, pilin proteins are polymerized to form a helical structure that protrudes from the peptidoglycan layer (PG) (Melville & Craig, 2013). As shown in Figure 1.1, the pilus is assembled at the base. Essential proteins for polymerization of the pilus include PilB (assembly ATPase) and PilC (inner membrane core protein) (Melville & Craig, 2013). In Gram-negative bacteria, the pilus is pushed through the outer membrane utilizing the secretin protein, and another important protein PilT (retraction ATPase) functions to help pull the pilus back (Melville & Craig, 2013). Also shown in Figure 1.1 is the protein PilA, which functions as the major pilin. Additionally, Gram-negative bacteria also have other proteins necessary for T4P assembly, including PilD (prepilin peptidase) which removed the prepilin peptide and allows for polymerization, as well as the inner membrane accessory proteins PilM, PilN, and PilO (Melville & Craig, 2013).

Assembly of T4P in Gram-positive bacteria with a focus on *C. perfringens*. In *Clostridia*, the T4P proteins are named and described in a similar way as Gram-negative bacteria; the major pilin is called PilA, the assembly ATPase PilB, the inner membrane core protein PilC, prepilin peptidase PilD, and the retraction ATPase PilT (Melville & Craig, 2013) (Fig. 1.2). *C. perfringens* has two sets of pilin genes that encode PilA, PilB, and PilC (Melville & Craig, 2013). However, since *C. perfringens* only has one set of genes that encode for PilM, PilN, and PilO, these proteins could be shared between more than one system (Fig. 1.2). Notably, *C. perfringens* also lacks secretin, which makes sense for a Gram-positive bacterium that lacks an outer membrane (Melville & Craig, 2013). Although *C. perfringens* technically does not have a secretin, there must be an analogous method or channel to move proteins across the thick peptidoglycan layer.

As shown in Figure 1.1, Gram-positive bacteria have the same kind of machinery as Gram-negative bacteria for their T4P (Melville & Craig, 2013), but it is still unknown how they get the pilus through the peptidoglycan. Gram-positive organisms have a small space (comparable to the periplasm in Gram-negative bacteria) between the cytoplasmic membrane and the peptidoglycan, represented by the inner wall zone in Figure 1.3, where proteins reside prior to being secreted across the PG layer (Zuber *et al.*, 2006). Gram-positive bacteria also have hypothetical predicted T4P-associated proteins, which may function in a way analogous to the secretins in Gram-negative bacteria (Melville & Craig, 2013).

The proteins that comprise T4P in *C. perfringens* strain HN13 are found in two operons (Soncini *et al.*, 2020), and those that were examined in this study, along with their roles, are listed in Table 1.2. While most of the T4P genes are in a large operon, there are other genes in a small operon referred to as the *pilB1* operon (since *pilB1* is the first gene in that nine gene operon), however the function each of the two TFP systems play is still yet to be understood (Melville & Craig, 2013). The *pilB1* operon was originally thought to be made up of four genes, *pilB1*, *pilC1*, and *pilA4*, and *CPE1841* (Soncini *et al.*, 2020). It is now thought that the *pilB1* operon contains nine genes in total, similarly to other strains of *C .perfringens*, five of which are downstream of the previously identified genes (Soncini *et al.*, 2020). The proteins encoded in the last five genes of the *pilB1* operon are small membrane-associated proteins including CPE1840, CPE1839, CPE1838, CPE1827, and CPE1836 (Soncini *et al.*, 2020). Although there is a clearer picture now of what genes are included in this operon (Soncini *et al.*, 2020), it still is currently unknown for certain what the *pilB1* operon is used for.

To briefly compare Gram-positive T4P versus Gram negative T2SS (see later section), they look exactly the same at the gene level (the variations observed are at the protein level) (Melville & Craig, 2013). The distinction between Gram-positive T4P and Gram-negative T2SS is that T4P extends all the way through the membrane, and makes a pilus outside of the cell, as opposed to T2SS which involves a short pilus that does not exit the cell, and is used to secrete proteins (Melville & Craig, 2013). The aim of

this study is to determine which of these proteins is involved in making a T4P and which may be involved in forming a T2SS.

Bacterial Protein Secretion

One of the problems that bacteria face, which mammalian cells do not, is that bacteria have a barrier(s) outside of their membrane. Both Gram-negative and Gram-positive bacteria have a peptidoglycan layer beyond the cytoplasmic membrane and Gram-negative bacteria have an outer membrane as well (Silhavy *et al.*, 2010). Both have evolved multiple complex secretion systems to overcome these barriers (Abby & Rocha, 2017). Figure 1.3 illustrates this problem, and the concept of what the bacteria are trying to overcome. By having an outer membrane and peptidoglycan barrier, this justifies why bacterial have evolved so many types of protein secretion systems.

Gram-negative protein secretion systems. To get proteins from the cytoplasm to the outside of the cell, Gram-negative bacteria can use translocation, export, or secretion (Fig. 1.3) (Desvaux *et al.*, 2009). To illustrate the secretion systems more clearly, Figure 1.4 shows the eight types of Gram-negative secretion systems, along with the Sec (secretion) and twin-arginine translocation (Tat) secretion systems (Desvaux *et al.*, 2009). As depicted in Figure 1.4, many secretion systems use the Sec pathway to transport substrates across the cytoplasmic membrane prior to secretion of a folded substrate from the periplasm; however, many of these secretion systems can operate independently of Sec (Green & Mecsas, 2016). Additionally, T2SS uses Tat to transport substrates that were already folded in the cytoplasm, while Sec primarily transports linearized proteins (Robinson & Bolhuis, 2004).

The Sec system is also commonly referred to as the general secretory protein (GSP) pathway. The pathway is made up of three main parts: a component that sorts and targets proteins, the motor protein SecA, and an integral membrane channel referred to as the SecYEG complex (Yan & Wu, 2015). Each protein that eventually gets transported is first made in the cytoplasm, and at this point is referred to as a proprotein that has a signal sequence. The protein then gets recognized by another protein, SecB, which is a chaperone that binds to the portion of the protein that will be conserved in the mature protein, near the C terminus (Freudl, 2018). At this point, SecA ATPase activation causes SecA to then bind to the Sec preprotein complex, and deliver the preprotein to SecYEG (Pugsley, 1993). Once the signal sequence gets cleaved by the signal peptidase, the functional form of the protein will end up in the periplasm (Pugsley, 1993). Proteins transported using the Sec system can exit the periplasm of Gram-negative bacteria using either T2SS or alternatively T4SS, T5SS, T7SS, or T8SS (Desvaux *et al.*, 2009).

The Tat system is a post translational process because it can transport proteins that have already been folded or fully assembled in the cytoplasm (Kudva *et al.*, 2013). The signal in the amino terminus contains two highly conserved arginine residues, and this signal gets recognized by Tat proteins that reside in the inner membrane (Frobel *et al.*, 2012). Once the Tat proteins bind to the substrate protein, the

proton motive force is utilized and the channel opens up to move the protein across the membrane and into the periplasm (Frobel *et al.*, 2012).

The T1SS, T3SS, T4SS, and T6SS all are able to operate independent of Sec and Tat, and can transport proteins across both the cytoplasmic membrane and the outer membrane in one step (Green & Mecsas, 2016). T3SS, T4SS, and T6SS also can move proteins across the host cell membrane as well, which often serves as a mechanism of virulence (Green & Mecsas, 2016). The T5SS appears to rely on Sec to transport proteins across the inner membrane to the periplasm (Green & Mecsas, 2016). The T5SS then transports the protein across the outer membrane (Green & Mecsas, 2016). Not as much is known about the mechanism by which T7SS and T8SS move substrates to the outside of the cell (Desvaux *et al.*, 2009). T2SS requires initial transport of their substrates to the periplasm, and can use either Sec or Tat to do so (Green & Mecsas, 2016). T2SS is the system of particular interest in this study.

T2SS in Gram-negative bacteria. Of the eight different types of secretion systems that exist in Gram-negative bacteria, the one that is being examined in this study specifically is T2SS, to determine if Gram-positive bacteria also have a similar apparatus (Desvaux *et al.*, 2009). T2SS is a two-step process; the first step involving transport from the cytoplasm, across the inner membrane, to the periplasm, and the second step involving transport from the periplasm, across the outer membrane, to the outside of the cell (Green & Mecsas, 2016). Given that T2SS secretes folded proteins, first step involves either the Sec or Tat secretion systems to move proteins across the cytoplasmic membrane (Green & Mecsas, 2016). The T2SS is used by Gram-negative bacteria to translocate many different kinds of proteins from the periplasm through the outer membrane, including various toxins and enzymes (Green & Mecsas, 2016).

While proteins transported using Tat are folded in the cytoplasm prior to crossing the cytoplasmic membrane, other proteins transported using Sec require folding in the periplasm; regardless of the method by which the protein reaches the periplasm in a folded state, the proteins can then be transported to the outside of the cell using the T2SS (Douzi *et al.*, 2012). Figure 1.5 illustrates the concept for what is going on with T2SS in Gram-negative bacteria (Desvaux *et al.*, 2009). T2SS machinery is thought to span both the inner and outer membranes of Gram-negative bacteria, as shown in Figures 1.4 and 1.5. While a basic understanding of T2SS components and overall structure is known, the details of how polymerization of the pseudopilus occurs is still an active area of research (Korotkov *et al.*, 2012).

There are four main parts thought to be associated with T2SS: the pseudopilus, the outer membrane complex, the secretion ATPase, and the inner membrane platform (Green & Mecsas, 2016). The pseudopilus is generated from the polymerization of pilin protein monomers in the periplasmic space (Douzi *et al.*, 2012). Secretin forms the outer membrane complex, which is a channel that will open for the protein to pass through to be secreted (Korotkov *et al.*, 2012). The secretion ATPase residing on the cytoplasmic side of the membrane binds ATP, providing the energy necessary for transport to occur

(Green & Mecsas, 2016). The inner membrane platform physically contacts each of the other three main portions, and because of this proximity is able to receive and relay information with the other three components to facilitate secretion (Korotkov *et al.*, 2012). The "piston model" proposes that the pseudopilus involved in this structure would be too large to fit through the pore, and functions by moving up and down within the channel, akin to a piston, without exiting the cell (Campos *et al.*, 2013). The pseudopilus could potentially act by pushing the proteins through a secretin channel from the periplasmic space across the outer membrane (Howard *et al.*, 2019). After protein transport, the pseudopilus would then depolymerize and retract (Wolfgang *et al.*, 2000). To summarize, mechanistically T2SS makes a short pseudopilus that is used to secrete proteins (Green & Mecsas, 2016). Related to T2SS is T4P; T2SS and the T4P systems have similar machinery when the two are compared structurally (McCallum *et al.*, 2019).

Gram-positive protein secretion systems. Gram-positive bacteria have the same problem as Gram-negative bacteria in that they need to fold a protein in the space between their cell membrane and peptidoglycan, and then need a way to transport this folded protein through the peptidoglycan (Desvaux *et al.*, 2004). There are several methods by which secretion can occur in Gram-positive bacteria (and some Gram-negative bacteria), including the Tat, flagellum mediated (FEA), holin (hole forming), fimbrilin-protein exporter (FPE), and Sec systems (Desvaux *et al.*, 2009).

C. perfringens has three known mechanisms for secretion, as shown in Figure 1.6. Because *C. perfringens* does not possess flagella or Tat proteins, flagellum mediated (FEA) and twin-arginine translocation (Tat) can be categorically eliminated as possible systems for secretion (Myers *et al.,* 2006). They are able to use the holin, competence pseudo-pili assembled by FPE, and the Sec secretion system (Desvaux *et al.*, 2009). What is still unknown is how the folded proteins can get across the peptidoglycan layer (CW) as shown in Figures 1.3 and 1.6. Since Gram-positive bacteria possess a thick peptidoglycan layer, it is probable that this transport necessitates a channel (Desvaux *et al.*, 2004). A potential T2SS-like system in Gram-positive bacteria is thought to look like the apparatus associated with T2SS observed in Gram-negative bacteria.

The individual pilins and their associated proteins for Gram-positive T4P have analogous components with T2SS in other prokaryotes, and it is possible that T4P and T2SS are related (Melville & Craig, 2013). It is possible that *C. perfringens* could use these other T4P-like proteins as a T2SS (Melville & Craig, 2013). Figure 1.7 shows a detailed structure of the proposed hypothetical model for T2SS in Gram-positive bacteria, such as *C. perfringens*.

Bacterial Biofilms

Another material that is used by many bacteria for survival is a biofilm matrix (Abraham, 2006). A biofilm is a community of microbial life that is contained in a matrix composed of polysaccharide, protein, and/or DNA (Hoiby *et al.*, 2010). Biofilms provide increased protection against antibiotics, antibodies, and physical forces (Hoiby *et al.*, 2010). This allows the bacteria existing in biofilms to persist after exposure to high levels of antibiotics and allows the bacteria to evade the host immune response, preventing the clearance of harmful bacterial infections in the host (Hoiby *et al.*, 2010). Most bacteria that exist in the environment are in this state where they are protected within the biofilm, allowing the bacteria to survive in nutrient poor surroundings (Abraham, 2006). The stage of biofilm development is extremely dependent on the microenvironmental factors and interactions that are occuring between the bacteria and surfaces at any given point in time (Guzman-Soto *et al.*, 2021).

There are multiple components that come together to comprise biofilms, including various types of polymers, that protect the bacteria and establish them in the environment. (Abee *et al.*, 2011). The main components of the extracellular polymeric substances in this matrix material include polysaccharides, as well as extracellular proteins and DNA (Obana *et al.*, 2020). These components, in general, provide structural advantages for the biofilm and allow for bacterial adhesion to surfaces (Flemming & Wingender, 2010). The polymers that form the biofilm vary significantly depending on what microorganism is being studied (Abee *et al.*, 2011). For example, *Bacillus subtilis* NCIB3610 creates an exopolysaccharide as its polymer, whereas *B. subtilis* RO-FF-1 makes poly-γ-D,L-glutamate instead to form a biofilm (Abee *et al.*, 2011). These polymers that create a biofilm matrix are distinct from the capsule, and some studies have shown that biofilm formation and capsule formation had an inverse relationship when observed in Gram-negative bacteria (Petruzzi *et al.*, 2017). Additionally, cells can communicate with one another while biofilm formation is occurring through quorum sensing, utilizing signaling molecules such as D-amino acids, cis-2-deconoic acid, and autoinducer 2 (Abee *et al.*, 2011). The D-amino acids indicate biofilm inhibition, whereas cis-2-deconoic acid and autoinducer 2 are both signals that promote dispersal of biofilms (Abee *et al.*, 2011). To summarize, there are many kinds of polymers that construct biofilms that help bacteria persist in their environment.

C. perfringens has been found to form biofilms as a survival strategy (Obana *et al.*, 2020). It was previously thought that this biofilm is made of amyloid proteins, however with respect to the type of filamentous structure being produced, there is evidence that suggests that the biofilm is not made of an amyloid protein. To test the stability of the BsaA protein Obana et al. exposed the BsaA protein to 350 mM SDS or 20% (v/v) formic acid, both of which would typically denature even an amyloid protein. However, even after such harsh treatments, a polymer still formed, indicating that the BsaA protein may in fact not be an amyloid (Obana *et al.*, 2020). Another important aspect observed in *C. perfringens* is that

T4P are needed for biofilm formation to occur effectively (Liu *et al.*, 2019). For this study, biofilms are significant because the components of this matrix material are studied in Chapter 2. The function of the biofilm was not investigated in this study, however more was learned about the secretion of biofilm matrix proteins.

Summary

If there is a relationship between biofilm secretion and T4P then this would shed light on many other aspects of pathogenesis, such as how certain toxins are secreted. Defining this potential relationship would allow for a more in-depth understanding of the secretion mechanisms that Gram-positive bacteria use to secrete the toxins that are imperative to infecting their host. In summary, it is known that *C. perfringens* has T4P but has two sets of T4P operons (Soncini *et al.*, 2020). Preliminary evidence showed that the pilin deletion mutant *pilA3* is needed to secrete a biofilm matrix material protein (Hartman, 2012). This preliminary evidence that a T4P protein might be involved in secretion of a biofilm matrix protein was the foundation for the experiments performed in Chapter 2. This project is meant to explore if the folded protein exits the cell via Gram-positive T2SS, and whether or not the modeled secretion system structure exists.

Tables and Figures for Chapter 1

Table 1.1. Relevant diseases and toxins associated with each toxinotype		
Toxinotype	**Disease produced**	**Major toxin(s)**
A	Gas gangrene	PLC (α-toxin)
B	Enterotoxemia of sheep, foals and goats	β-toxin, ε-toxin
C	Human enteritis necroticans, necrotic enteritis in animals	β-toxin
D	Enterotoxemia of sheep	ε-toxin
E	Enterotoxemia of calves and rabbits	ι-toxin
F	Food poisoning	CPE (enterotoxin)
G	Necrotic enteritis in birds	NetB

Table 1.2. TFP Proteins in *C. perfringens* that were analyzed in this study and their putative roles	
Protein	**Role**
PilA2	Pilin; Aids in attachment to muscle cells
PilA4	Pseudopilin
PilT	Retraction ATPase
PilA1	Pilin
PilD	Prepilin peptidase
PilC2	Inner membrane core protein
PilC1	Inner membrane core protein
PilA3	Major pilin/pseudopilin
PilB1	Assembly ATPase
PilB2	Assembly ATPase
PilM	Inner membrane accessory protein
PilN	Inner membrane accessory protein
PilO	Inner membrane accessory protein
CPE1841	Unknown
CPE2277	Unknown
CPE2279	Large pilin, involved in secretion?
CPE2280	Large pilin, involved in secretion?

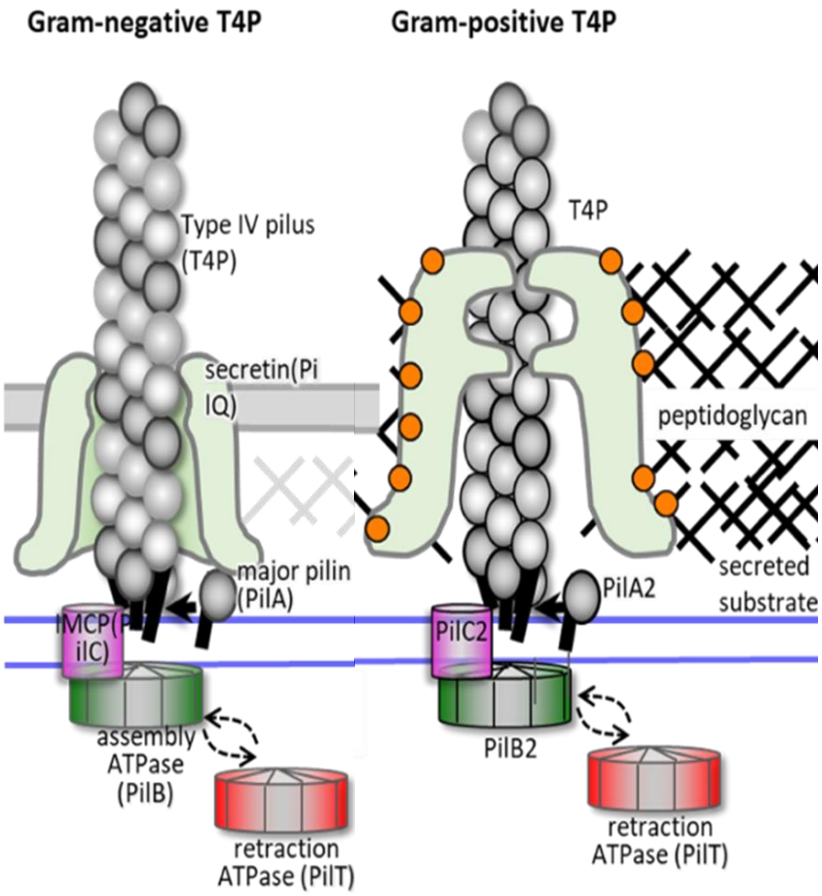


Figure 1.1. Structure of T4P in both Gram-negative and Gram-positive bacteria. Essential proteins involved in polymerization of the T4P include PilC, the inner membrane core protein (IMCP), PilB, the assembly ATPase, and PilA, the major pilin. Secretin is notably only found in Gram-negative bacteria to assist in secretion. The retraction ATPase aids in retracting the pilus after secretion occurs, although it is not known exactly how this happens. Figure is modified from reference (Melville & Craig, 2013) .

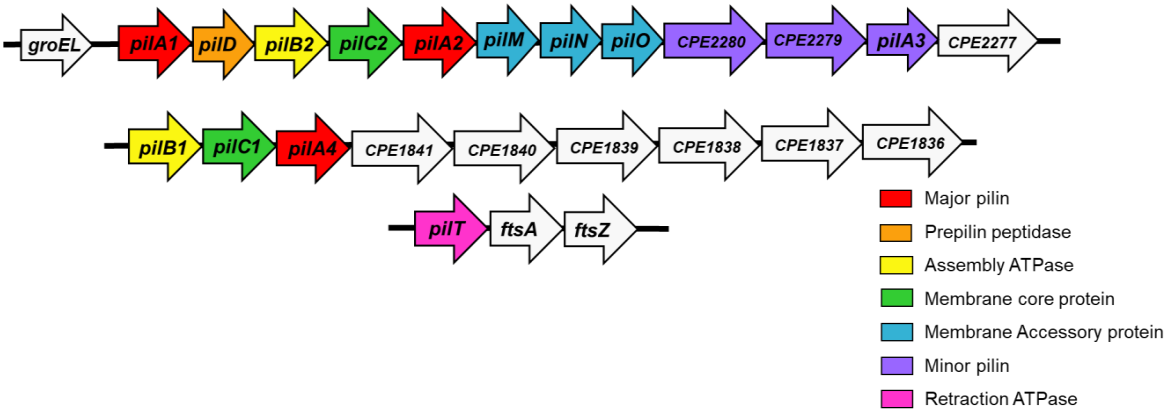


Figure 1.2. *C. perfringens* T4P operons. There are two operons containing T4P genes; The primary TFP operon for *C. perfringens* (top) and the secondary *pilB1* operon in *C. perfringens* strain HN13, including the five transmembrane proteins located downstream (middle). A single *pilT* gene is found in a third operon. (bottom). Predicted protein functions are indicated by color coding. White color means the protein is not related to TFP or the function is unknown. Figure modified from reference (Melville & Craig, 2013).

