Characterizing the Secretion and Function of TcfA: A Unique Autotransporter and Virulence Factor in *Bordetella pertussis*

by

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Abstract

Autotransporters (ATs) are an important family of proteins that are essential for the virulence of a variety of Gram-negative bacteria. The vast majority of ATs possess a classical right handed β-helical structure which facilitates the vectorial secretion of the protein. However, not all ATs possess this classical structure. Tracheal colonization factor (TcfA) of *B. pertussis* is one of only a few ATs that are predicted to be relatively unstructured or to possess a coil structure. It is not known what factors are important for the secretion of non- β-helical ATs. This study sought to characterize the secretion of TcfA which could also reveal more broadly applicable requirements for the secretion of ATs and other surface exposed proteins. This thesis characterized the secretion of TcfA both in *E. coli* as well as in *B. pertussis*. The study determined that TcfA has special secretion requirements that are not met when the protein is expressed in *E. coli*. The unique *B. pertussis* chaperone Par27 was identified as an important factor for secretion via both its disruption in *B. pertussis* as well as via its insertion in *E. coli*. However, it was also determined that there are additional factors that *E. coli* is lacking that are important for the secretion of TcfA. The study also sought to characterize potential virulence functions of TcfA. It is known that TcfA contributes to the pathogenesis of *B. pertussis*, but its specific role remains to be elucidated. This study used modeling to provide support for the theory that TcfA binds Factor H in *B. pertussis*. However, a factor H surface binding assay determined that TcfA is not the only factor that binds the complement regulatory protein Factor H in *B. pertussis*. Another uniquely structured AT, BapB, was hypothesized as the potential additional factor that binds Factor H. However,

additional studies are required to determine the importance of BapB. Furthermore, the study determined that TcfA does not play a large role in the serum survival of *B. pertussis*. In summary, this thesis characterized the secretion and some potential virulence functions of TcfA, but it also raised many additional questions.

Lay Summary

The thesis aims to increase our knowledge of the respiratory pathogen *Bordetella pertussis*, and to increase our understanding of how microorganisms secrete proteins. *B. pertussis* is the causative agent of whooping cough which is a highly contagious respiratory disease. A vaccine for whooping cough does exist, but it does not grant lifelong immunity. This thesis worked to understand how one of the virulence factors of *B. pertussis* contributes to disease. Increasing our understanding of how the virulence factors of *B. pertussis* function could lead to improved vaccines as well as improved treatment options for *B. pertussis* infections. The focus of this thesis is TcfA. TcfA is a uniquely structured protein, and its mechanism of secretion is not known. In addition to looking at function, this thesis also worked to characterize the secretion of TcfA in order to increase our understanding of the requirements for the secretion of bacterial proteins.

Preface

I designed and performed all of the relevant experiments for this thesis. I also analyzed all of the research data.

Shengjuan Xu provided the pBAD24 full-length TcfA clone which was used in Figures 9-14 as well as the *E. coli* chaperone knockout strains used in Figure 14.

The research was conducted under the following biohazard approval certificates issued by the Biosafety Committee at the Office of Research Services at UBC: B12-0002, B12-0004, B16-0006 and B16-0007.

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List of Abbreviations

ACT	adenylate cyclase toxin
AT	autotransporter
BG agar	Bordet-Gengou agar
BrkA	*Bordetella* resistance to killing
HI-NHS	heat-inactivated normal human serum
IM	inner membrane
MAC	membrane attack complex
MASPs	MBL-associated serine proteases
MBL	mannose-binding lectin
MOMP	major outer membrane porin of B. pertussis
NHS	normal human serum
OM	outer membrane
PAMPs	pathogen associated molecular patterns
PPiA	peptidylprolyl isomerase A
PPiD	peptidylprolyl isomerase D
Ptx	pertussis toxin
TAA	trimeric autotransporter adhesins
TCA	trichloroacetic acid
TcfA	tracheal colonization factor
Tps	two-partner secretion systems
WT	wild type

Acknowledgements

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Chapter 1: Introduction

1.1 *Bordetella pertussis*

Bordetella pertussis is a Gram-negative bacterium which is the causative agent of the infectious respiratory disease known as whooping cough or pertussis. Pertussis must be transferred directly from human to human because it does not have an environmental reservoir [2]. Pertussis is most deadly in children because they have not had a chance to build up any immunity, and their small airways are easily constricted. In the most extreme cases, pertussis causes fits of rapid coughs, vomiting, exhaustion and can ultimately result in death. It is difficult to estimate the total burden of pertussis, but there are approximately 48.5 million yearly cases of pertussis worldwide and about 295,000 deaths [3]. Currently two vaccines for pertussis exist, but both have significant drawbacks.