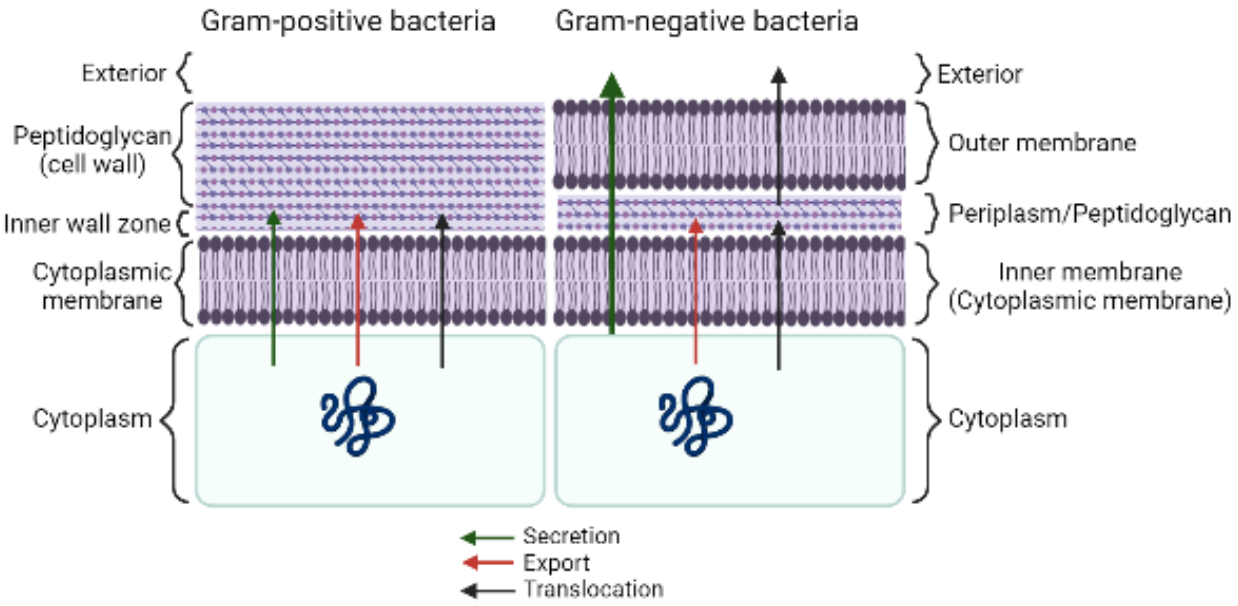


Figure 1.3. Comparison of how proteins are translocated, exported, and secreted for Gram-positive and Gram-negative bacteria. Figure based on image from reference (Desvaux *et al.*, 2009). This figure was generated using BioRender software.

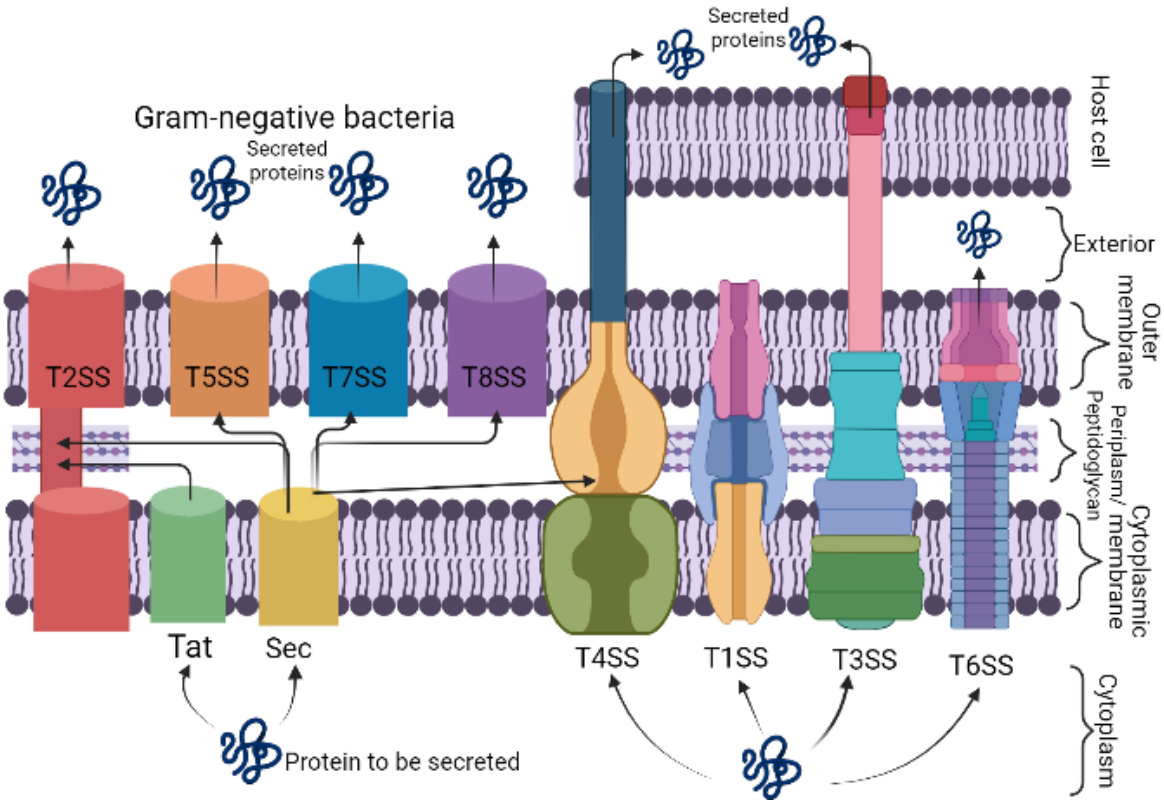


Figure 1.4. Known Gram-negative bacterial protein export systems. The eight Gram-negative bacterial secretion systems used as methods of transport to the outside of the cell are shown, along with which system uses Sec and Tat. Figure modified from references (Green & Mecsas, 2016) and (Desvaux *et al.*, 2009). This figure was generated using BioRender software.

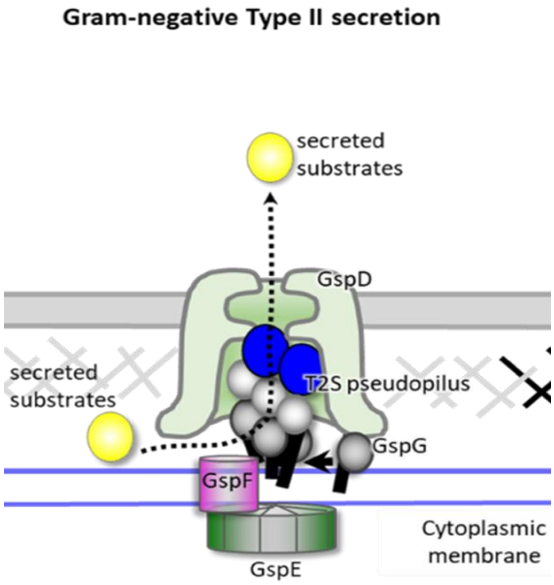


Figure 1.5. Gram-negative T2SS model. The proteins associated with Gram-negative T2SS have homologs comparable to T4P proteins. GspD is a homolog of the outer membrane secretin PilQ, GspF is a homolog of PilC, GspE is a PilB homolog, and GspG is a PilA homolog. Figure is modified from reference (Melville & Craig, 2013).

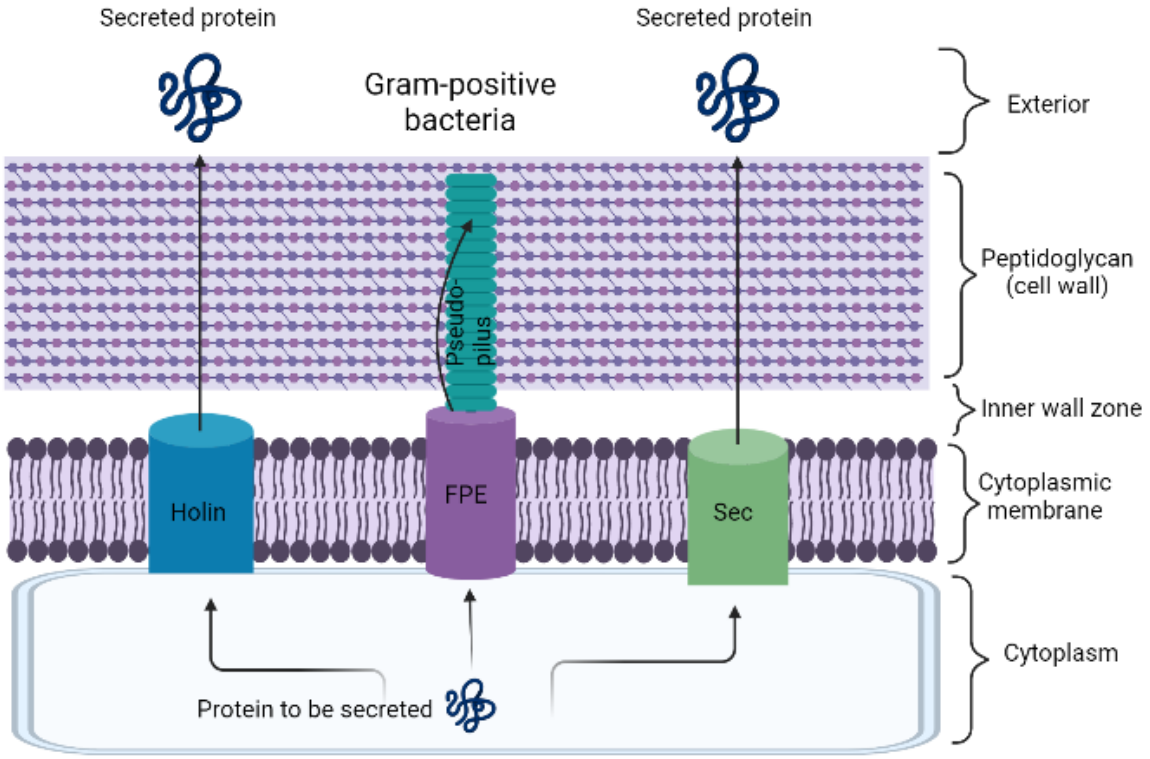


Figure 1.6. Methods of transport from the cytoplasm to the outside of the cell in Gram-positive bacteria. *C. perfringens* can use the fimbrilin-protein exporter (FPE) to assist in assembling the competence pseudo-pili. *C. perfringens* additionally can use holin and Sec for secretion. Figure derived from reference (Desvaux *et al.*, 2009). Figure was generated using BioRender software.

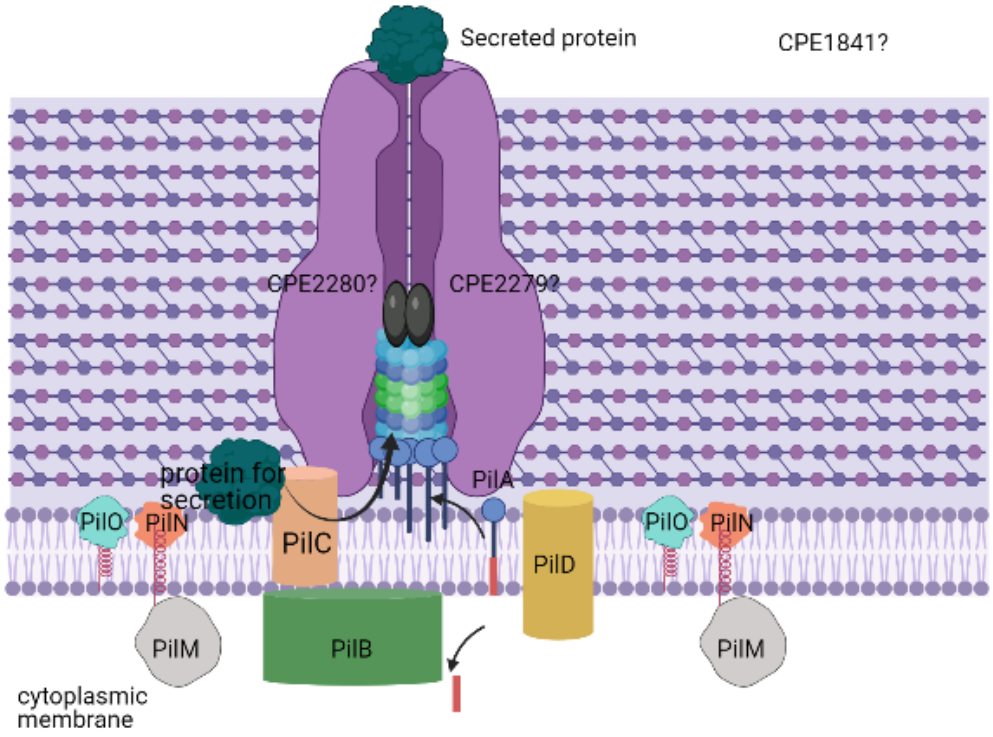


Figure 1.7. Proposed structural machinery for T2SS in Gram-positive bacteria. PilD cleaves the prepilin signal peptide allowing the pilin to polymerize into the pilus. This Figure was generated using BioRender software.

Chapter 2: Characterize the mechanism of secretion of biofilm matrix material in *Clostridium perfringens*

Abstract

The goal of these experiments was to identify pilin mutants that showed a defect in secretion of biofilm matrix material protein. In these experiments, individual proteins encoded in the biofilm operon were tagged with antigenic peptides, to measure the levels of biofilm matrix material protein being secreted in a quantitative manner using immunoblots and slot blots. The qualitative data collected gave insight as to which T4P proteins might be needed for secretion of biofilm matrix material. However, a lack of statistically significant differences in secreted proteins detected between the wild-type and mutant strains made it impossible to build a complete system model.

Contributions from Andrea Hartman and Gary Camper were key to developing and accomplishing the experiments in Chapter 2, especially results shown in Figure 2.3 and Figure 2.4.

Introduction

Biofilm matrix material formation observed in *C. perfringens* requires T4P pili (Varga *et al.*, 2008). As depicted in Fig. 1.2, the genes that encode for T4P proteins are found in two operons. The protein PilA3 appears to be especially important for secretion of the protein CPE0517 (Hartman, 2012). The gene encoding CPE0517 is found in another key operon, depicted in Figure 2.2, which is referred to as the biofilm operon. The gene *CPE0514* codes for a protein that is a SipW signal peptidase (Obana *et al.*, 2020). *CPE0515* codes for the main matrix material protein (also known as BsaA) (Obana *et al.*, 2020) . *CPE0516* codes for the minor matrix material protein (also known as BsaB) (Obana *et al.*, 2020). The gene *CPE0517* codes for a protein with a von Willebrand A domain (VWA) (also known as BsaC) (Obana *et al.*, 2020).

To identify TFP proteins that are required for secretion of biofilm matrix material, experiments done by previous students indicated a potential relationship between pili and biofilm secretion (Hartman, 2012). Specifically, it was noticed that the protein CPE0517 was not secreted in a *pilA3* mutant (Fig.2.3) (Hartman, 2012). In addition, the presence of a large quantity of biofilm matrix material was seen in the supernatant when this biofilm operon was overexpressed (Fig. 2.4). These experiments served as initial evidence that the biofilm operon encodes proteins needed for biofilm matrix material. Because it was observed that a protein in the biofilm operon needs a pilin (PilA3) for secretion (Hartman, 2012), in addition to the evidence that this operon encodes proteins for biofilm, it was inferred that PilA3 might be

a part of a T2SS. This information laid the foundation for the reasoning behind the experiments done in this chapter.

Obana et al. demonstrated that the biofilm operon (*CPE0514-CPE0517*) encodes proteins required for biofilm matrix material. Specifically, they identified that the protein BsaA (CPE0515) is needed for the construction of a pellicle biofilm. They determined this by creating mutations and deleting the genes involved in biofilm formation (Obana *et al.*, 2020).

Results

Preliminary evidence and experiments. Andrea Hartman and Gary Camper, previous graduate students in the Melville laboratory, contributed to the results in Figure 2.3, which illustrates that a T4P protein is needed for secretion of biofilm. Obana et. al. also contributed supporting evidence for the rationale of Chapter 2 (Obana *et al.*, 2020). Andrea Hartman examined the secretome of a *pilA3* mutant, and found that it was missing a band (panel A, Fig. 2.3) (Hartman, 2012). Mass spectrometry analysis demonstrated that the band was CPE0517 (Hartman, 2012). She complemented the *pilA3* mutant, and the band reappeared in the secretome (panel A, Fig. 2.3) (Hartman, 2012). Gary Camper did the same thing with a western blot using anti-CPE0517 antibodies, looking at expression of CPE0517 in the *pilA3* mutant in the secretome (panel B, Fig. 2.3). When the *pilA3* mutant did not secrete CPE0517, he complemented the *pilA3* mutant and CPE0517 secretion was restored, using pure VWA as the positive control (panel B Fig. 2.3). This indicated that PilA3 was required for secretion of CPE0517. Examination of *CPE*0517 showed it was the fourth gene in an operon (Fig. 2.2), and it was clear that there were some proteins that had a SipW signal peptidase, and so it was thought that this was a biofilm operon at this time.

Gary Camper contributed the images shown in Figure 2.4. He overexpressed the entire biofilm matrix operon, and he saw matrix material in the supernatant (Fig. 2.4). When he purified the matrix material, shown on the right in Figure 2.4, it was a viscous material such that it would not flow within the tube. Gary Camper was previously studying secretion of CPE0517 alone, however when the entire biofilm matrix operon was overexpressed, this material was unique from the consistency of CPE0517 by itself. Because of the difference in consistency when the entire biofilm matrix operon was overexpressed, it became more apparent that other proteins were being secreted besides CPE0517, but he did not pursue this further. Obana et. al. clarified in more detail what the biofilm proteins are and made the connection between CPE0517 and biofilm matrix material (Obana *et al.*, 2020).

Because PilA3 is associated with secretion of biofilm matrix (Hartman, 2012), in addition to the evidence of other biofilm proteins being secreted, it was inferred that the T4P used might be similar to a T2SS. Based on all of the results from Andrea Hartman, Obana et al., and Gary Camper, we hypothesized that the T4P protein PilA3 is needed for biofilm secretion. Based on what is known about *C. perfringens*

T4P operons, and the evidence that has been observed in previous studies, it is possible that there are two different pili systems, which do different things, one for assembling a pilus and one for T2SS.

Preliminary characterization of secreted matrix material. Preliminary characterization of the matrix material ladder was done to see what the secretome of the entire matrix operon might look like. Gary Camper transformed the plasmid pHLL65, created by a previous graduate student Hualan Liu, with the regulated biofilm operon cloned behind the lactose-inducible promoter in the pKRAH1 plasmid (Hartman *et al.*, 2011), into strain HN13. The pKRAH1 plasmid-based promoter system was used to deliver regulated expression as lactose was added continuously until the cells grew to stationary phase. After reaching stationary phase, the culture was pelleted in a centrifuge. The supernatant was removed (S1) and the proteins in the supernatant were collected using trichloro-acetic acid (TCA) precipitation. The cell pellet (P1) resulting from the initial centrifugation was resuspended in 1x Dulbecco's Phosphate Buffered Saline (DPBS) without calcium or magnesium. and this solution was centrifuged for 30 minutes at 20,000 x *g*. The supernatant resulting from this centrifugation (S2) was subjected to TCA precipitation. A 10% (gradient) SDS acrylamide gel was used, and it was stained using Sypro Ruby, a fluorescent protein stain. A ladder of oligomeric protein was observed in the culture supernatants, indicating the formation of long fibers when the entire operon is expressed (data not shown). It appeared that the biofilm matrix material was found abundantly in both the (S1) and (S2) supernatant samples. Since it was observed that CPE0517 secretion itself is dependent on *pilA3*, it would be helpful to know if this ladder is present in the pilin mutants or not (data not shown).

Additionally, an experiment to precipitate the matrix material using $MgCl_2$ and NaCl was done, however, when SDS-PAGE was done it appears that some of the matrix material was stuck to the cells, and some of it was free floating. The real problem here was that the proteins did not fractionate well; while the biofilm proteins could be precipitated by magnesium, it was only a fraction of the total. A fraction of the material came down using the TCA precipitation as well. Therefore, the magnesium precipitate was not the best method to fractionate all of the biofilm material. From these results, it appears that it was possible that a lot of matrix material protein was being made but it did not precipitate with magnesium chloride (data not shown). These results were not significant, so this approach was not pursued.

Preliminary determination of which TFP proteins are required for secretion of biofilm matrix. Gary Camper put the plasmid pHLL65 with the regulated biofilm operon into most of the TFP gene mutant strains. He made all the strains except for *ΔpilT*, and this was completed by the author (S. Kivimaki) by transforming the pHL65 plasmid into a *ΔpilT* mutant that was made by previous students in the lab. From this point forward, experiments and figures were generated by the author (S. Kivimaki). The TFP mutants were screened for secretion using the biofilm operon expression system, as shown in Figure

2.5. For the gel in Figure 2.5, and many others performed to test other mutants, these results gave a general idea of what pilin proteins might be involved, however they did not lead to quantitative results. It was thought that the biofilm matrix material was visible in the upper part of the gel, and for certain individual experiments it appeared that there was a decrease in secretion observed in pilin deletion mutants. The problem with this qualitative analysis is the amount of protein cannot accurately be determined using the Sypro ruby stain by densitometry of the gels because the quantitative results were inconsistent. Aside from the fact that the secretome for each of the mutant replicates varied from experiment to experiment, the intensity was also difficult to measure because the image detection of the fluorescent signal of this protein was saturated for the *pilA2* deletion mutant specifically (data not shown).

Creation of antigen-tagged proteins in the biofilm operon. As an alternative method that would allow for quantitative data to be gathered, a FLAG tag was put on the CPE0515 protein to look for the protein in supernatants using western blots. Overlapping PCR was performed for *CPE0515* and *CPE0516*; a FLAG-tagged version of CPE0515 and a HA-tagged version of CPE0516 were made. For CPE0515-FLAG experiments, the PCR products were digested, and ligated into the plasmid pCRBluntITOPO before transforming into *E. coli*. For CPE0515-FLAG and CPE0516-HA experiments, transformation procedures for *C. perfringens* were performed using the overlapping PCR products (see experimental procedures). The digested overlapping PCR products were ligated into pKRAH1, and then transformed into *C. perfringens*. The FLAG-tagged version of CPE0515 and HA-tagged version of CPE0516 (both expressed on pKRAH1) were detected by western blots. *CPE0515*-FLAG plasmid DNA was transformed into 18 pilin deletion mutant strains, to make the FLAG-tagged version in every mutant strain.

CPE0515-FLAG. The goal of the experiments pertaining to CPE0515-FLAG was to see which of the T4P proteins are required for secretion of CPE0515, by detecting CPE0515-FLAG in supernatants. An example of the results from the Sypro ruby stained protein gel shows the expression of CPE0515-FLAG, along with the negative control containing the empty plasmid vector is shown in Figure 2.6. The wild-type strain expressing CPE0515-FLAG shows what is inferred to be the presence of matrix material (ladder), as indicated by the area of secretome encompassed by the red box in Figure 2.6. An example of the western blot results for CPE0515-FLAG is shown in Figure 2.7, along with the negative control and six examples of the pilin mutant deletion strains. The ladder of CPE0515-FLAG in the wild-type strain HN13 can be observed in the upper part of the secretome. However, as clearly shown in Figure 2.7 by "WT variation", the wild type secretion levels vary significantly for each blot performed. The difference between the wild-type ladders shown in Figure 2.6 and Figure 2.7 could be attributed to a variability in recovery and loading of the WT secretome protein samples.

CPE0515-FLAG western blots. The results shown in Figure 2.8 were determined using densitometry from the Image Lab software package (Bio-Rad Corp.). The densitometry results for CPE0515-FLAG in various mutants were collected by taking the mean (average) pixel intensity for boxes drawn around each individual secretome from the bottom of the wells all the way down to ~50 kD (an example of how the boxes were drawn is indicated in Figure 2.7 by the red rectangle). The densitometry gave a readout, which indicated the mean pixel intensity of the entire rectangle being measured. For these results, each individual mutant had a minimum of five replicates (the positive and negative controls had 38 replicates). Figure 2.8 depicts the mean pixel intensity and the standard error of the mean (SEM) for each of the pilin deletion mutants tested with the CPE0515-FLAG construct. These results show that for the CPE0515-FLAG western blots, *pilA1* was the only mutant with a significant P value (i.e, $P \leq .05$) for being lower than the wild type, as indicated by the asterisk in Figure 2.8. Due to the lack of statistically significant values lower than the WT according to the student's t-tests performed, in addition to variability between the wild-type samples, a different method was pursued in order to examine the levels of secretion of construct CPE0515-FLAG.

CPE0515-FLAG slot blots. Slot blots were performed as a way of determining which T4P proteins might be involved in secretion of the biofilm matrix protein CPE0515-FLAG. The slot blots collect all the protein in the supernatant, but do not sort them by size, and therefore are not as selective as a western blot. The slot blot was to identify CPE0515-FLAG proteins that were trapped on the membrane. An example of the slot blots performed for CPE0515-FLAG in various pilin mutants is shown in Figure 2.9**.** Figure 2.9 only shows the results for the *pilA1*, *pilA2*, and *pilA3* deletion strains, however, raw intensity data for each of the pilin deletion mutant strains was collected as well in repeated slot blots. For the CPE0515-FLAG slot blots, the wild-type replicates appeared to have varying levels for the raw intensity data values compared to the mutant replicates. Plots for each individual slot blot were made, where the mean and standard deviation are shown, as depicted in Figure 2.10. The raw intensity data points were taken in the linear range, using the 10% supernatant sample values.

The mean and standard error of the mean (SEM) for the raw intensity data using the 10% supernatant sample values was graphed for the slot blots, as shown in Figure 2.11. For the results shown, this represents a minimum of at least six independent replicates for each mutant strain. Both the raw and normalized data are shown in Figure 2.11. The raw data shows the mean and the SEM. The normalized data was calculated as such: the raw data for each WT replicate was averaged, and then the raw data for each mutant replicate was divided by the average of the WT replicates. For each mutant, the normalized values were averaged, and the mean and SEM for all mutant replicate normalized intensity values are shown for all 18 pilin mutants tested. A significant amount of variation was observed in general, but it was observed that the actual variation coming from the wild-type samples was at a high level . The raw

intensity data for the mutants themselves do not vary as much. The mutants that showed statistically significant values lower than the WT are indicated by an asterisk in Figure 2.11. The slot blot densitometry results collected for CPE0515-FLAG showed that the mutants with statistically significant values from the WT were *pilA1*, *pilA2*, *CPE1841*, and *CPE2280* deletion mutants. The *pilA1* and *CPE2280* deletion mutants showed a statistically significant decrease in secretion compared to the wild type, whereas *pilA2* and *CPE1841* showed a statistically significant increase in secretion compared to the wild type.

Expression of CPE0515-FLAG with CPE0514. The purpose of creating the construct CPE0514_CPE0515-FLAG tag was to examine the secretion profile of CPE0515 in the absence of CPE0516 and CPE0517, to see whether or not CPE0516 and CPE0517 are important to secretion of matrix material. PCR was performed and a clone was made where CPE0514 and CPE0515-FLAG were expressed, and CPE0515 was detected with a FLAG tag antibody. On the left in Figure 2.12 is a western blot of the wild-type (WT) version for the CPE0514_CPE0515-FLAG; WT meaning that the expression plasmid was in a WT strain background, so that all of the operon genes are present, but perhaps not strongly expressed. Shown is CPE0515-FLAG, however CPE0514 is also being expressed, but was not tagged. These images look very similar to the entire operon expression in the wild-type strain for CPE0515-FLAG. This expression vector was transformed into the pilin deletion mutants *pilA1* and *pilA2*, and western blots were performed, shown on the right in Figure 2.12, to show whether or not secretion is affected by the absence of *pilA1* and *pilA2*. The secretion of CPE0515 does not appear significantly different after deleting *pilA1* and *pilA2* individually.

CPE0516-HA. The initial goal of the experiments pertaining to CPE0516-HA was to verify that CPE0516 was secreted, and eventually determine if CPE0516 required the T4P proteins. To do this, an HA tag was put on the CPE0516 protein, using overlapping PCR, to detect this protein in the supernatants using western blots (Fig. 2.13). What resulted was CPE0516-HA was seen as a monomer (~28 kDa, predicted mol wt of 24 kD) and a potential dimer (~45 kDa). Because the protein was treated with SDS, DTT, heated, and subjected to electrophoresis via SDS-page, it is likely based on these results that this ~45 kDa dimer is heat stable.

Discussion

From the CPE0515-FLAG immunoblot densitometry results, there was no statistically significant difference between the secretion of CPE0515-FLAG in the mutants and wild-type strains, except for the *pilA1* deletion mutant. This interpretation was drawn because when the *pilA1* gene is mutated, it consistently shows defects in CPE0515-FLAG secretion in comparison to the wild type, and had a statistically significant value lower than the WT with respect to the densitometry analysis. One issue with

performing accurate statistical analysis from the quantitative data was the fact that the wild type results were inconsistent from day to day, and the fact that the mutant replicates also varied significantly on a daily basis. To get a clearer picture of which T4P proteins are required and acquire more accurate statistics for the CPE0515-FLAG secretion, slot blot experiments were also performed for CPE0515-FLAG.

The densitometry results collected for the slot blots performed for CPE0515-FLAG revealed that four of the mutants showed significantly different secretion: *pilA1*, *pilA2*, *CPE1841*, and *CPE2280*. The deletion mutants *pilA1* and *CPE2280* were significant for being lower than the wild type, while deletion mutants *pilA2* and *CPE1841* were significant for being higher than the wild type. The *pilA1* mutant was consistent for being lower than the wild type in both the immunoblot and slot blot data. While a phenotype has not been identified for the *pilA1* mutant, it is clear that it does not secrete as much of this proteinaceous material. The *CPE2280* mutant showing a significant decrease indicates that the protein CPE2280 likely also plays a major role in secretion. The *pilA2* mutant on the other hand showed a significant increase in CPE0515-FLAG secretion in comparison to the wild type, which was not surprising, given previous studies. While it is possible that PilA2 could be involved in the secretion process, that is not what is suspected to be happening. Obana et al. found that PilA2 is a regulator of transcription for the synthesis of the biofilm operon materials, because a *pilA2* mutant was upregulated for the biofilm operon expression (Obana *et al.*, 2020). The role of PilA2 is still not entirely understood, but likely is not involved in the secretion system itself based on current knowledge. Additionally, the *CPE1841* deletion mutant also showed a significant increase in comparison to the wild type. The fact that there are higher levels of this protein being secreted could indicate that the mutant might be enhancing the activity of another protein, or something of that nature. Based on this data, there was not a consistent relationship emerging when the intensities were examined between the remaining mutant deletion strains and the wild type. The fact that *pilB* and *pilC* mutants did not appear to significantly affect biofilm secretion indicates pilus functions do not appear to be involved, since a pilus or T2SS cannot form without at least one PilB and one PilC protein.

What could be some possible sources of variation with respect to the immunoblot and slot blot results for CPE0515-FLAG? The main issue (for both types of experiments, but western blots especially) was that a wild-type sample is always needed for normalization since the western blots themselves are extremely variable. However, because the densitometry values for the wild type itself also varied from experiment to experiment, this made maintaining any level of consistency extremely difficult. The wild type (HN13 CPE0515-FLAG) was analyzed 38 individual times for the slot blot experiments, however the SEM still remained high despite the large number of trials. It is not understood why there is so much variation in the levels of CPE0515-FLAG in the wild type when all other variables are kept consistent.

Additional sources of variation could have occurred through negative selection. If there was a negative selection on overexpressing CPE0515, that is, it was toxic to the cells, then it could be that the expression was affected by this negative selection for mutants that did not make or secrete the CPE0515-FLAG tagged protein. Another factor is that the lactose-inducible promoter (BgaR) on the pKRAH1 is not totally silent (Hartman *et al.*, 2011). Because it is little bit leaky, this also could have affected expression levels.

Potential sources of error that could have occurred in the lab are related to the method of preparation of the TCA precipitation samples; by solubilizing the proteins in sterile Milli-Q water this could have affected the TCA samples, making them more acidic (which was observed) and potentially causing variability. Additionally, if the proteins were left to dry for too long they were very difficult to resolubilize. Due to the sticky nature of the protein, it is possible that some of the protein ended up stuck in the tips when preparing the TCA precipitation aliquots, and this also could explain the differences in intensities if some of the protein was being lost in the tips. Also, it is possible that the amount of biofilm is dependent on the speed of centrifugation based on preliminary observations. There are several reasons why there was so much variation in the results, and it is hard to pinpoint which one specifically explains the inconsistencies in the data.

For the experiments using the CPE0514_CPE0515-FLAG construct, the results indicated that CPE0516 and CPE0517 are not necessary for secretion of the protein CPE0515. Because this looked very similar to the wild-type strain secretion of CPE0515-FLAG, it is likely that CPE0516 and CPE0517 are not necessary for secretion of the matrix material protein CPE0515. There was still a copy of the entire biofilm operon (*CPE0514-CPE0517*) on the chromosome, but in this expression vector system it looked as if CPE0516 and CPE0517 were not needed to secrete CPE0515.

For the construct where CPE0516 was tagged with HA, the qualitative data collected showed that polymerization is not occurring given the presence of only a monomer and a dimer in the immunoblot performed. From these results in Figure 2.13, the lack of polymerization is clear. The presence of the dimer is also interesting; given that this was analyzed on SDS-PAGE, any non-covalently bound dimer would not have shown up in the acrylamide gel after being subjected to DTT, SDS-PAGE, and heating treatments. This indicated that the dimer showing up in Figure 2.13 was covalently bound, which is an interesting finding.

Future experiments which could be done could include generating a western blot for the CPE0517-His_6 construct. Additionally, examining secretion kinetics of fluorescently tagged CPE0515 protein would be helpful. Other experiments could include testing the CPE0514_CPE0515-FLAG, CPE0516-HA, and CPE0517-His_6 constructs in various pilin mutants. Another experiment that was attempted, but could be tried again, was to delete the chromosomal *CPE0514-CPE0517* operon. For the

experiments done in this study, the biofilm operon was being expressed on a plasmid, and it would be helpful to delete the chromosomal copy for two reasons. The first of which is that there might be competition with the *pilB1* operon system. It is also possible that the FLAG-tag version being expressed might be interacting with the wild-type copies of the proteins, therefore deleting the chromosomal operon would be extremely helpful to see if this is occurring.

C. perfringens biofilm matrix material forms long filaments, that Obana et. al suggested might be covalently linked (Obana *et al.*, 2020). Biofilm could theoretically be polymerized in the periplasmic-like space, but then would need a T2SS, or something similar, to continue to the outside of the cell. It is possible that CPE0516 and CPE0517 could have a relationship akin to that of TasA and TapA proteins found in *B. subtilis*, where one protein is a major component and the other is an accessory protein. TapA protein is covalently linked to the cell, and so based on evidence gathered for CPE0516-HA showing a heat stable dimer forming, TapA could be analogous to CPE0516 in some ways (El Mammeri *et al.*, 2019). Given that there were not enough significant results to form a complete T2SS, there is plenty of investigation which can be continued to potentially answer the questions initially posed in this study.

Conclusions

Individual proteins in the biofilm operon were tagged to detect levels of matrix material protein being secreted in a quantitative manner using western blots and slot blots. Constructs created included CPE0515-FLAG, CPE0514_CPE0515-FLAG, and CPE0516-HA. For the western blot densitometry, it was determined that only the *pilA1* deletion mutant had a statistically significant decrease in CPE0515-FLAG secretion, while the remaining 17 deletion mutants did not have statistically significant changes. This indicates that the PilA1 protein is likely needed for secretion, however it does not point to any other proteins being important. To confirm what was seen in the western blots, slot blots were also performed. For the slot blot densitometry, it was determined that the *pilA1* and *CPE2280* deletion mutants had statistically significant decrease in CPE0515-FLAG secretion relative to the wild type, indicating these are likely to be involved in secretion of the biofilm matrix material protein CPE0515. Additionally, it was determined that *pilA2* and *CPE1841* deletion mutants had statistically significant increases in CPE0515-FLAG secretion relative to the wild type. For the *pilA2* mutant, the reason for this increase in intensity compared to the wild type could be because PilA2 might be a transcriptional regulator of the biofilm matrix operon (Obana *et al.*, 2020). The qualitative data collected for CPE0515-FLAG using Sypro stained protein gels gave preliminary insight as to which T4P proteins might be needed for secretion of biofilm matrix material. However, a lack of statistically significant decrease for most of the T4P proteins, compared to the WT, makes it impossible to build a functional model based on this data. For the

experiments examining the construct CPE0514_CPE0515-FLAG on western blots, this showed that CPE0516 and CPE0517 are not needed for secretion of CPE0515. Additionally, when the construct CPE0516-HA was observed on a western blot, this showed the presence of a dimer and a monomer, and indicated a lack of polymerization occurring. The dimer formed is heat stable, and covalently bound, which is an interesting finding. While two pilin deletion mutants, *pilA1* and *CPE2280*, showed a statistical defect in secretion of biofilm matrix material protein, it could not be confirmed which other proteins are necessary for secretion, and therefore a functional model for this system was not able to be constructed. It is possible that *C. perfringens* could be using T4P as something similar to T2SS as seen in Gram-negative bacteria, however based on the data collected there is not a T2SS that exists in *C. perfringens*.

Experimental Procedures

Strains of bacteria and growth environments. The strains of bacteria, in addition to the plasmids that were utilized for Chapter 2, are itemized in Table 2.1. The primers for Chapter 2 are itemized in Table 2.2. For all experiments using *E. coli*, strain DH10B was cultivated using Luria-Bertani (LB) broth (1% sodium chloride, 1% tryptone, and 0.5% yeast extract) and incubated for a minimum of six hours at 37°C. When *C. perfringens* strain HN13 was grown, the media used included brain heart infusion (BHI) liquid medium (VWR International), TY liquid medium (made of 2% yeast extract, 3% tryptone, 0.1% sodium thioglycolate), or PGY medium (3% glucose, 1% peptone, 0.1% yeast extract, 0.1% sodium thioglycolate). All growth for *C. perfringens* occurred using an anaerobic chamber (Coy Laboratory Products, Inc.); all incubations occurred at 37°C, under anaerobic conditions (approximately 85% nitrogen, 10% carbon dioxide, and 5% hydrogen). Chloramphenicol (at a concentration of 20 µg/µL) was included in the media inoculated with strains of *C. perfringens* containing pKRAH1. Kanamycin (at a concentration of 100 µg/µL) was included in the media inoculated with strains of *E. coli* carrying pCRBluntIITOPO.

In all experiments pertaining to protein secretion, the strains of *C. perfringens* were prepared inside the anaerobic chamber initially using TY, and later in BHI medium, and incubated at 37°C. The overnight cultures were grown for a minimum of six hours anaerobically. Using a 1:50 dilution, experimental cultures were inoculated using the overnight cultures. In order to induce the lactose-inducible promoter on pKRAH1 (Hartman *et al.*, 2011), lactose at a concentration of 500 mM was added one hour after inoculation (approximately late in the logarithmic growth phase) to achieve a final concentration of approximately 10 mM using a 1:50 dilution, thereby expressing the proteins of interest. Following the initial induction one hour after inoculation, 500 mM lactose was added using a 1:50

dilution every 30 minutes for five hours. After five hours, the culture samples were pelleted in a centrifuge at 3,500 x *g* to remove the supernatant for TCA precipitation and SDS-PAGE analysis.

Isolation of matrix material. Gary Camper put the plasmid pHLL65 (created by Hualan Liu) with the regulated biofilm operon into HN13. The pKRAH1 plasmid-based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously, until the cells grew to stationary phase. After induction, this was subjected to centrifugation for 30 minutes at 14,000 x *g*.

The resulting supernatant was subjected to trichloroacetic acid (TCA) precipitation (see "TCA precipitation of supernatants" section on page 29 for details). The resulting pellet from this 30-minute centrifugation at 14,000 x *g* was resuspended in DPBS, and this solution was pelleted in a centrifuge for 30 minutes at 20,000 x *g*. The resulting supernatant from the 30-minute centrifugation at 20,000 x *g* was subjected to TCA precipitation. The resulting pellet from this 30-minute centrifugation at 20,000 x *g* was discarded. The TCA precipitations for supernatant (1), and supernatant (2) were loaded onto a protein gel which was subjected to electrophoresis and then stained with Sypro Ruby. This allowed the visualization of the secretome of purified matrix material (data not shown).

Methods for preliminary determination of which TFP proteins are required for secretion of biofilm matrix. Gary Camper transformed plasmid pHLL65 into 17 TFP gene mutant strains. pHLL65 was transformed into the *pilT* deletion mutant by the author (S. Kivimaki). Colonies for this mutant were grown in PGY and chloramphenicol (20 µg/ml) to prepare frozen stocks. For all 18 mutant strains, see "TCA precipitation of supernatants" section below for next steps. Protein samples from TCA precipitation for these experiments were separated using SDS-PAGE and were developed with the fluorescent dye Sypro Ruby. For protein gels, a 10% acrylamide gel was used for these experiments.

TCA precipitation of supernatants. After centrifugation (3,500 x *g*, five minutes) and extraction of supernatants, a protein precipitation of the supernatants was performed. A volume of 750 µL of cold 20% TCA/acetone (1:1, v/v, with a final 10% TCA/50% acetone) was added to 750 µL of supernatant per sample. The mixtures were placed on ice for 5-10 minutes, and then pelleted in a centrifuge (13,300 x g, three minutes). The supernatants were then discarded, and the protein precipitates were washed with 800 µL of 80% acetone, followed by centrifugation (13,300 g, three minutes). This wash step was repeated twice. After washing, the protein precipitates were air-dried for approximately four minutes, and then dissolved into sterile Milli-Q water. All protein precipitates were suspended in 8.3 µL of 4x SDS page buffer, followed by 3.7 µL of 100 mM dithiothreitol (DTT) per sample. To adjust the pH to neutral for the TCA precipitation replicates, approximately 0.5 µL -1 µL of 1M Tris base pH 9 was included in each sample. These samples were heated at 95°C for 20 minutes.

SDS-PAGE and gel staining. For protein experiments, centrifugation (3,500 x *g* for five minutes) of culture samples (five mL) was done to separate the pellet fractions and supernatant. The supernatant was stored at -80°C until used. The supernatants were subjected to TCA precipitation (as described above "TCA precipitation of supernatants"). TCA precipitation protein samples for these experiments were separated using SDS-PAGE and were developed with the fluorescent dye Sypro Ruby. For protein gels, a 10% or 12.5% acrylamide gel was used for these experiments.

Slot blot and immunoblotting. Proteins for these experiments were also analyzed using immunoblots and slot blots, developed using either immunoblotting method one or immunoblotting method two. For the immunoblot experiments, the proteins were moved onto a polyvinylidene difluoride (PVDF) membrane submerged in cold buffer which contained three mM Na_2CO_3 and 10 mM $NaHCO_3$ with 20% methanol.