Both a whole-cell pertussis vaccine and an acellular pertussis vaccine are currently used to prevent disease. The whole-cell vaccine is the original vaccine which is used predominantly in the developing world. The whole-cell vaccine consists of inactivated suspensions of the entire microorganism. Despite resulting in long-lasting immunity, the whole-cell vaccine has reactogenicity issues which can result in extremely high fevers [4]. Due to these reactogenicity issues, the acellular pertussis vaccine was developed and is currently used in most of the developed world. The acellular pertussis vaccine consists of specific antigens purified from *B. pertussis* strains. While the acellular vaccine avoids the reactogenicity issues of the whole-cell vaccine, it has some shortcomings. The acellular vaccine protects against the disease but it does not grant lifelong immunity, and requires frequent boosters to maintain efficacy [5]. Furthermore, a recent study in a baboon model showed that the acellular pertussis vaccine protects against disease, but not transmission or colonization [6]. Unsurprisingly, the current

immunization programs have not been successful at preventing pertussis epidemics in the last ten years [7]. The shortcomings of both the whole-cell and acellular pertussis vaccines highlight the need for additional research into the mechanism of *B. pertussis* pathogenesis.

Infection with *B. pertussis* is initiated by the adherence of bacteria to the epithelium of the nasopharynx and trachea [8]. The adherent bacteria are capable of surviving innate host defenses and can therefore multiply locally. Seven to 14 days after adherence, pertussis progresses to what is known as the paroxysmal phase which is characterized by coughing fits followed by inspiratory whoops. In most cases, the convalescent phase follows in which adaptive immunity eventually clears the bacteria. However, symptoms frequently take a month or longer to subside after bacterial clearance [8]. In infants, an extreme course of pertussis can occur where the bacteria disseminates into the lungs to cause necrotizing bronchiolitis, intraalveolar haemorrhage and fibrinous edema[8]. In the most severe cases, extreme lymphocytosis occurs, resulting in respiratory failure and death. *B. pertussis* virulence factors play essential roles throughout the course of infection.

B. pertussis has evolved a variety of virulence factors that enable it to adhere, persist and replicate in its host. All of the protein virulence factors are under control of the BvgAS two component system. BvgA is the response regulator protein and BvgS is the sensor kinase[9]. While inhabiting a host, the transcription of virulence factors is active. However, under laboratory conditions, *B. pertussis* can be grown in the presence of magnesium sulfate or nicotinic acid to repress the Bvg system [10]. The adhesins are the virulence factors that are responsible for the initial binding of *B. pertussis* to the respiratory tract.

FHA and Fim are the two major adhesins which facilitate the direct binding of *B. pertussis* to the epithelial cells of the respiratory tract. The deletion of FHA from *B. pertussis*

almost completely abrogates cell binding [11]. The deletion of the major or minor subunits of Fim results in a greater than 50% reduction in adherence in multiple adhesion models [12, 13]. Although FHA and Fim are the principle adhesins in *B. pertussis*, they are not the only adhesins present. Several other virulence factors including Prn, BrkA and TcfA have been implicated in *B. pertussis* adherence [14-16].

B. pertussis also produces several toxins that are important for both adherence and persistence. Adenylate cyclase toxin (ACT) supports *B. pertussis* adhesion by inducing cytotoxicity that leads to the exposure of cryptic receptors on the basement membrane [17]. Pertussis toxin (Ptx), which is unique to *B. pertussis*, was originally thought to be an adhesin. However, further studies showed that adherence of a Ptx deletion mutant strain to nonciliated human bronchial and laryngeal cells was not attenuated [12]. It is now believed that Ptx facilitates adhesin-driven binding by modulating the host's immune responses [18]. ACT, Ptx and tracheal cytotoxin play additional roles in modulating the host immune response[10]. Additionally, *B. pertussis* secretes several proteins from the class of autotransporters (ATs) which play multiple roles in pathogenesis including serum resistance [8].