Immunoblotting method one. The PVDF membranes were laid into a Snap i.d. 2.0 system (Millipore Sigma). Once set up, a solution containing Tris-buffered saline (TBS) (pH 7.4), 1% Tween 20, and 1% bovine serum albumin was added as a blocker so that the surface of the membrane was submerged. Both primary and secondary antibodies were added to separate aliquots of the solution containing TBS, 1% Tween 20, and 1% BSA. First, the primary antibody solution was left to sit for 10 minutes, followed by washings with TBS- Tween 20. Lastly the secondary antibody solution was left to sit for 10 minutes, followed by washings with TBS- Tween 20. The primary antibody utilized was OctA-probe H-5 (1:250 dilution) used to detect FLAG (from Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody utilized for these experiments was StarBright700 anti-mouse (Bio-Rad) (1:10,000 dilution). The pictures of the immunoblots were taken using a ChemiDoc MP imaging system (Bio-Rad), to measure the intensity of the fluorescence detected using densitometry. For the statistical analysis of the western blot densitometry results for CPE0515-FLAG in various pilin mutants, the secretome was quantified from the bottom of the well, all the way down to ~50 kD.

Immunoblotting method two. Membranes were transferred to a sealed container and blocked with EveryBlot blocking Buffer (Bio-Rad). Antibodies were diluted with EveryBlot blocking buffer and applied to membranes, followed by a minimum of five washes with Tris-buffered saline (TBS) (pH 7.4) containing 1% Tween 20. The primary antibodies utilized included OctA-probe H-5 (1:227 dilution) used to detect FLAG, and HA-Tag F-7 (1:227 dilution) used to detect HA (both from Santa Cruz Biotechnology, Santa Cruz, CA). Membranes submerged in EveryBlot blocking buffer with primary antibody were incubated at 4°C overnight. The secondary antibody utilized for these experiments was StarBright700 anti-mouse (Bio-Rad) (1:13,333 dilution), incubated for one hour at room temperature. The pictures of the immunoblots were taken using a ChemiDoc MP imaging system (Bio-Rad), to measure

and observe the intensity of the fluorescence for densitometry analyses. For the statistical analysis of the western blot densitometry results for CPE0515-FLAG in various pilin mutants, the secretome was quantified from the bottom of the well, all the way down to ~50 kD.

Slot blotting method. Supernatant samples were prepared by diluting supernatants with sterile BHI. For initial experiments, samples of 100% pure culture supernatant, 50% culture supernatant and 50% blank medium, 25% culture supernatant and 75% blank medium, 10% culture supernatant and 90% blank medium, 1% culture supernatant and 99% blank medium, and 100% blank medium were prepared. For later experiments, samples of 50% culture supernatant and 50% blank medium, 20% culture supernatant and 80% blank medium, 10% culture supernatant and 90% blank medium, 5% culture supernatant and 95% blank medium, 1% culture supernatant and 99% blank medium, and 100% blank medium were prepared. The 10% culture supernatant samples were used to collect the raw intensity data for quantitative results.

A polyvinylidene difluoride (PVDF) membrane was soaked in Tris-buffered saline (TBS) (pH 7.4) containing 1% Tween 20 prior to being loaded into the slot blot while still damp. The slot blot was loaded with TBS-Tween 20 in each slot, and the buffer was vacuumed for 10 minutes while being exposed to air. This buffer was vacuumed off completely, and then the diluted supernatants for the slot blots were loaded into the individual slots of the device and the vacuum was applied for 10 minutes while the device was exposed to air. The supernatants were vacuumed off, and then lastly the slot blot was loaded with TBS- Tween-20 in each slot and the vacuum was applied for another 10 minutes while being exposed to air. After washing with buffer, this buffer was vacuumed off as well, and the membrane was developed using immunoblotting method two.

Cloning strategy to create FLAG tagged version of CPE0515 utilizing overlapping PCR

Overlapping PCR. A FLAG tag was added to CPE0515 so that the protein could be detected using western blots. This was done using overlapping PCR. The primers OHL145 and OHL146 were utilized, and the primers OSK1 and OSK2 (Table 2.2) were designed for this purpose. OHL145 and OHL146 were designed to flank the coding sequence of *CPE0515*, and primers OSK1 and OSK2 with introduce the FLAG tag into the amplified product of *CPE0515*. Two PCR reactions were performed initially to acquire the two halves of the DNA, and then a second PCR reaction using the flanking primers OHL145 and OHL146, and the products from the first two PCR reactions was performed. This second PCR reaction yielded a product the same size as *CPE0515*, but with a FLAG tag.

Plasmid construction of pSK1. The PCR product from the second PCR reaction was used to ligate into pTOPO, and the resulting ligation was transformed into *E. coli* strain DH10B, and plated on LB plates containing kanamycin (100 µg/ml). The positive colonies were regrown, and then plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit, and the plasmids were checked

through a restriction enzyme digest for the correct insert size. The plasmid pKRAH1 was also digested with the same restriction enzymes, and the insert for *CPE0515*-FLAG was ligated into pKRAH1. The ligation of CPE0515 with a FLAG tag in pKRAH1 was transformed into *C. perfringens*, and was plated on BHI plates containing chloramphenicol (20 µg/ml). The colonies on this initial BHI chloramphenicol (20 µg/ml) plate were not isolated, so the initial colonies were struck out again on a fresh BHI chloramphenicol (20 µg/ml) plate to achieve isolation. After growing individual colonies in liquid PGY with chloramphenicol (20 µg/ml), a frozen stock was prepared, and the plasmids were then purified using a GeneJET Plasmid Miniprep Kit. The frozen stocks were used to inoculate overnight cultures. The overnight cultures were used to inoculate experimental cultures using a 1:50 dilution. The pKRAH1 plasmid- based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. Protein gels were loaded using the CPE0515-FLAG TCA precipitation samples and were stained using Sypro Ruby. This was followed by western blots to see which samples were producing matrix material. The clone producing matrix material was grown, and the plasmids were purified using a GeneJET Plasmid Miniprep Kit. These purified plasmids were transformed into 18 pilin deletion mutants. Colonies for each mutant were grown in PGY and chloramphenicol (20 µg/ml) to prepare frozen stocks. After growing the frozen stock in BHI and chloramphenicol (20 µg/ml), the pKRAH1 plasmid- based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. These TCA precipitation samples were tested on western blots to see which proteins are necessary for secretion. Densitometry results were obtained after imaging the western blots. For the statistical analysis of the western blot densitometry results for CPE0515-FLAG in various pilin mutants, the secretome was quantified from the bottom of the well, all the way down to ~50 kD.

Cloning Strategy to Create HA Tagged Version of CPE0516 utilizing overlapping PCR.

Overlapping PCR. To track the protein using western and slot blots, an HA tag was added to CPE0516. This was done using overlapping PCR. The primers OHL145 and OHL146 were utilized, and the primers OGC22 and OGC23 were designed for this purpose by Gary Camper. OHL145 and OHL146 were designed to flank the coding sequence of *CPE0516*, and primers OGC22 and OGC23 would introduce the HA tag into the amplified product of *CPE0516*.

Two PCR reactions were performed initially to acquire the two halves of the DNA using OHL145 and OGC23, and OHL146 and OGC22, and then a second PCR reaction using the flanking primers OHL145 and OHL146, and the products from the first two PCR reactions. This second PCR reaction yielded a product the same size as *CPE0516*, but with an HA tag.

Plasmid construction of pSK2. The PCR product from the overlapping PCR reaction was used to ligate into pCRBluntITOPO, and the resulting ligation was transformed into *E. coli* strain DH10B, and plated on LB plates containing kanamycin (100 µg/ml). The positive colonies were regrown, and then plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit, and these plasmid preps were checked through a restriction enzyme digest using BamHI and Pst1 for the correct insert size. The insert and the plasmid were determined to be the same size of around 4.5 kbp, where the plasmid pCRBluntITOPO would normally be seen around 3.5 kbp. The presence of the 4.5 kbp insert was also confirmed with a digest using the restriction enzyme EcoRI. The plasmid pKRAH1 was also digested with the same restriction enzymes, and the insert for *CPE0516*-HA was ligated into pKRAH1. The ligation of *CPE0516* with an HA tag in pKRAH1 was transformed into *C. perfringens*, and was plated on BHI plates containing chloramphenicol (20 µg/ml). Isolated colonies were grown in liquid BHI with chloramphenicol (20 µg/ml), to prepare frozen stocks. Frozen stocks were used to inoculate overnight cultures in liquid BHI with chloramphenicol (20 µg/ml). The overnight cultures were used to inoculate experimental cultures at a 1:50 dilution in liquid BHI with chloramphenicol (20 µg/ml). The pKRAH1 plasmid-based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. A western blot was performed using these CPE0516-HA tagged TCA precipitation samples, to see which sample was producing matrix material. The clone producing matrix material was grown, and the plasmids were purified using a GeneJET Plasmid Miniprep Kit.

Method to Create FLAG Tagged Version of CPE0514-CPE0515 utilizing PCR

PCR. In order to see the activity of the genes *CPE0514* and *CPE0515* in the absence of the influence of *CPE0516* and *CPE0517* in the expression vector pKRAH1, pSK1 plasmid DNA was utilized and primer OSK20 (Table 2.2) was designed. Primer OSK20 was the reverse complement of the first portion of gene *CPE0516*'s coding sequence, with a BamHI site added, and was paired with primer OHL145 (Table 2.2) for this PCR. The PCR product for this FLAG Tagged Version of *CPE0514-CPE0515* was approximately 1.3 kb.

Plasmid construction of pSK4. The PCR product was used to ligate into pTOPO, and the resulting ligation was transformed into *E. coli* strain DH10B, and plated on LB plates containing kanamycin (100 µg/ml). The positive colonies were regrown, and then plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit, and these plasmid purifications were checked through a restriction enzyme digest for the correct insert size, initially using BamHI and Pst1, and then later the restriction enzyme EcoRI. The plasmid pKRAH1 was also digested with the same restriction enzymes (BamHI and Pst1), and the insert for *CPE0514_CPE0515*-FLAG was ligated into pKRAH1. The ligation of

CPE0514_CPE0515-FLAG in pKRAH1 was transformed into *C. perfringens*, and was plated on BHI plates containing chloramphenicol (20 µg/ml). The colonies on this initial BHI chloramphenicol (20 µg/ml) plate were not isolated, so the initial colonies were struck out again on a fresh BHI chloramphenicol (20 µg/ml) plate to achieve isolation. Isolated colonies were grown in liquid BHI with chloramphenicol (20 µg/ml) for overnight cultures. Frozen stocks were prepared from the overnight cultures. In order to analyze this using PCR, the chromosomal and plasmid DNA was extracted from the overnight cultures using a NaOH heating method (Yonogi *et al.*, 2016). To screen for colonies which potentially had the correct inserts, a GoTaq PCR was done using the DNA from the six NaOH samples as a template. To identify the correct inserts, primers OSK20 and OAHI (Table 2.2) were utilized, which yielded a band approximately 1.7 kb in size. Once three correct stocks were identified; they were grown in BHI and chloramphenicol (20 µg/ml), and the pKRAH1 plasmid- based promoter system was then used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. A western blot was performed to see if CPE0514_CPE0515-FLAG was producing matrix material, and to see whether or not CPE0516 or CPE0517 are part of the complex seen on westerns. The plasmid pSK4 was also transformed into in-frame deletion strains Δ*pilA1* and Δ*pilA2* in HN13, to see how the absence of these genes affected protein expression.

Statistics. Means, standard deviations, and SEMs were determined and graphed with Prism 6 (GraphPad Software). To calculate densitometry (MPI) for western blots, Image lab 6.1 (Bio-Rad Corp.) was used. To calculate the densitometry values (raw intensity data) for the slot blots, ImageJ 1.52a was used.

Tables and Figures for Chapter 2

Table 2.1: Strains of bacteria and the plasmids utilized in this study for Chapter 2		
Strain	**Relevant characteristics**	**Reference or source**
Clostridium perfringens		
type A strain HN13	Δ*galKT*	(Nariya *et al.*, 2011)
type A strain HN13 Δ*pilA1*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilA2*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilA3*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilA4*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilB1*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilB2*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilB1B2*	Δ*galKT*	Andrea Hartman
type A strain HN13 Δ*pilC1*	Δ*galKT*	Hualan Liu
type A strain HN13 Δ*pilC2*	Δ*galKT*	William Hendrick
type A strain HN13 Δ*pilD*	Δ*galKT*	Samantha Soncini
type A strain HN13 Δ*pilM*	Δ*galKT*	Hualan Liu
type A strain HN13 Δ*pilN*	Δ*galKT*	Hualan Liu
type A strain HN13 Δ*pilO*	Δ*galKT*	Hualan Liu
type A strain HN13 Δ*pilT*	Δ*galKT*	Stephen Melville
type A strain HN13 Δ*CPE1841*	Δ*galKT*	Samantha Soncini
type A strain HN13 Δ*CPE2277*	Δ*galKT*	Hualan Liu
type A strain HN13 Δ*CPE2279*	Δ*galKT*	Hualan Liu
type A strain HN13 Δ*CPE2280*	Δ*galKT*	Hualan Liu
type A strain HN13 pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilA1*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilA2*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilA3*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilA4*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilB1*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilB2*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilB1B2*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilC1*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilC2*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilD*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilM*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu

type A strain HN13 Δ*pilN*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilO*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*pilT*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*CPE1841*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*CPE2277*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*CPE2279*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 Δ*CPE2280*pHL65	Δ*galKT*, pKRAH1 with *CPE0514-517*	Hualan Liu
type A strain HN13 pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilA1*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilA2*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilA3*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilA4*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilB1*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilB2*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilB1B2*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilC1*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilC2*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilD*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilM*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study

type A strain HN13 Δ*pilN*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilO*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilT*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*CPE1841*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*CPE2277*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*CPE2279*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*CPE2280*pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study
type A strain HN13 pSK2	ΔgalKT, pKRAH1 with *CPE0514-517*, HA tag on CPE0516	This study
type A strain HN13 pSK4	Δ*galKT*, pKRAH1 with *CPE0514-515*, FLAG tag on CPE0515	This study
type A strain HN13 Δ*pilA1*pSK4	Δ*galKT*, pKRAH1 with *CPE0514-515*, FLAG tag on CPE0515	This study
type A strain HN13 *ΔpilA2*pSK4	ΔgalKT, pKRAH1 with *CPE0514-515*, FLAG tag on CPE0515	This study
Escherichia coli		
Strain DH10B	F− mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL nupG λ−	(Grant *et al.*, 1990)
Strain DH10B pSK1	F− mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL nupG λ− FLAG tag on CPE0515	This study
Plasmid	**Relevant Characteristics**	**Source**
pSK1	Δ*galKT*, pKRAH1 with *CPE0514-517*, FLAG tag on CPE0515	This study

pSK2	Δ*galKT*, pKRAH1 with *CPE0514-517*, HA tag on CPE0516	This study
pSK4	Δ*galKT*, pKRAH1 with *CPE0514-515*, FLAG tag on CPE0515	This study
pHL65	pKRAH1 with *CPE0514-CPE0517*	Hualan Liu
pKRAH1	Cm^r; lactose-inducible expression system plasmid for use in *C. perfringens*	(Hartman *et al.*, 2011)
pCRBluntITOPO	PCR cloning vector	Invitrogen

Table 2.2. Primers used for the experiments described in Chapter 2.	
Primer Name	**Sequence (5'- 3')**
OHL145	5' - CTGCAGGAAAAGGAAGAAAAGGAATAATTATGAAAAAAG – 3' (Pst1 site)
OHL146	5' - GGA TCC GTT TTC CAT CTT AAT TTA ATT TTA - 3' (BamHI site)
OAHI	5'-GGCGATAAGTCGTGTCTTACCG-3'
OGC22	5' - GATAAGTATATAGAAATTTTATCTAAATACCCATACGACGTCCCAGACTA CGCTTAGTTAGGT - 3'
OGC23	5' - ACCTAACTAAGCGTAGTCTGGGACGTCGTATGGGTATTTAGATAAAATTTC TATATACTTATC - 3'
OSK1	5'- GCAGGTACTAATGCACATAAAGATTACAAGGATGACGATGACAAGTAAGT AATA TTTTAAATTAAGTTATAACC- 3'
OSK2	5'- GGTTATAACTTAATTTAAAATATTACTTACTTGTCATCGTCATCCTTGTAAT CTTTATGTGCATTAGTACCTGC- 3'
OSK20	5' – GGATCCAAAAGAAATGCCATAGTAAATAAAAAGGATTTTATTAGATTCTT CAT- 3' (BamHI site)

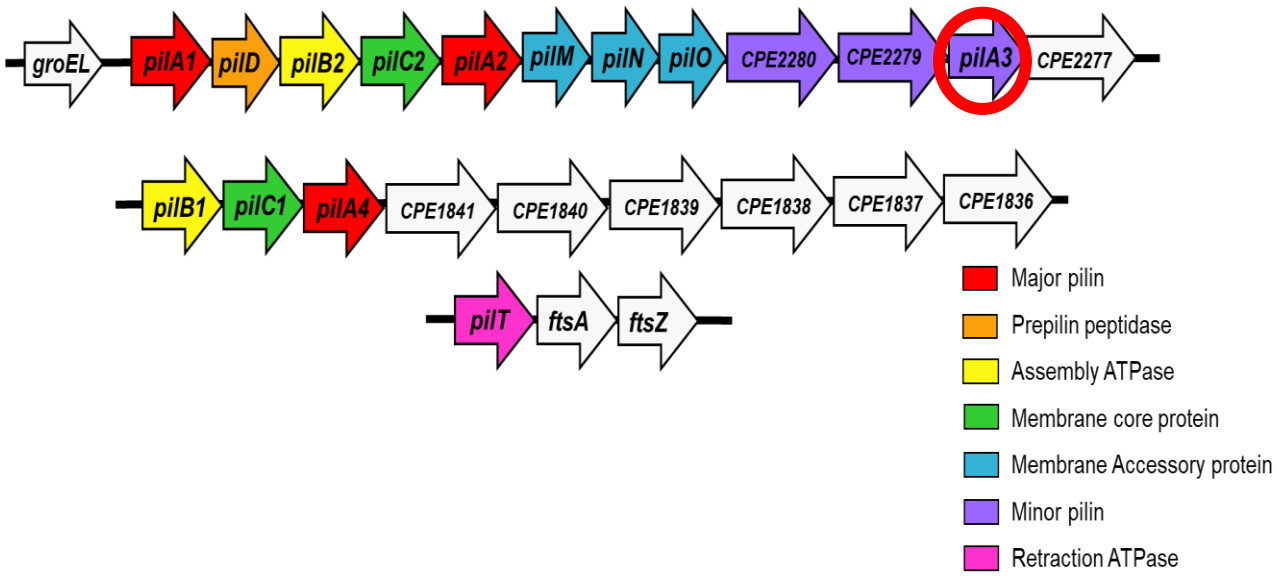


Figure 2.1. *C. perfringens* T4P operons, highlighting *pilA3*. There are two operons containing T4P genes, the primary TFP operon for *C. perfringens* (top) and the secondary *pilB1* operon in *C. perfringens* strain HN13, including the 5 transmembrane proteins located downstream (middle). A single *pilT* gene is found in a third operon. (bottom). Predicted protein functions are indicated by color coding. Circled is the gene *pilA3*, which encodes the protein PilA3 that was found to be significant to biofilm matrix material secretion in studies performed by Andrea Hartman and Gary Camper (Hartman, 2012). Figure modified from reference (Melville & Craig, 2013) .

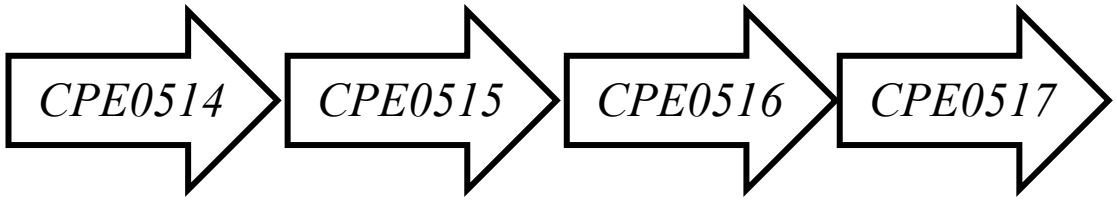
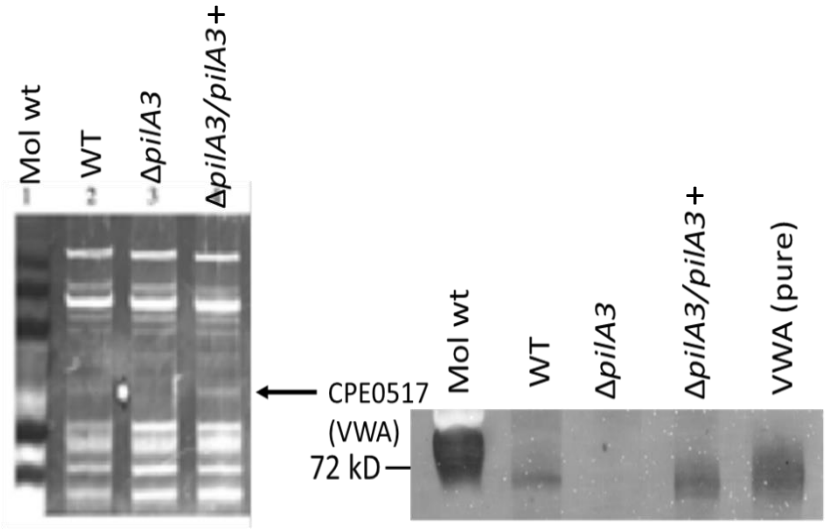


Figure 2.2. Biofilm matrix operon of *C. perfringens*. This operon includes genes *CPE0514, CPE0515, CPE0516,* and *CPE0517. CPE0514* gene encodes the SipW signal peptidase protein, *CPE0515* gene encodes the main matrix material protein, *CPE0516* gene encodes the minor matrix material protein, and *CPE0517* encodes a protein which contains a von Willebrand A domain.



A. B.

Figure 2.3. The protein CPE0517 is not secreted in a *pilA3* mutant. **A.** An SDS-PAGE of the secretome for a *pilA3* mutant, using the fluorescent Sypro ruby stain (results courtesy of Andrea Hartman) (Hartman, 2012). Note the absence of a 70 kD protein CPE0517 (VWA), shown by the arrow. **B**. Western blot of the secretome using anti-VWA antibodies (results courtesy of Gary Camper).

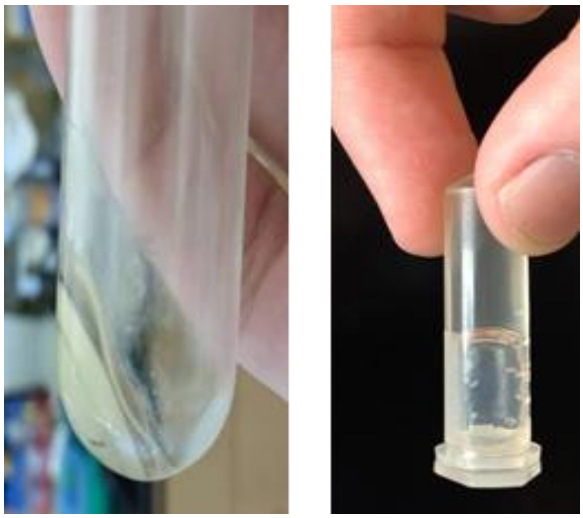


Figure 2.4. Potential biofilm matrix material in the supernatant of *C. perfringens* cultures. Left panel, pellet showing gel-like biofilm material separated from cells. Right panel, the viscous pellet contains a mixture of protein, carbohydrates, and DNA in the matrix. Figures courtesy of Gary Camper.

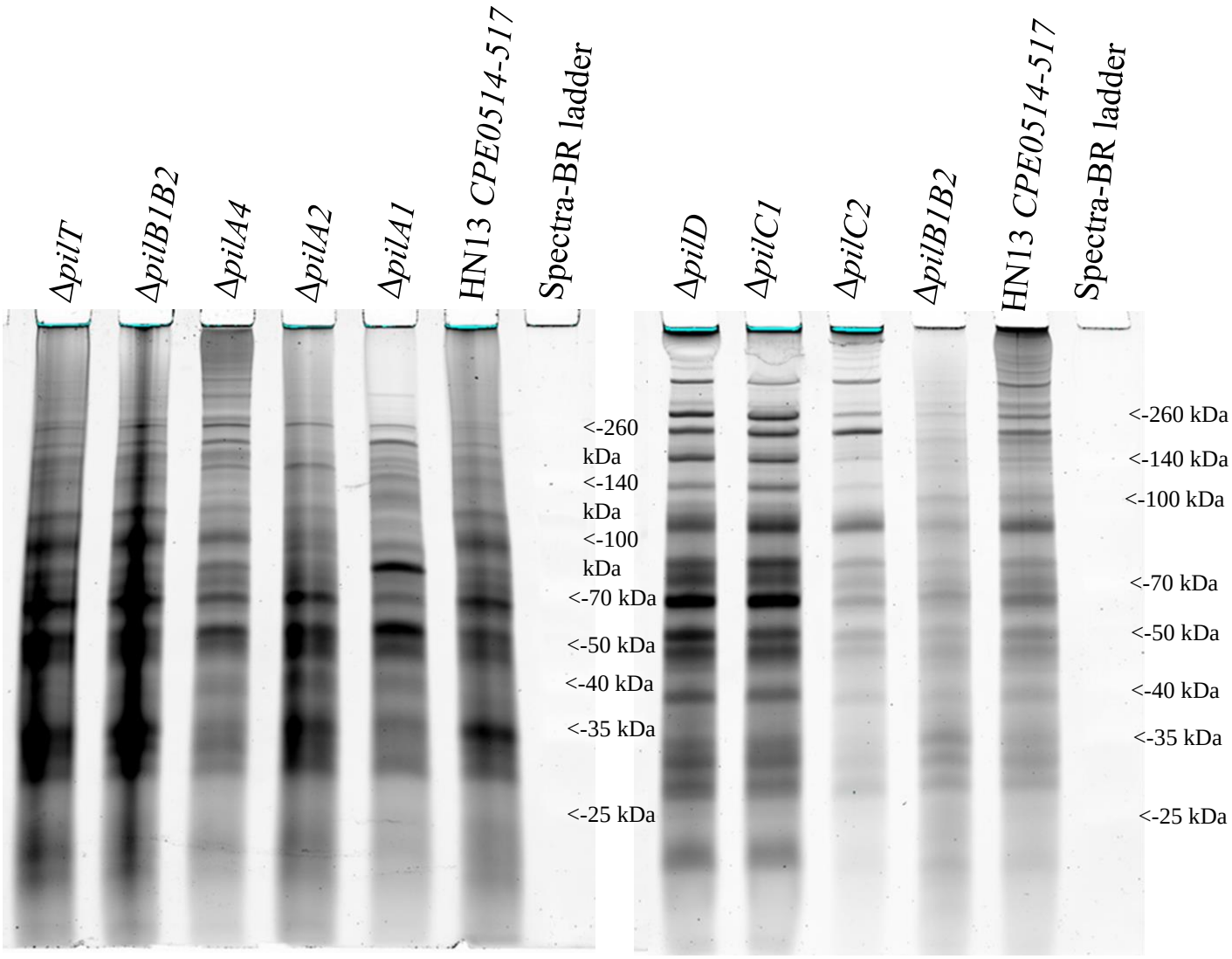


Figure 2.5. Effects of T4P mutants on secretion of biofilm matrix material proteins. Protein experiments screening for T4P deletion mutants expressing the entire biofilm operon (*CPE0514-CPE0517*). Each strain contains the plasmid pHL65. SDS-page gel was used to separate the proteins by size, showing entire biofilm operon expressed in deletion mutants. The 10% acrylamide gel was stained with the fluorescent dye Sypro Ruby. Left: Secretome for the following mutants and wild type; *pilT* deletion, *pilB1* and *pilB2* deletion, *pilA4* deletion, *pilA2* deletion, *pilA1* deletion, HN13 (WT), ladder. Right: Secretome for the following mutants and wild type: *pilD* deletion, *pilC1* deletion, *pilC2* deletion, *pilB1* and *pilB2* deletion, HN13 (WT), ladder.

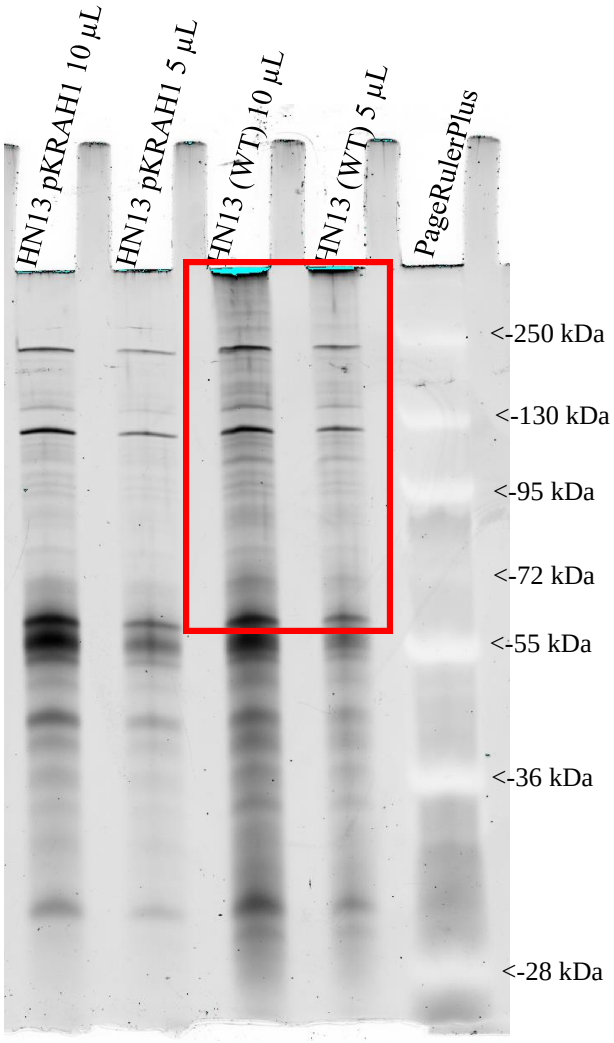


Figure 2.6. Potential matrix material in the WT strain containing plasmid pSK1, compared to a negative control. SDS-PAGE gel stained Sypro ruby showing CPE0515-FLAG tag in HN13 (WT) as the positive control and pKRAH1 in HN13 as the negative control. The WT strain carries the pSK1 plasmid and the negative control HN13 pKRAH1 has an empty vector. The bands that are encompassed by the red box are the ones that are thought to be part of the CPE0515 ladder, from just below the well to approximately 50 kDa.

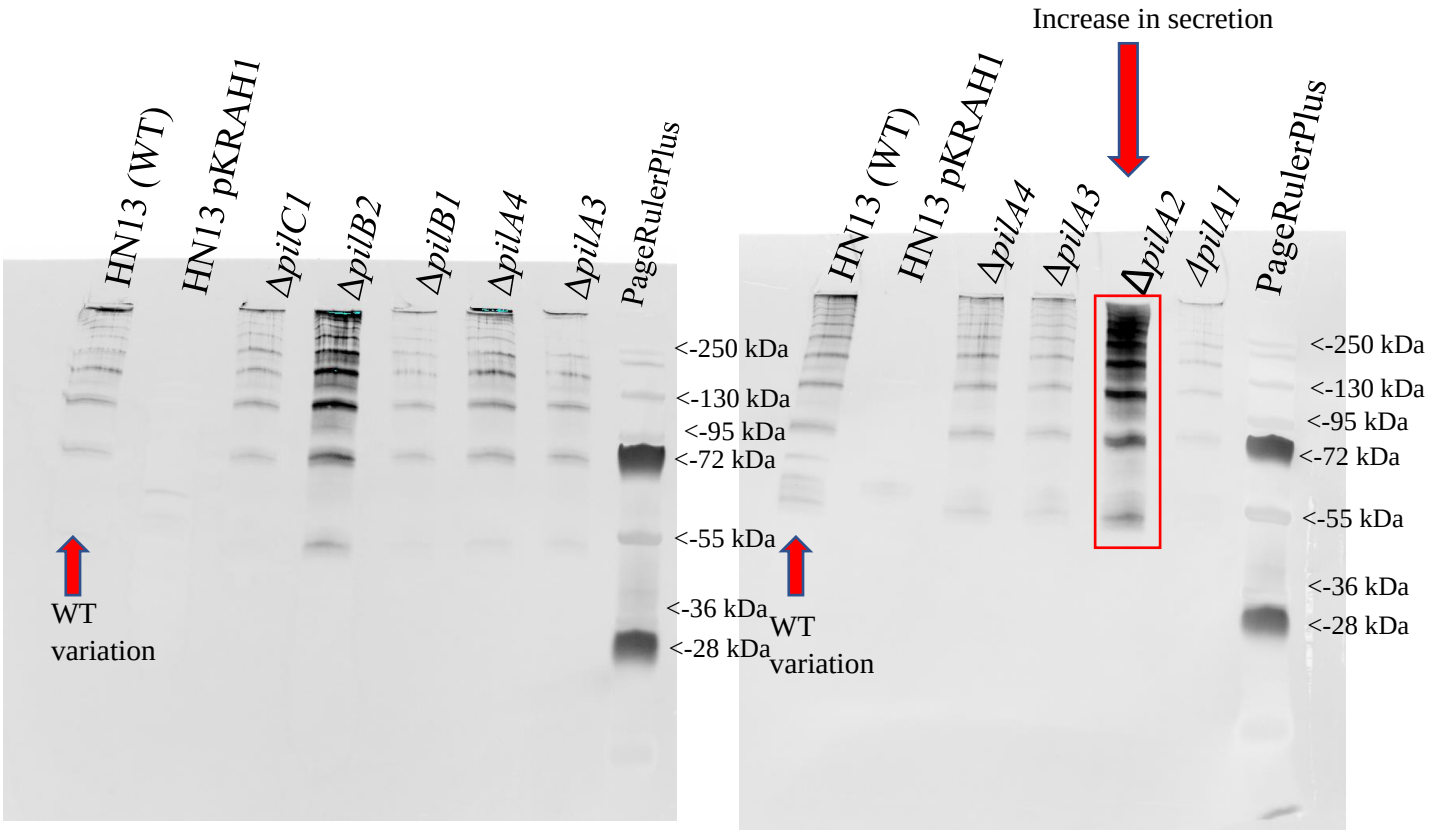


Figure 2.7. Secretion variations for large proteins in the CPE0515-FLAG secretome. Immunoblots of CPE0515-FLAG are shown in six pilin mutant deletion strains. Each strain (including the WT) carries the pSK1 plasmid, except for the negative control HN13 pKRAH1 which has an empty vector. The red box around the *pilA2* deletion mutant is showing how the densitometry was measured, from the bottom of the well to around ~50 kD, resulting in a mean pixel intensity representing an average of all pixels within the box. The red box representing the area the densitometry was measured is where we are interpreting the ladder of FLAG-tagged protein is showing up. The upward facing arrows illustrate the different levels of CPE0515-FLAG seen in the wild type control supernatants. There is an obvious increase in secretion observed for the *pilA2* deletion mutant (red arrow showing increase in secretion).

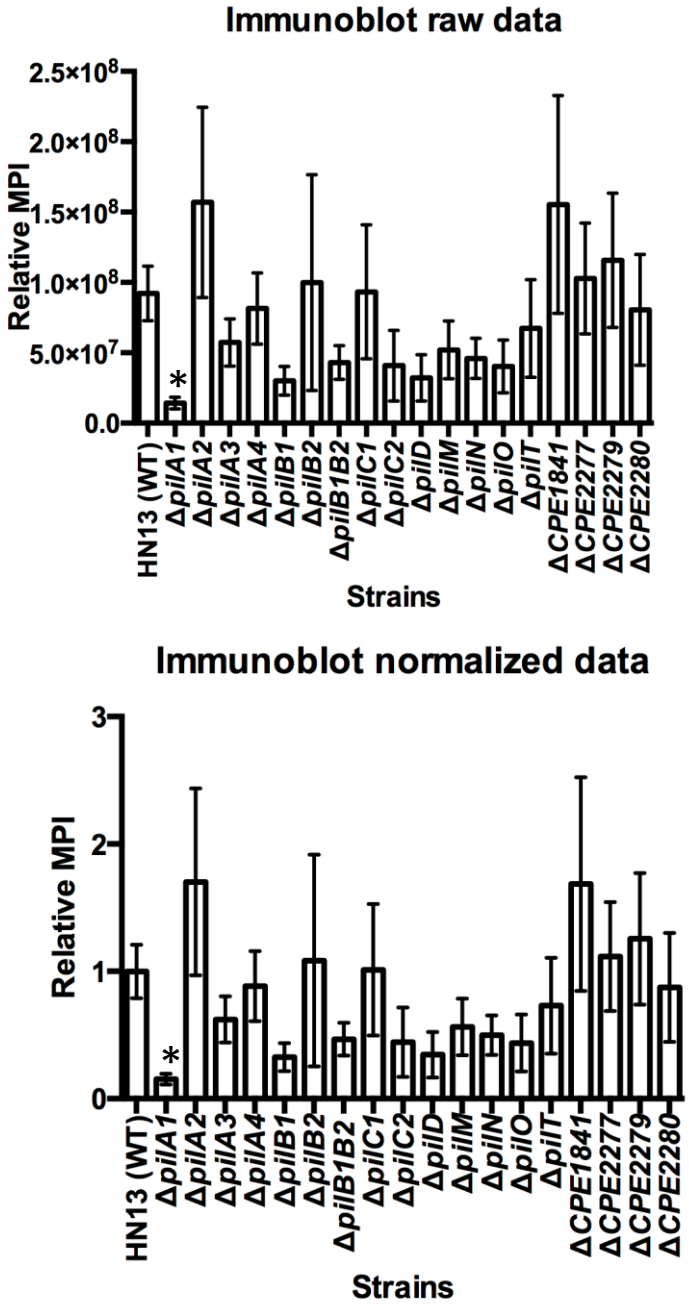


Figure 2.8. Secretion of CPE0515-FLAG in all T4P mutant strains tested. Each strain depicted in the graph carries the pSK1 plasmid. The bar graph represents the mean pixel intensity (MPI) and SEM of the densitometry measurements taken from the western blots performed using CPE0515-FLAG in 18 pilin deletion mutants. A minimum of five replicates were done for each mutant, and 23 replicates were done for the WT. The asterisk represents that the mutant Δ*pilA1* had a statistically significant value from the WT, determined using the student's t-test. To summarize, the relative MPI and SEM are shown for each mutant strain and the positive control HN13 (WT) for the CPE0515-FLAG construct.

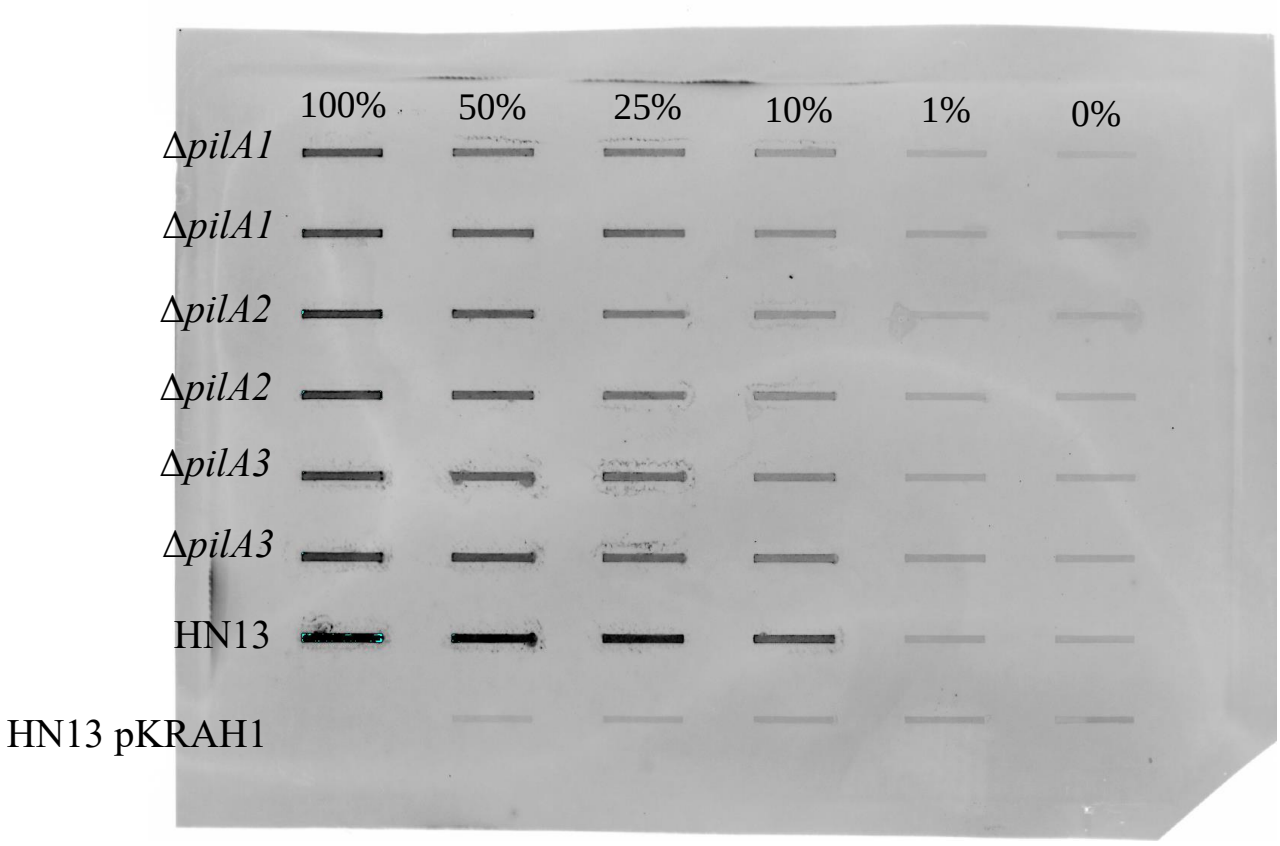


Figure 2.9. Detection of entire secretome intensities for CPE0515-FLAG in T4P mutant strains. Membrane developed using the slot blot method to identify CPE0515-FLAG proteins. Each strain carries the pSK1 plasmid, except for the negative control HN13 pKRAH1 which has an empty vector. The dilution for the supernatant samples is as follows: The 100% sample represents pure culture supernatant, 50% represents 50% culture supernatant and 50% blank medium, 25% contains 25% culture supernatant and 75% blank medium, 10% contains 10% culture supernatant and 90% blank medium, 1% contains 1% culture supernatant and 99% blank medium, and the 0% sample represents pure blank medium. This slot blot depicts the results for CPE0515-FLAG in three pilin mutant deletion strains, Δ*pilA1*, Δ*pilA2*, and Δ*pilA3*. The mutants are listed on the left side, along with the controls. For the CPE0515-FLAG construct, HN13 is the positive control in the WT strain and HN13 pKRAH1 contains an empty vector, serving as the negative control.

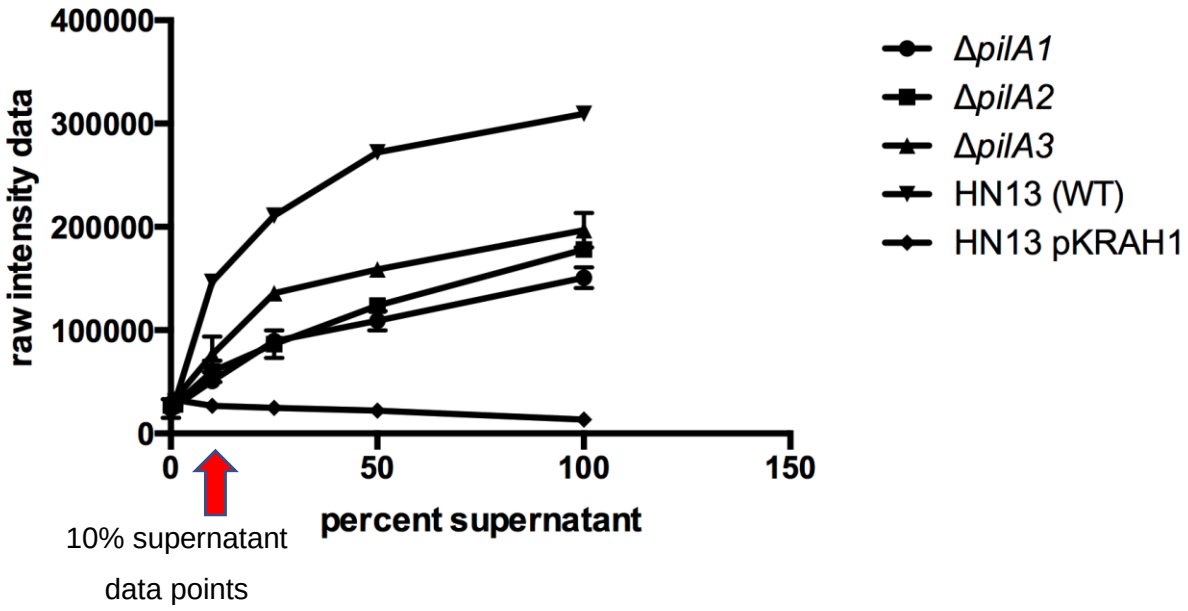


Figure 2.10. Determination of linear range for slot-blot detection. Each strain carries the pSK1 plasmid, except for the negative control HN13 pKRAH1 which has an empty vector. Slot blot raw intensity data for CPE0515-FLAG in three pilin mutant deletion strains, Δ*pilA1*, Δ*pilA2*, and Δ*pilA3*. The mean and standard deviation for all three mutant strains is shown for each of the data points (for each slot blot, at least two replicates of each mutant were performed). There were a minimum of six replicates done for each mutant, and 38 replicates done for the WT. The data points used to generate the values shown in Figure 2.11 were taken at the 10% supernatant data point (indicated by the red arrow).

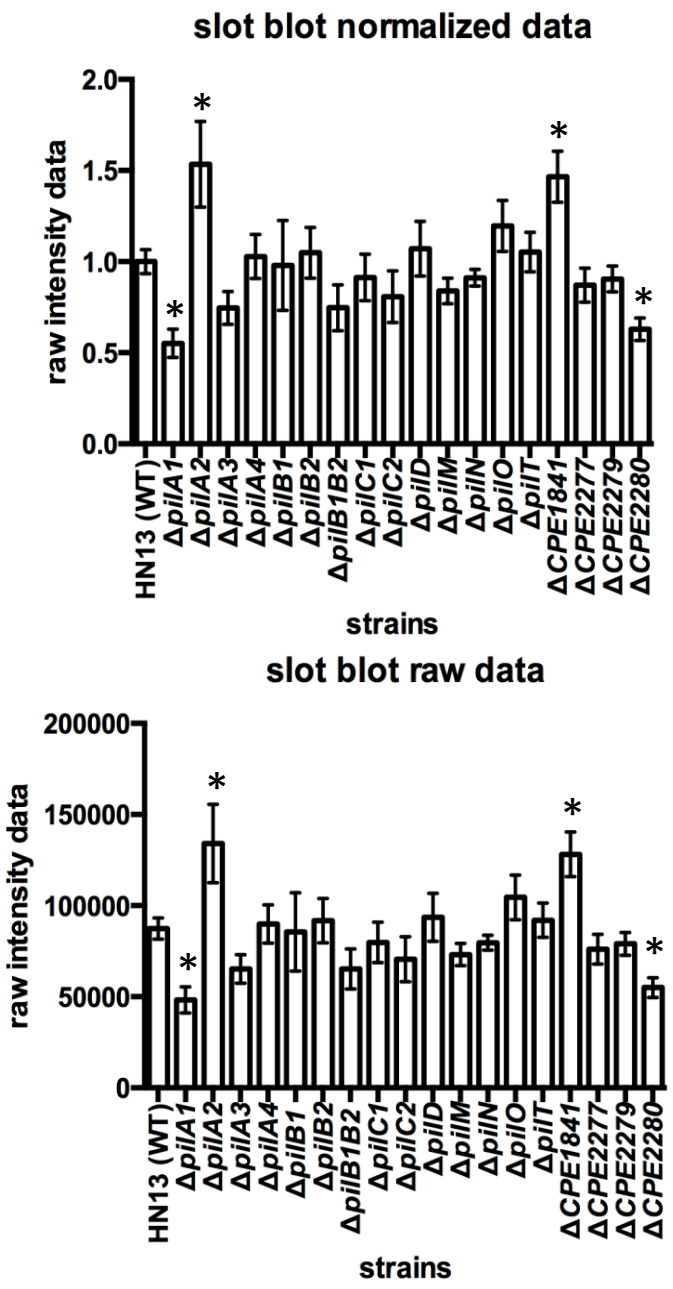


Figure 2.11. Confirmation of secretion of CPE0515-FLAG in T4P mutant strains. Each strain carries the pSK1 plasmid. The bar graph represents the mean and SEM for the raw intensity data taken for the slot blots developed for CPE0515-FLAG in 18 pilin deletion mutants, for the 10% supernatant data points. The normalized calculations were done by taking the average of the raw WT values, and then dividing each raw mutant value by the average of the raw WTs. These normalized values for each mutant were averaged, and the SEM accounts for all the replicates for each 10% supernatant sample done on different days, where a minimum of six replicates were done for each mutant, and 38 replicates for the WT. The four mutants that had statistically significant values from the wild-type (HN13), as determined using the student's t-test, are indicated by the asterisks.

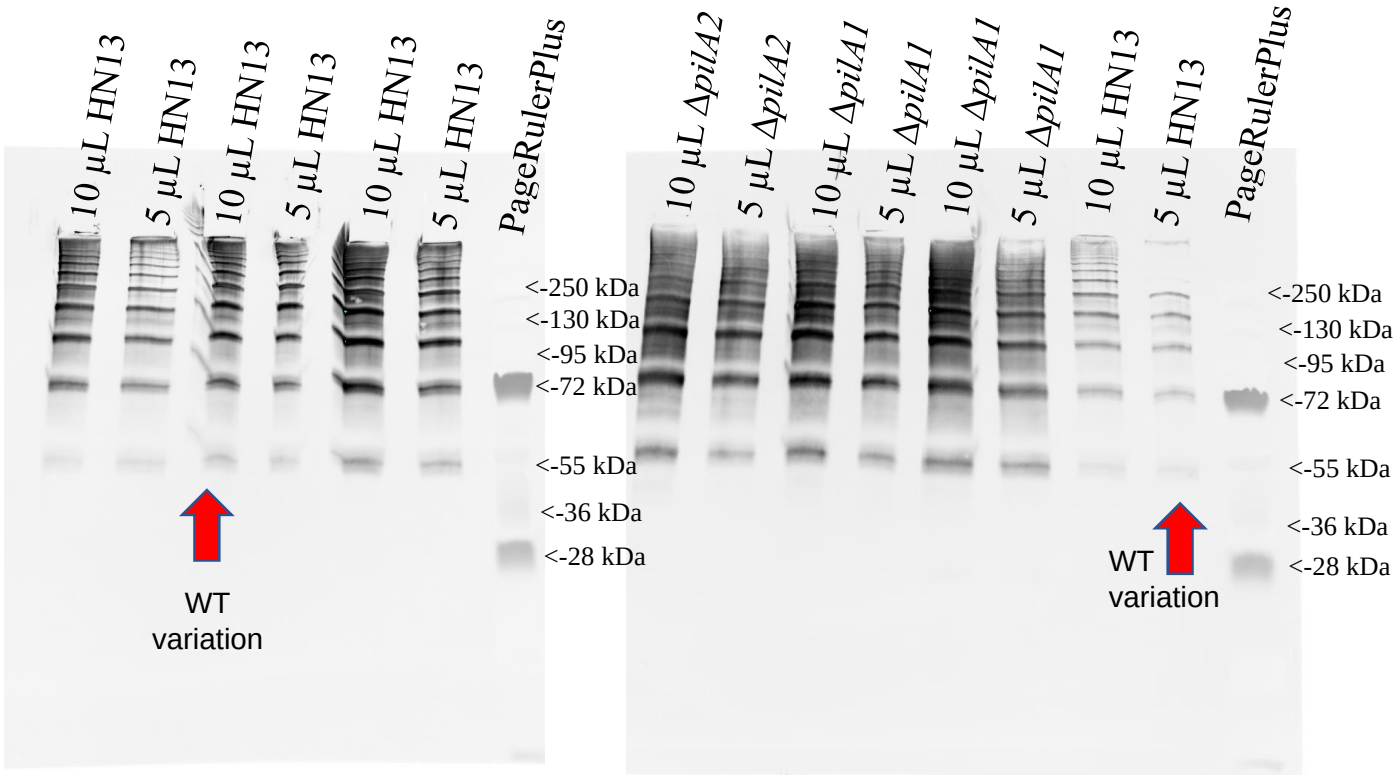


Figure 2.12. Immunoblot of CPE0514_CPE0515-FLAG in two pilin deletion mutant strains, Δ*pilA1* and Δ*pilA2*. Each strain carries the pSK4 plasmid. On the left is a western blot for CPE0514_CPE0515-FLAG wild-type colony samples. On the right is a western blot for CPE0514_CPE0515-FLAG tag in two pilin deletion mutants. Δ*pilA1* and Δ*pilA2* represent the *pilA1* and *pilA2* deletion mutant strains using the CPE0514_CPE0515-FLAG construct. The red arrows indicating "WT variation" are to illustrate how much variation was seen from experiment to experiment for the WT strain HN13 for the CPE0514_CPE0515-FLAG wild type construct, as similarly observed in the CPE0515-FLAG wild-type construct.

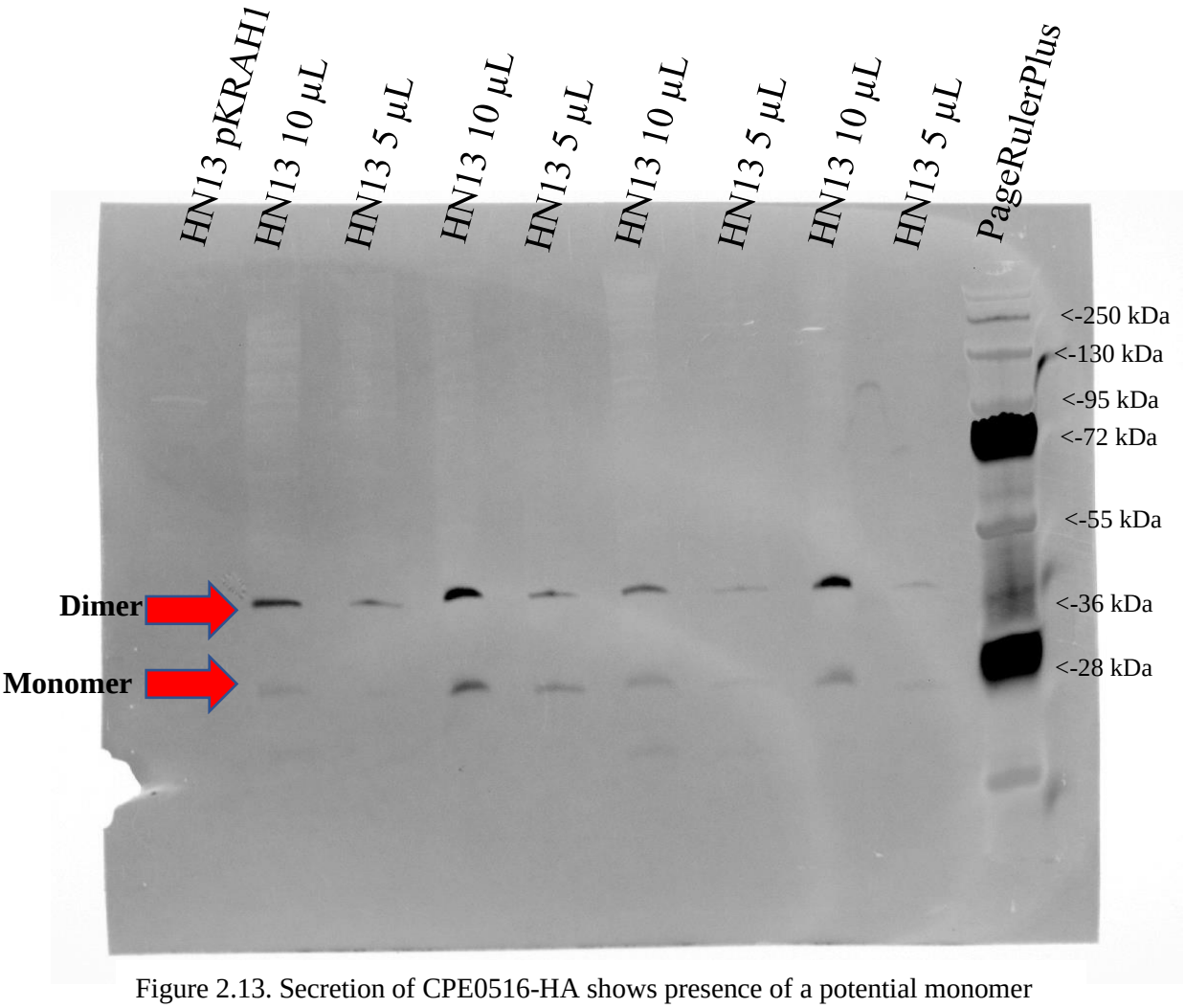


Figure 2.13. Secretion of CPE0516-HA shows presence of a potential monomer and dimer. Immunoblot showing the wild type CPE0516-HA construct. The WT strain contains the pSK2 plasmid, and the negative control HN13 pKRAH1 has an empty vector. The arrows indicate a potential monomer (~28 kDa, predicted mol wt of 24 kD) and a potential dimer (~45 kDa).

Chapter 3: Concluding Remarks

The initial goal of this study was to create a model for a possible pilus-like (or T2SS-like) system that might be used to secrete biofilm matrix material. There must be a method for the biofilm material to get through the peptidoglycan layer, and creating a model for this would give a physical explanation for the mechanism *C. perfringens* would need for secretion of large substrates. The reasoning and process behind how *C. perfringens* can secrete this biofilm in the first place is not well understood. However ,we hypothesized that there might be T4P proteins involved in secretion of biofilm matrix material, based on results observed by previous students in the lab and preliminary results performed by the author (S. Kivimaki). After investigating 18 T4P genes, only two, Δ*pilA1* and Δ*CPE2280,* mutants displayed statistically significant values that were lower than the WT. It is interesting that Δ*pilA1* was the only mutant that had a significant decrease for both the immunoblot and slot blot assays performed. The *pilA1* gene is not transcribed along with the other genes (Soncini *et al.*, 2020), and it is possible that the *pilA1* mutant could have a significant function that is yet to be defined. For the results of CPE0516-HA shown in Figure 2.13, it is interesting how there appears to be a covalently bound, heat stable dimer of CPE0516 formed. This also begs the question, what exactly is this biofilm matrix material made of? Obana et al. showed that the biofilm might not be made of amyloid proteins, as previously thought (Obana *et al.*, 2020). The polymers that compose the biofilm matrix material *C. perfringens* produces appears to be extremely resilient, and it would be interesting to learn more about the exact components and molecular interactions occurring during polymerization and secretion of the biofilm. We haven't proven any specific proteins to be involved in a pilus or T2SS system for this purpose, however, there must be a way they are secreting large proteins through the peptidoglycan. Because we were not able to identify enough proteins to build a functional model, we were not able to yet understand how this polymerization is occurring.

Many of the questions that were posed initially about T2SS were left unresolved based on the results gathered, however, there is much room for continued investigation. There might be other proteins that are involved in secretion that were not identified in this screening examining pilin mutants. Since *C. perfringens* has a competence system, and possesses all of the competence proteins, this could also potentially be involved in secretion. Alternatively, there could be other kinds of secretion methods involved such as a holin, or another method that is yet to be discovered. Perhaps another unidentified set of genes exists that functions to aid in secreting large substrates such as biofilm through the thick peptidoglycan layer *C. perfringens* possesses. One way or another, the bacteria must overcome this barrier to secrete biofilm, however how this occurs is still a mystery which is yet to be solved.

Appendix A: Additional Experiments with Tables and Figures

Introduction

To identify additional proteins that are secreted by T4P, this would help in understanding how *C. perfringens* might be using T4P for secretion, and if there is more than one type of secretion system that exists. Previously the protein VWA was studied for this purpose, however because it is expressed at such low levels further experiments were done to identify better candidates to confirm whether there is more than one type of secretion system involved. To find a protein to confirm if there is more than one type of secretion system, proteins that were secreted only by the wild type HN13 were examined, in addition to three other proteins tested, which were only present in the deletion mutant *pilB2.* Another approach was also attempted by examining the secretome of the *pilB1* operon overexpressed in pKRAH1, however none of the evidence gathered from this study points to a good protein to examine.

Results

Identifying additional proteins secreted by T4P. To prepare the samples shown in Figure A.1, a *pilB2* deletion mutant and the wild type HN13 were grown, and their supernatants were subjected to TCA precipitation. SDS-PAGE was performed to separate the protein by size, and the 3-8% acrylamide gel was stained using Sypro ruby (Fig. A.1). Four of the bands shown in Figure A.1 were analyzed by mass spectrometry for the HN13 and *pilB2* mutant strains.

A band was present in the WT but not the *pilB2* mutant strain. The proteins that are observed in the WT, but not *pilB2* are proteins of interest, indicated by the large band present in the WT secretome (Figure A.1) where the extremely dark band for the WT is indicated by a vertical line. When mass spectrometry was performed for this band, the most abundant protein detected was exo-alpha-sialidase. While many proteins were observed in the mass spectrometry results, sialidase was tested since it was the first enzyme in the list. This was analyzed by doing a sialidase assay (Mi *et al.*, 2018), where the goal of this assay was to test a protein which was only present in the WT, to measure the activity of WT. The *pilB2* deletion mutant was screened for secretion of this enzyme, however, not even the positive control was showing any activity. Therefore, this avenue was not further pursued.

Proteins secreted by a *pilB2* mutant, but not the WT strain. In total, there were three proteins that were secreted by the *pilB2* mutant, but not the WT strain, which were examined based on the mass spectrometry results. Two of the extra proteins in the *pilB2* mutant strain matched using mass spectrometry with (1) pyruvate ferredoxin oxidoreductase and (2) 1 glyceraldehyde – 3 – phosphate dehydrogenase, indicated by the arrows shown in Figure A.1. These enzymes are the most highly expressed metabolic proteins in the whole cell. However, they should be present in the cytoplasm instead of the secretome. While it would be helpful to understand why these proteins are showing up in the secretome as opposed to the cytoplasm, no further assays were pursued to study their activity.

The last band tested using mass spectrometry was the *pilB2* mutant deletion band that was the same size as the band which was discussed in the previous section (present in the WT, but not the *pilB2* mutant). The goal was to test a protein which was only in the *pilB2* mutant, but not present in the WT. When this band was detected by mass spectrometry, the most abundant protein secreted only in the *pilB2* mutant, was the enzyme D-lactate dehydrogenase. The *pilB2* deletion mutant was screened for secretion of this enzyme using a D-LDH assay to monitor the enzymatic activity of D-LDH. There was no activity that could be described even for the wild type; therefore, this assay was not pursued further.

Overexpression of the *pilB1* operon. In addition, a screening was done where the *pilB1* operon was overexpressed in pKRAH1, and a lack of secretion of a large protein was observed as shown in Figure A.2. The question here is, why does overexpression of the *pilB1* operon block secretion of this protein? At the time of these experiments, a proteomics analysis was not available, so an educated guess was made for the extra protein's identity. It was systematically determined that the missing protein was around 192 kDa in size. Based on previous knowledge about *C. perfringens* proteins, the only protein that is transcribed at high levels and matches the size is CPE1281 (190 kDa). A CPE1281-His_6 construct was made to examine in 18 pilin deletion mutants. However, there was no obvious change in the CPE1281 phenotype when immunoblots were performed using a His_6 tag as depicted in Figure A.3. In one western blot in particular (Fig. A.4), the *pilB1* mutant showed reduced extraction of the full-length protein (indicated by the black arrow), and the appearance of a C-terminal fragment in the supernatant. Unfortunately, the *pilB1* mutant did not show this phenotype consistently. Additionally, the band that was missing in Figure A.2 reappeared in later repetitions of this experiment, and therefore the *pilB1* operon may not actually be showing a blockage in secretion. An example of one of these repetitions is shown in Figure A.5. Since this ~192 kDa protein was only absent in one single experiment, and was present in the secretome for many subsequent experiments, it was inferred that this initial result in Figure A.2 could have been artifactual from that specific experiment.

Discussion

After examining the secretome of both the deletion mutant *pilB2* and the wild type for differences, a mass spectrometry experiment was done to identify differentially expressed proteins. For the sialidase found in the WT, but not the *pilB2* mutant, the sialidase assay did not work (Mi *et al.*, 2018). Since the positive control did not generate expected results it is likely the assay was performed incorrectly. For the extra proteins which were found in the *pilB2* mutant which were not present in the WT, pyruvate ferredoxin oxidoreductase and 1-glyceraldehyde – 3 – phosphate dehydrogenase, these were not tested with any assays in these experiments, but there are future studies that could be done. Because these cytoplasmic proteins are showing up outside of the cell, there are two possibilities for how this could be occurring: secretion or lysis. In the analyses of the other protein found only in the *pilB2*

mutant, D-LDH, is hard to say specifically what error caused the enzyme assays to go wrong. This experiment presented technical challenges, including that the kinetics did not turn out as expected for the wild type.

The *pilB1* operon overexpressed in pKRAH1 was observed to have a large protein missing, and at the time of these experiments, mass spectrometry was not available. Therefore, an educated guess was made for the extra protein's identity. The suspected protein CPE1281 was tagged with His_6 to observe its expression in 18 deletion mutants. From this screening none of the deletion mutants had a significant difference in phenotype consistently. In later repetitions of the initial experiment when the *pilB1* operon was overexpressed in pKRAH1 and a band was missing, the band consistently appeared where it was previously missing. Since the result shown in Figure A.2 could not be repeated, it was concluded that the missing band was due to a factor from that single experiment, and that the suspicion for the protein identity turned out not to be correct. For future experiments, if a replicate of Figure A.2 was able to be generated, mass spectrometry could be done on this band to find out its identity. While there could be potential proteins to study that use T4P for secretion of large proteins through the peptidoglycan, none of the evidence gathered from these experiments points to a good one to examine.

For additional figure experiments, the CPE0517-His_6 construct could be performed on western blots, in addition to creating pilin deletion mutants using CPE0517-His_6. The experiments for deleting the chromosomal biofilm operon (*CPE0514-CPE0517*) could be reattempted, as well as modified experiments to mutagenize the genes in the *pilB1* operon.

Experimental procedures

Strains of bacteria and plasmids utilized. The strains of bacteria, in addition to plasmids that were utilized for Appendix A, are itemized in Table A.1. The primers for Appendix A are listed in Table 2.2. For all experiments using *E. coli*, strain DH10B was cultivated using Luria-Bertani (LB) broth (1% sodium chloride and tryptone and 0.5% yeast extract) and incubated for a minimum of six hours at 37°C. Erythromycin (at a concentration of 400 µg/µL) was included in the media inoculated with strains of *E. coli* with the pSM300 plasmid.

When *C. perfringens* strain HN13 was grown, the media used included brain heart infusion (BHI) liquid medium (VWR International), TY liquid medium (made of 2% yeast extract, 3% tryptone, 0.1% sodium thioglycolate), PGY medium (3% glucose, 1% peptone, 0.1% yeast extract), or FTG medium. All growth for *C. perfringens* occurred using an anaerobic chamber (Coy Laboratory Products, Inc.); all incubations occurred at 37°C, under anaerobic conditions (approximately 85% nitrogen, 10% carbon dioxide, and 5% hydrogen). Chloramphenicol (at a concentration of 20 µg/µL) was included in the media inoculated with strains of *C. perfringens* containing pKRAH1. Kanamycin at a concentration of (100 µg/µL) was included in the media inoculated with strains of *E. coli* carrying pCRBluntIITOPO.

In all experiments pertaining to proteins, the strains of *C. perfringens* were prepared inside the anaerobic chamber initially using FTG or TY, and later in BHI medium, and incubated at 37°C. The overnight cultures were grown for a minimum of six hours anaerobically. Using a 1:50 dilution, experimental cultures were inoculated using the overnight cultures. In order to induce the lactose-inducible promoter on pKRAH1 (Hartman *et al.*, 2011), lactose at a concentration of 500 mM was added one hour after inoculation (approximately late in the logarithmic growth phase) to achieve a final concentration of approximately 10 mM using a 1:50 dilution, thereby expressing the proteins of interest. Following the initial induction one hour after inoculation, 500 mM lactose was added using a 1:50 dilution every 30 minutes for five hours. After five hours, the culture samples were pelleted in a centrifuge at 3,500 x *g* to remove the supernatant for TCA precipitation and SDS-PAGE analysis.

Preparation of *pilB2* deletion mutant and HN13 samples for the experiment in Figure A.1. These two strains of *C. perfringens* were prepared inside the anaerobic chamber initially using FTG medium, and incubated at 37°C. The overnight cultures were grown for a minimum of six hours anaerobically. Using a 1:50 dilution, experimental cultures were inoculated using the overnight cultures, and grown until stationary phase. See TCA precipitation below for remaining methods for this experiment.

TCA precipitation of supernatant. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for protein precipitation. A volume of 750 µL of cold 20% TCA/acetone (1:1, v/v, with a final 10% TCA/50% acetone) was added to 750 µL of supernatant per sample. The mixture was placed on ice for 5-10 minutes, and then the mixture was pelleted in a centrifuge (13,300 x *g*, three minutes). The supernatant was then discarded, and the protein precipitate was washed with 800 µL of 80% acetone, followed by centrifugation (13,300 g, three minutes). This wash step was repeated twice. After washing, the protein precipitates were air-dried for approximately four minutes, and then dissolved into sterile Milli-Q water. All protein precipitates were suspended in 8.3 µL of 4x SDS page buffer, followed by 3.7 µL of 100 mM dithiothreitol (DTT) per sample. To adjust the pH to neutral for the TCA precipitation replicates, approximately 0.5 µL -1 µL of 1M Tris base pH 9 was included. These samples were heated at 95°C for 20 minutes.

SDS-PAGE and staining. For protein experiments, centrifugation (3,500 x *g* for five minutes) of culture samples (five mL) was done to separate the pellet fractions and supernatant. The supernatant was stored at -80°C until used. The supernatants were subjected to TCA precipitation (as described above). TCA precipitation protein samples for these experiments were separated using SDS-PAGE and were developed with the fluorescent dye Sypro Ruby. For protein gels, a 3-8% or 10% acrylamide gel was used for these experiments.

Mass spectrometry summary. The supernatant samples subjected to TCA precipitation were subjected to electrophoresis on an SDS-page gel and stained with Coomassie prior to mass spectrometry. Mass spectrometry was used to identify the content of the secretome in the sample based on the mass-to-charge ratio of the proteins.

Sialidase assay summary. Sialidase enzyme activity measurement of supernatant (Mi *et al.*, 2018). A 0.1 mL aliquot of an overnight FTG culture of *pilB2* deletion mutant strain, and HN13 were both transferred to five mL of FTG. Cultures were incubated for five hours at 37°C. After centrifugation for five minutes, 20 µL of supernatant was removed from each culture and added to 20 µL of 0.05 M Tris HCL buffer (pH 7.2) in a microtiter plate. A 20 µL aliquot of substrate (four mM 5-bromo-4-chloro-3-indolyl-α-D-N-acetylneuraminic acid) was added, and the solution was incubated at 37°C for 30 minutes. The absorbance was measured at 620 nm using a Bio-Rad microtiter reader.

Immunoblotting. Proteins for these experiments were also analyzed using immunoblots and slot blots, developed using either immunoblotting method one or immunoblotting method two. For the immunoblot experiments, the proteins were moved onto a polyvinylidene difluoride (PVDF) membrane submerged in cold buffer which contained three mM Na_2CO_3 and 10 mM $NaHCO_3$ with 20% methanol.

Immunoblotting method one. The PVDF membranes were laid into a Snap i.d. 2.0 system (Millipore Sigma). Once set up, a solution containing Tris-buffered saline (TBS) (pH 7.4), 1% Tween 20, and 1% bovine serum albumin was added as a blocker so that the surface of the membrane was submerged. Both primary and secondary antibodies were added to separate aliquots of the solution containing TBS, 1% Tween 20, and 1% BSA. First, the primary antibody solution was left to sit for 10 minutes, followed by washings with TBS- Tween 20. Lastly the secondary antibody solution was left to sit for 10 minutes, followed by washings with TBS- Tween 20. The primary antibodies utilized included His-H8 (1:250 dilution) used for detection of polyhistidine tags (from Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody utilized for these experiments was StarBright700 anti-mouse (Bio-Rad) (1:10,000 dilution). The pictures of the immunoblots were taken using a ChemiDoc MP imaging system (Bio-Rad), to observe the intensity of the fluorescence, and size of proteins.

Immunoblotting method two. Membranes were transferred to a sealed contained and blocked with EveryBlot blocking Buffer (Bio-Rad). Antibodies were diluted with blocking buffer and applied to membranes, followed by a minimum of five washes with Tris-buffered saline (TBS) (pH 7.4) containing 1% Tween 20. The primary antibodies utilized included His-H8 (1:227 dilution) used for detection of polyhistidine tags (from Santa Cruz Biotechnology, Santa Cruz, CA) which was incubated at 4°C overnight. The secondary antibody utilized for these experiments was StarBright700 anti-mouse (Bio-Rad) (1:13,333 dilution), incubated for one hour at room temperature. The pictures of the

immunoblots were taken using a ChemiDoc MP imaging system (Bio-Rad), to observe the intensity of the fluorescence, and size of proteins.

Cloning strategy to create a His_6 tagged version of CPE1281 utilizing PCR

PCR. In order to track the protein using western and slot blots, a His_6 tag was added to CPE1281. This was done using PCR. The primers OSK3 and OSK4 (Table A.2) were designed to flank the coding sequence of *CPE1281*, and introduce the His_6 tag into the amplified product of *CPE1281* respectively. One PCR reactions was performed to acquire a product the same size as *CPE1281* but with a His_6 tag.

Plasmid Construction. The PCR product from the second PCR reaction was used to ligate into pTOPO, and the resulting ligation was transformed into *E. coli* strain DH10B, and plated on LB plates containing kanamycin 100 µg/ml. The positive colonies were regrown, and then plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit, and these plasmid preps were checked through a restriction enzyme digest for the correct insert size. The plasmid pKRAH1 was also digested with the same restriction enzymes, and the insert for *CPE1281*-His_6 tag was ligated into pKRAH1. The ligation of *CPE1281* with a His_6 tag in pKRAH1 was also transformed into *C. perfringens*, and was plated on BHI plates containing chloramphenicol (20 µg/ml). The colonies on this initial BHI chloramphenicol (20 µg/ml) plate were not isolated, so the initial colonies were struck out again on a fresh BHI chloramphenicol (20 µg/ml) plate to achieve isolation. After growth in liquid PGY with chloramphenicol (20 µg/ml), a frozen stock was prepared, and plasmids were then purified using a GeneJET Plasmid Miniprep Kit. The frozen stocks were used to inoculate overnight cultures. The overnight cultures were used to inoculate experimental cultures using a 1:50 dilution. The pKRAH1 plasmid- based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. A protein gel was performed using these CPE1281 His_6 tagged TCA precipitation samples, and was stained using Sypro Ruby. This was followed by a western blot to see which sample was producing matrix material. The clone producing matrix material was grown, and the plasmids were purified using a GeneJET Plasmid Miniprep Kit. These purified plasmids were transformed into 18 pilin deletion mutants by electroporation. Colonies for each mutant were grown in PGY and chloramphenicol (20 µg/ml) for frozen stocks. After growing the frozen stock in BHI and chloramphenicol (20 µg/ml), the pKRAH1 plasmid- based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. Western blots were performed using these TCA precipitation samples to see if there were any changes in phenotype.

Tables and Figures for Appendix A

Table A.1. Strains of bacteria in addition to the plasmids for experiments in Appendix A		
Strain or plasmid	Relevant characteristics	Reference or source
Clostridium perfringens		
type A strain HN13	Δ*galKT*	(Nariya *et al.*, 2011)
type A strain HN13 Δ*pilB2*	Δ*galKT*	A. Hartman
type A strain HN13 pSM399	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed	S. Melville
type A strain HN13 pSK3	Δ*galKT*, pKRAH1 with *CPE0514-517*, His_6 tag on CPE0517	This study
type A strain HN13 pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 *Δpil A1*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilA2*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilA3*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilA4*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilB1*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilB2*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilB1B2*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study

type A strain HN13 Δ*pilC1*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilC2*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilD*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilM*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilN*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilO*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*pilT*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*CPE1841*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*CPE2277*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*CPE2279*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
type A strain HN13 Δ*CPE2280*pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
Escherichia coli		
strain DH10B	F− mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL nupG λ−	(Grant *et al.*, 1990)

strain DH10B pSK5	F− mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu)7697 galU galK rpsL nupG λ− His_6 tag on CPE1281	This study
Plasmids		
pSM300	A suicide plasmid for insertional mutagenesis in C. perfringens carrying an erythromycin resistance gene	(Varga *et al.*, 2004)
pCM-GalK	Vector used for making in-frame deletions in C. perfringens strain HN13.	(Nariya *et al.*, 2011)
pSM399	Δ*galKT*, pKRAH1 with *pilB1* operon under control of a lactose-inducible promoter	Stephen Melville
pSK3	Δ*galKT*, pKRAH1 with *CPE0514-517*, His_6 tag on CPE0517	This study
pSK5	Δ*galKT*, pKRAH1 with *pilB1* operon overexpressed, His_6 tag on CPE1281	This study
pKRAH1	Cm^r; lactose-inducible expression system plasmid for use in *C. perfringens*	(Hartman *et al.*, 2011)
pCRBluntITOPO	PCR cloning vector	Invitrogen

Table A.2. Primers used in experimental procedures for Appendix A	
Primer Name	**Sequence (5'- 3')**
OSRM8	5'-CATTTATAACCTTTATTTCATAGTAAGTATCCCTAGAAAGTCCAC-3'
OSK3	5' – CGTCGACATCAGGAGGAGAGAAAAAATGAATAAGAGAAAAATAGCAGCT ATAA 3' (Sal1 site)
OSK4	5'CGGATCCTTAATGATGATGATGATGATGTACGTTAAATAAATAGAACAT TACTCCACTAAAACC 3' (BamHI site)
OSK5	5' GGA TCC TTA ATG ATG ATG ATG ATG ATG ATT TAA TTT TAA TAT ACC AAA ATC ATT AAT TTT TTC TTT ATT ATC ATA GAA GTA TTT TTC 3' (BamHI site)
OSK10	5' - GGG GGG TCG ACT ATG AAT TAA AAG TTG AAG GAG AAA TAA G -3' (Sal1 site)
OSK11	5' - GCT AAT AAT AAA TGC AAT TAT TTT CAT TAA ATT TAC AAG ACT ATA CAA CTA AAT ACA TTT CTG CAT CCA ACA AGT AGT TTA ATT CTT TCT C – 3'
OSK12	5' - GAG AAA GAA TTA AAC TAC TTG TTG GAT GCA GAA ATG TAT TTA GTT GTA TAG TCT TGT AAA TTT AAT GAA AAT AAT TGC ATT TAT TAT TAG C - 3 '
OSK13	5' - CTC TTC CTG CAG AAC CTT ATC AAC TAA AGA TGA AAT TAC ATT AAG TAA – 3' (Pst1 site)
OSK14	5' - GGA TCC ATA TAT AGG CCT TTA ATC AAT TTC TTT TGC TAC CAC – 3' (BamHI site)
OSK15	5' - CTT TAA AGT CAT AAA ACA AAA ATA AAA AGT TTT CCA TCT TAA TTT AAT TTT TTC ATA ATT ATT CCT TTT CTT CCT TTT CAA CTT TAC AG – 3'
OSK16	5' CTG TAA AGT TGA AAA GGA AGA AAA GGA ATA ATT ATG AAA AAA TTA AAT TAA GAT GGA AAA CTT TTT ATT TTT GTT TTA TGA CTT TAA AG -3'
OSK17	5' - GTC GAC ATA TTA CAT AAA ATA ATT CTA TAA CAT TCC GTA CAT ACA TAA AAT TAC ATA TCG CC -3' (Sal1 site)
OSK18	5'- GGATCCGAAGACTATATTGTAGTTAAGTTTTGGAGAAAGGG -3' (BamHI site)
OSK21	5' GTCGACGTTGAAGAATTTGATGTGGTAGAAAAGAAGAAC -3' (Sal1 site)

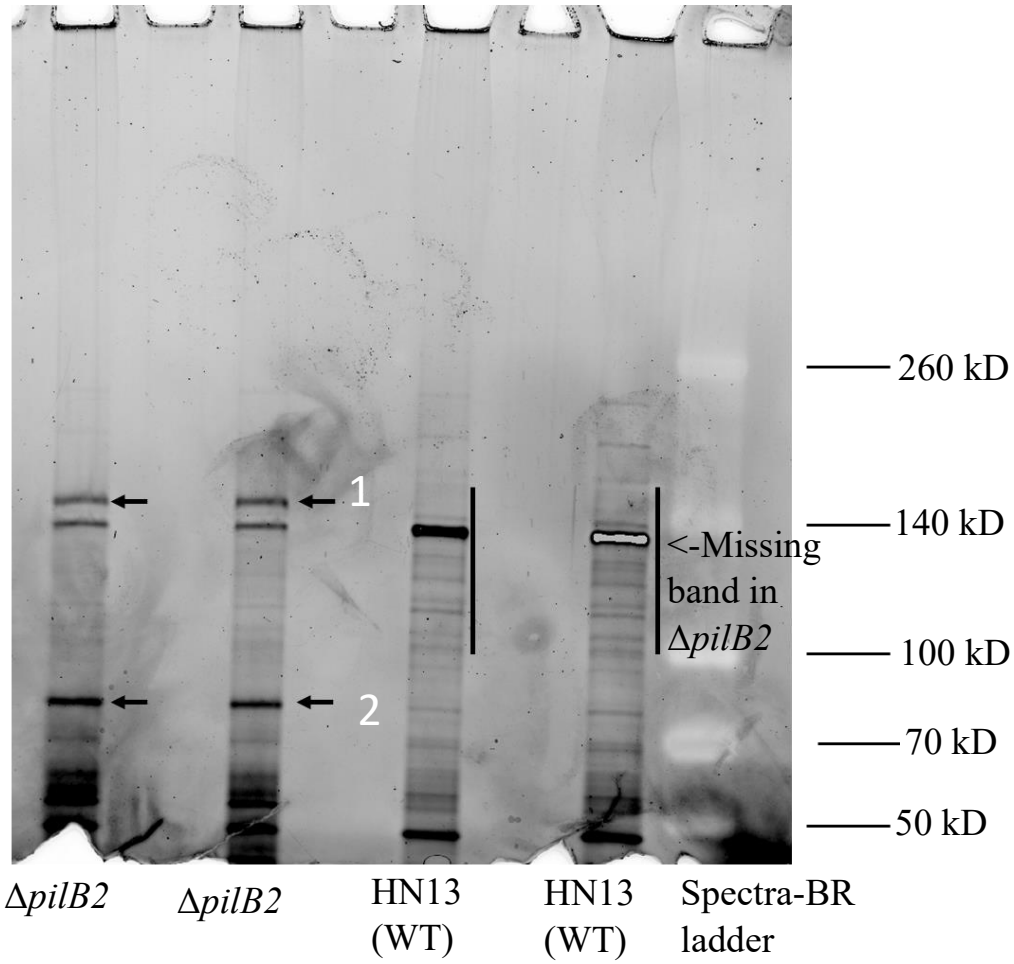


Figure A.1. Secretome of Δ*pilB2* mutant and WT. The arrows indicate additional proteins in Δ*pilB2*. Arrow 1 indicates pyruvate ferredoxin oxidoreductase and arrow 2 indicates 1-glyceraldehyde – 3 – phosphate dehydrogenase. The vertical line shows a missing band in Δ*pilB2*, which was found to be exo-alpha-sialidase according to the mass spectrometry analysis. Samples of a *pilB2* deletion strain and HN13 (WT) were subjected to TCA precipitation of the supernatants.

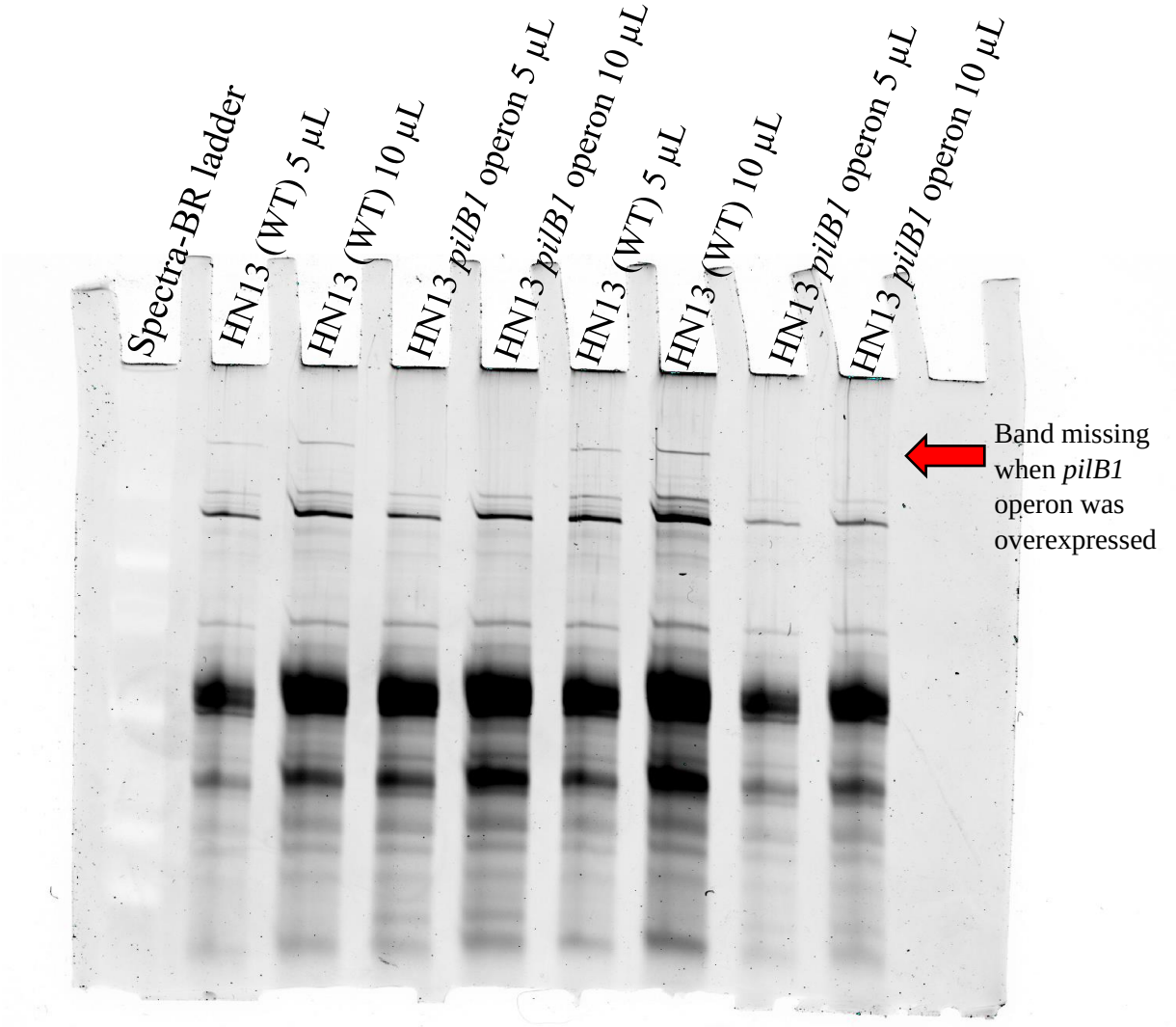


Figure A.2. Lack of secretion when the *pilB1* operon is overexpressed. Overexpression of the *pilB1* operon using plasmid pSM399, compared to the wild type HN13. Note the absence of a band at ~192 kD when the *pilB1* operon was overexpressed, as indicated by

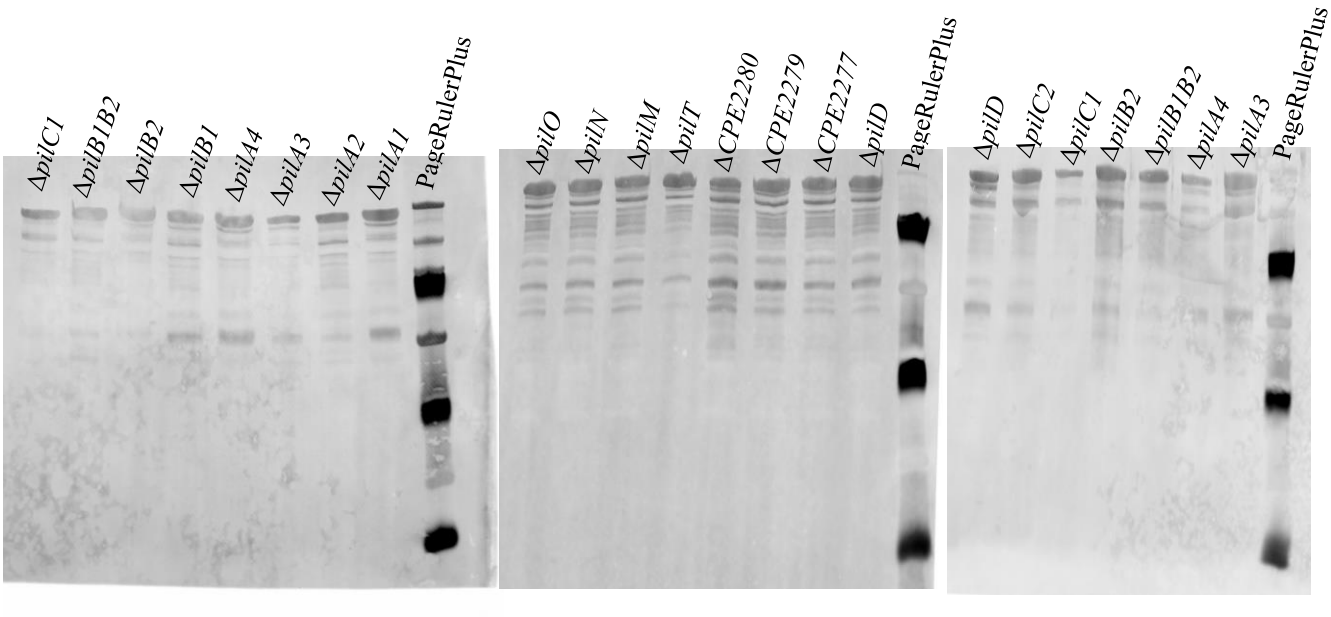


Figure A.3. Secretion of CPE1281-His_6 in T4P deletion mutants. Western blots of CPE1281-His_6 in various pilin deletion mutant strains. All strains have the pSK5 plasmid. From this screening, there was not a significant difference in the secretion levels of CPE1281 (~190 kD sized protein) in TFP-associated mutant strains.

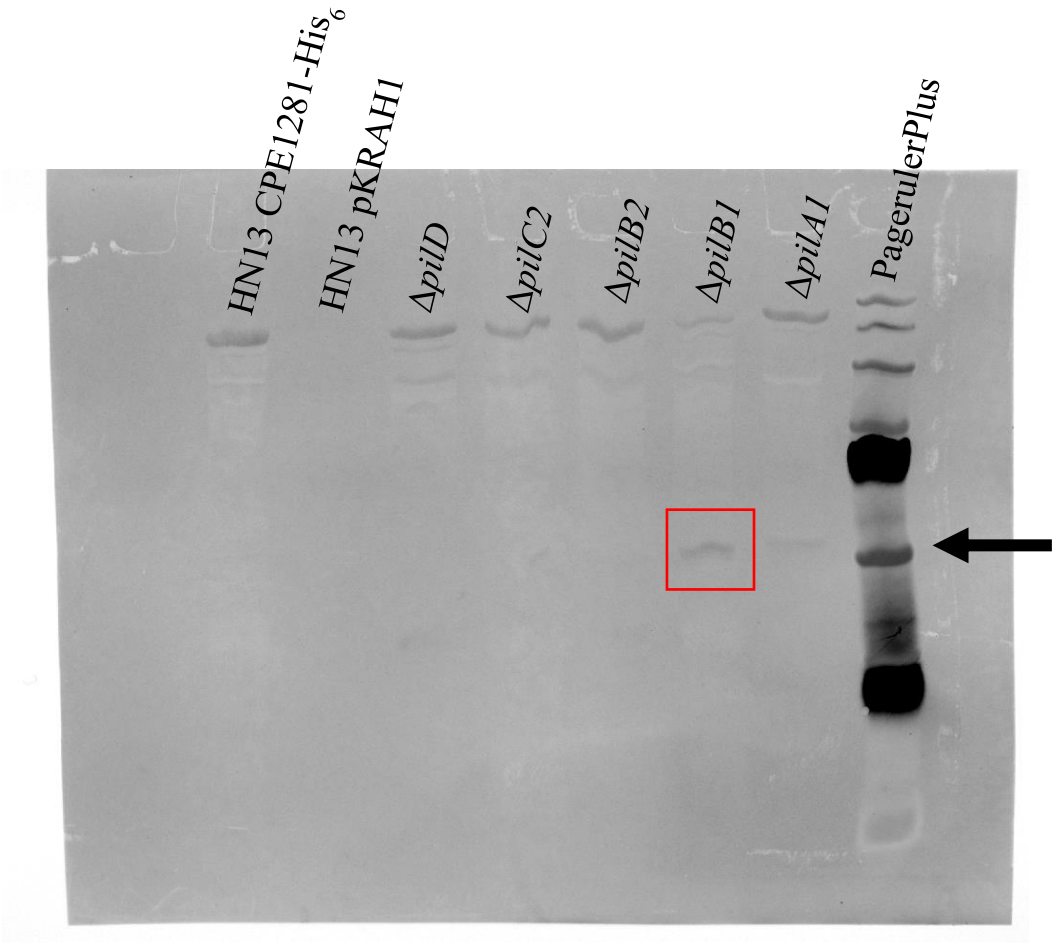


Figure A.4. Altered phenotype for Δ*pilB1* mutant for secretion of CPE1281-His_6.Western blot of CPE1281- His_6 in five pilin deletion mutant strains. All strains have the pSK5 plasmid. The black arrow and red box indicates a reduced extraction of the full-length protein for the Δ*pilB1* mutant.

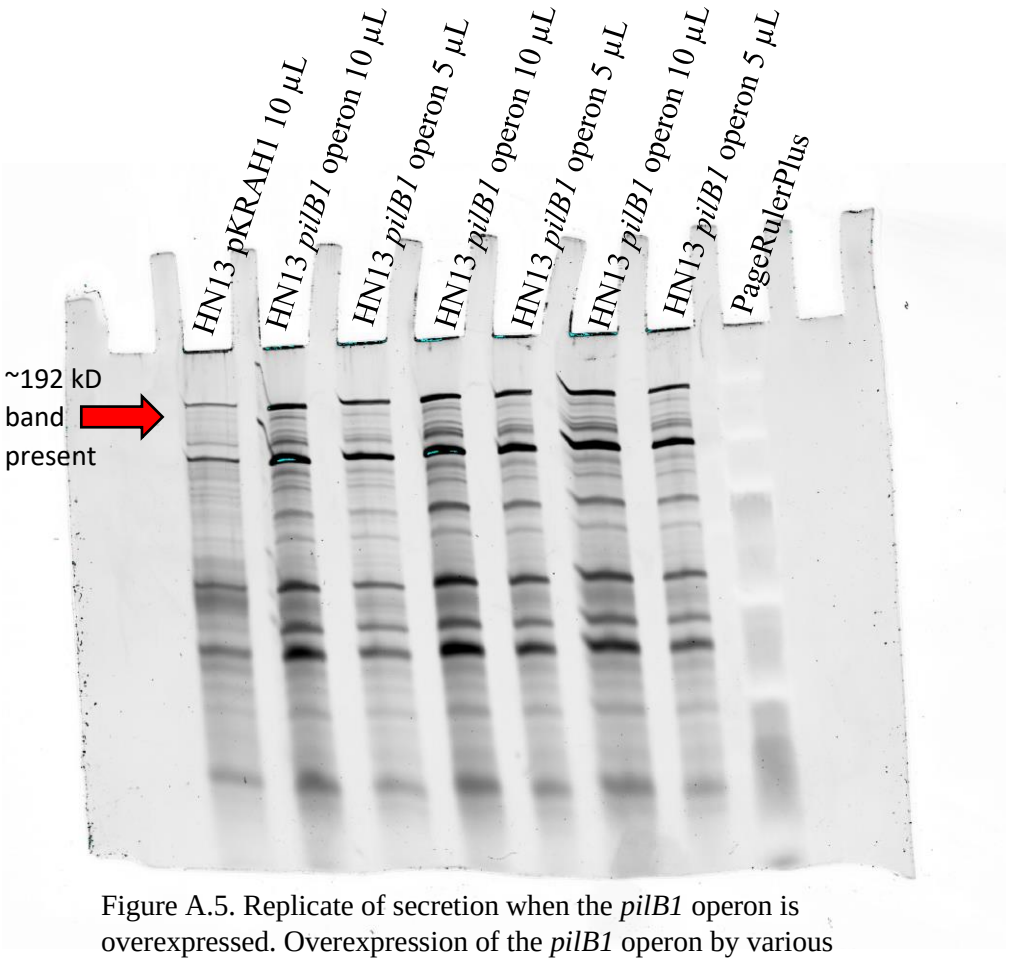


Figure A.5. Replicate of secretion when the *pilB1* operon is overexpressed. Overexpression of the *pilB1* operon by various colonies, compared to HN13. The strain where the *pilB1* operon is overexpressed has the pSM399 plasmid. The negative control HN13 pKRAH1 has an empty vector. The red arrow indicates that the band which was missing in Figure A.2 is consistently present in later replicates such as this one.

Appendix B: Supplementary Experiments

Introduction

There were also additional experiments performed which were designed with the purpose of identifying T4P proteins needed for biofilm secretion, but they did not generate any meaningful results to add to Chapter 2. Separately, a number of additional other experimental procedures were performed, although the results are not shown. The magnesium chloride precipitation experiments were done to purify the biofilm matrix material, however because the biofilm matrix material would not fractionate optimally this method was not pursued. The CPE0517-His_6 construct was made, but western blots were inconclusive. Efforts were made to delete the chromosomal biofilm operon (*CPE0514-CPE0517*). Because the biofilm operon is being expressed on a plasmid, deleting the chromosomal copy would not only eliminate potential competition with the *pilB1* operon, it also would allow us to understand how the WT copy might be interacting with the FLAG-tag version. Lastly, to check for function of the *pilB1* operon, the five genes at the end of the *pilB1* operon were examined, and a deletion of all five of these genes was attempted. Since all the *CPE1836-CPE1840* deletion colonies ended up having WT phenotypes, a strategy was designed to use the pSM300 suicide plasmid and then mutate the *CPE1840* gene to create transcription block, theoretically resulting in a polar effect. Unfortunately, no correct inserts were ever detected. Since the experiments for examining the *pilB1* operon never came to fruition, this was not pursued.

Experimental Procedures

Magnesium chloride and salt precipitation of matrix material. The entire biofilm operon was expressed in HN13 and secretion was induced using the pKRAH1 plasmid. The pKRAH1 plasmid-based promoter system was used to deliver regulated expression as lactose was added continuously until the cells grew to stationary phase, and was subjected to centrifugation at 10,000 x *g* for 10 minutes at 4°C. The supernatant (S1) was removed subjected to magnesium precipitation, and the cell pellet (P1) was set aside for later treatment.

For the magnesium precipitation of supernatant (S1): One hundred mM $MgCl_2$ and 150 mM NaCl were added to this supernatant (S1), and this solution was incubated on ice, then refrigerated overnight. This solution incubated overnight (S1 treated) was pelleted in a centrifuge at 10,000 x *g* for 10 minutes at 4°C, into supernatant (S1-MTS2) and magnesium precipitate (S1-MP2). The supernatant (S1-MTS2) was removed and subjected to TCA precipitation. The magnesium precipitate (S1-MP2) was resuspended in 1x Dulbecco's Phosphate Buffered Saline (DPBS) without calcium or magnesium (BioWhittaker). S1-MP2 suspended in DPBS was put into snakeskin dialysis tubing

, was floated in diluted DPBS for two hours, the buffer was changed, then this solution was refrigerated overnight. An aliquot of the treated pellet (P2) solution was removed, then 8.3 µL of 4x SDS

sample buffer and 3.7 µL of DTT were added to each sample, followed by heating at 95°C for 20 minutes. Both the supernatant (S1-MTS2) and magnesium precipitate (S1-MP2) samples were screened using a protein gel stained with the fluorescent dye Sypro ruby (data not shown).

The cell pellet (P1) that was set aside after the initial centrifugation at 10,000 x *g* for 10 minutes at 4°C was resuspended in DBPS. This solution was then pelleted in a centrifuge at 20,000 x *g* for 10 minutes at 4°C, into supernatant (P1-MS2) and the cell pellet (P1-BP2) was discarded. The supernatant (P1-MS2) was removed from this centrifugation, and 100 mM $MgCl_2$ and 150 mM NaCl were added to this supernatant, then refrigerated overnight. After the supernatant (P1-MS2) was subjected to magnesium precipitation, the solution was pelleted in a centrifuge at 20,000 x *g* for 10 minutes at 4°C, into the supernatant (P1-MS2-TS3) and the magnesium precipitate (P1-MS2-BP3). The supernatant (P1-MS2-TS3) was subjected to TCA precipitation. The magnesium precipitate (P1-MS2-BP3) was resuspended in DBPS, put into snakeskin dialysis tubing, was floated in diluted DPBS for two hours, the buffer was changed, then this solution was refrigerated overnight. An aliquot of the treated magnesium precipitate (P1-MS2-BP3) was removed, then 8.3 µL of 4x SDS page and 3.7 µL of DTT were added to each sample, followed by heating at 95°C for 20 minutes. The supernatant (P1-MS2-TS3) and the magnesium precipitate P1-MS2-BP3 samples were screened using a protein gel stained with the fluorescent dye Sypro ruby (data not shown).

Additional Constructs, Cloning and Mutagenesis for Appendix B

Cloning Strategy to Create His_6 Tagged Version of CPE0517 utilizing PCR

PCR. PCR was performed for CPE0517 – a His_6-tagged version of CPE0517 was made, to track the protein using western blots. The primers OHL145 (Table 2.2) and OSK5 (Table A.2) were designed to flank the coding sequence of *CPE0517* and introduce the His_6 tag into the amplified product of *CPE0517* respectively. One PCR reactions was performed to acquire a product the same size as CPE0517, but with a His_6 tag. This was initially done using GoTaq PCR, followed by an amplification using Phusion PCR, recognizing the potential for sequence errors.

Plasmid construction. The PCR product from the overlapping PCR reaction was used to ligate into pCRBluntITOPO, and the resulting ligation was transformed into *E. coli* strain DH10B, and plated on LB plates containing kanamycin (100 µg/ml). The positive colonies were regrown, and then plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit, and these plasmids were checked through a restriction enzyme digest using BamHI and Pst1 for the correct insert size. The insert and the plasmid were observed at the same size of around 4.5 kb, where the plasmid pCRBluntITOPO would normally be seen around 3.5 kb. The presence of the 4.5 kb insert was also confirmed with a digest using the restriction enzyme EcoRI. The plasmid pKRAH1 was also digested with the same restriction enzymes, and the insert for CPE0517- His_6 was ligated into pKRAH1. The ligation of CPE0517 with a His_6 tag in

pKRAH1 was transformed into *C. perfringens*, and was plated on BHI plates containing chloramphenicol (20 µg/ml). After growing in liquid BHI with chloramphenicol (20 µg/ml), a frozen stock was prepared. The frozen stocks were used to inoculate overnight cultures. The overnight cultures were used to inoculate experimental cultures using a 1:50 dilution. The pKRAH1 plasmid- based promoter system was used to induce secretion to deliver regulated expression by adding lactose continuously until the cells grew to stationary phase. After centrifugation (3,500 x *g*, five minutes), the supernatant was removed for TCA protein precipitation. A western blot was performed using these CPE0517 His_6 tagged TCA precipitation samples, to see which sample was producing matrix material. The clone producing matrix material was grown, and the plasmids were purified using a GeneJET Plasmid Miniprep Kit.

Method to delete the Chromosomal biofilm operon (*CPE0514-CPE0517*)

Overlapping PCR. To remove background levels of secretion, a deletion of the chromosomal biofilm operon (*CPE0514-CPE0517*) was attempted so that this digested PCR product could be cloned into the suicide plasmid pCM-GalK. This was done using overlapping PCR, and by keeping three codons of each gene at both the 5' and 3' ends, leaving six codons in place for in-frame deletions. The primers OSK14, OSK15, OSK16, and OSK17 (Table A.2) were designed for this purpose. The primers OSK14 and OSK17 were designed 1 kbp upstream/downstream of the genes, and primers OSK15 and OSK16 were designed to prime at the end of genes *CPE0514* and *CPE0517*, such that the initial PCR reaction would yield two individual one kbp sized fragments on either side of those four genes in the chromosomal biofilm operon (*CPE0514-CPE0517*). The overlapping PCR reaction with the correct deletion yielded a product approximately two kbp in size.

Plasmid construction and screening. The PCR product was cut with Sal1 and BamHI and ligated to pCM-GalK cut with the same enzymes. It was unclear whether they were the correct sized inserts due to the presence of an extra, large PCR product approximately five kbp band that was undefined but was not used for cloning. An electroporation was done to transform the approximately 2 kbp sized inserts into *C. perfringens*, and the cells were then grown in galactose (10 µL of ON and 200 µL 30% galactose added to two mL TY), and then plated on a TY galactose 3% plate. From this TY galactose 3% plate 25 colonies were picked and then grown on both BHI with chloramphenicol (20 µg/ml), and a new TY galactose 3% plate to select for colonies which were chloramphenicol sensitive. To screen for colonies which potentially had the correct inserts, a GoTaq colony PCR was done, but only wild type constructs were recovered.

Method to delete *CPE1836-CPE1840* using overlapping PCR to check for function.

Overlapping PCR. A deletion the five genes at the end of the *pilB1* operon was attempted, so that this digested PCR product could be cloned into the suicide plasmid pCM-GalK. This was done using

overlapping PCR, and by keeping three codons of each gene at both the 5' and 3' ends, leaving six codons in place for in-frame deletions. The primers OSK10, OSK11, OSK12, and OSK13 (Table A.2) were designed for this purpose. The primers OSK10 and OSK13 were designed one kbp upstream/downstream of the genes respectively, and primers OSK11 and OSK12 were designed to prime at the end of genes *CPE1836* and *CPE1840*, such that the initial PCR reaction would yield two individual one kbp fragments on either side of those five genes in the *pilB1* operon. The overlapping PCR reaction with the correct deletion yielded a product approximately two kbp in size.

Plasmid construction and screening. This overlapping PCR product was cut with Sal1 and Pst1 and was successfully ligated to pCM-GalK cut with the same enzymes, however it was unclear whether they were the correct sized inserts due to the presence of other larger bands. Using the approximately two kb sized inserts, a transformation into *C. perfringens* was done, and the cells were then grown in galactose (10 uL of ON and 200 uL 30% galactose added to two mL TY), and then plated on a TY gal 3% plate. From this TY Gal 3% plate 25 colonies were picked and then grown on both BHI Cm^r20 and a new TY gal 3% plate to select for colonies which were chloramphenicol sensitive. To screen for colonies which potentially had the correct inserts, a GoTaq colony PCR was done, however none of the inserts were the expected size of approximately two kb. To exclude WT clones more effectively, the primer OSRM8 was utilized for GoTaq PCR screening, since OSRM8 (Table A.2) anneals in the middle of *CPE1839*. By using OSRM8 in combination with OSK10, this would yield a band approximately 1.5 kbp in size, indicating wild type samples. At that point all the samples tested ended up being wild type. From there, it was decided that primers needed to be designed to instead amplify the middle of the *CPE1840* gene. Using the restriction sites in pSM300, a strategy was designed for this.

Experiment to create an insertion in *CPE1840*

PCR. Since none of the *CPE1836-CPE1840* deletion colonies ended up being mutants, it was decided that primers needed to be designed to instead amplify the middle of the *CPE1840* gene. Using the restriction sites in pSM300, a strategy was designed to use the pSM300 suicide plasmid and then mutate the *CPE1840* gene to create transcription block, resulting in a polar effect. Primers OSK18 and OSK21 (Table A.2) were designed for this purpose, and the resulting PCR fragment of this insertion into *CPE1840* was approximately 250 bp in size. After successfully getting the correct sized PCR fragment, pSM300 was prepared for cloning by cutting with the restriction enzymes BamHI and Sal1. The expected size of the fragment for pSM300 is approximately 2.7 kbp, which was cut out and purified.

Plasmid construction. The ~250 bp *CPE1840* PCR product with an insertion was cut with Sal1 and BamHI, and ligated to pSM300 cut with the same enzymes. This was transformed into *E. coli* strain DH10B and plated on LB plates containing erythromycin (400 µg /mL). This resulted in individual colonies which were overgrown initially. After being struck out, the individual colonies were checked for

inserts using the restriction enzymes Sal1 and BamHI, and additionally using a triple digest with Sal1, BamHI, and Pst1. For both digests there did not appear to be any inserts present.

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