B. pertussis uses several different strategies to avoid complemented-mediated removal from the respiratory tract. The complement system will be discussed in detail later in this introduction, but it consists of three terminal effector pathways which work together to protect the host from pathogenic invasions[1]. As a result, pathogenic microorganisms such as *B. pertussis* have evolved ways to dampen the effect of the complement system. For example, the AT protein *Bordetella* resistance to killing A (BrkA) inhibits complement-induced phagocytosis. In addition to preventing direct cell lysis, BrkA also interferes with the deposition of C2a, C3b, C4b and C5b on the C1 complex which prevents downstream phagocytosis and killing by

neutrophils [19]. *B. pertussis* also recruits and binds C4b-binding protein which inhibits the classical complement pathway, but this binding has been shown to not have an effect on serum killing of *B. pertussis* [20]. The *Bordetella* AT Vag8 is also important for serum resistance. Vag8 binds C1 esterase inhibitor which interferes with the formation of the C1 complex of the classical pathway. It is not known if other *B. pertussis* virulence factors play roles in inhibiting the complement system.

1.2 Protein secretion in gram-negative bacteria

Protein secretion is essential for the ability of bacteria to survive, acquire nutrients, and communicate with other microorganisms and of course for virulence. In order for a protein to be secreted by Gram-negative bacteria, the protein must traverse two different membranes prior to ultimately being secreted into the extracellular space. The first membrane that must be traversed is the inner membrane (IM) which borders the cytoplasm and the periplasm. The second membrane that must be traversed is the outer membrane (OM) which borders the periplasm and the extracellular space. Gram-negative bacteria have evolved a variety of specialized secretion systems for secreting proteins across these two membranes. Currently, at least 6 different secretion systems have been characterized in gram-negative bacteria. These secretion systems are named Type I through Type VI, and each system has unique characteristics and specific factors that aid in secretion.

The most relevant secretion system for this project is the Type V secretion system. Each of the type V systems is a different variation on a homologous secretion mechanism. Type Va systems consist of the classical ATs. This system consists of an N-terminal passenger domain and a C-terminal translocation unit. In the type Va systems, the translocation unit forms a pore through which the N-terminus is trafficked to the cell surface. After being exported to the

surface, the N-terminus is either cleaved and released into the extracellular milieu or remains attached to the cell surface. The type Va system will be discussed in detail later in this thesis because it is believed to be the mechanism for TcfA secretion, but understanding the other type V secretion systems could be essential for deciphering the nuances of the secretion of TcfA.

Type Vb systems are known as the two-partner secretion (Tps) systems. Unlike classical ATs, in Tps systems the passenger domain and translocation domain are two distinct protein chains but are expressed in the same operon. The translocator proteins are known as TpsB proteins and the transported proteins are known as TpsA proteins. The TpsA frequently remains on the cell surface attached to the TpsB, which led to the classification of these systems as being part of Type V secretion. Type Vc systems are obligate trimmers and virtually all of them are bacterial adhesins which led to their alternative name, trimeric AT adhesins (TAAs). Type Vc systems are unique in that the translocation pore is actually an oligomeric structure. Type Vd systems are similar to type Va systems, except they possess an additional periplasmic domain which connects the C-terminal translocation domain and the N-terminal passenger domain. This additional periplasmic domain is homologous to the type Vb translocation pores. Type Ve systems are known as the inverse autotransporters because the principle domains are switched such that the N-terminal part is the translocation unit and the C-terminal part is the passenger domain. The passenger domains of Type Ve autotransporters possess Ig-like and lectin-like domains that are distinct from the other Type V systems and are more similar to adhesins in gram-positive bacteria. The Type Ve ATs also possess an additional periplasmic domain at the end of the N-terminus that in many cases contains a peptidoglycan-binding motif[21].

1.2.1 Autotransporter secretion

ATs were originally named based on the assumption that everything that was needed for the secretion of these proteins was encoded within the protein itself. Although we now know this is not the case, these proteins do to a large degree facilitate their own secretion. As mentioned before, classical ATs possess a conserved domain consisting of an N-terminal signal peptide, N-terminal passenger domain and C-terminal translocation unit. The signal peptide targets the protein for secretion across the inner membrane via the Sec apparatus. The passenger domain is the functional part of the protein which frequently encodes a virulence factor. The translocation unit facilitates the secretion of the passenger domain through the outer membrane. In the classical model of AT secretion, after the AT is translocated into the periplasm, the protein is targeted to the outer membrane for secretion. It is thought that the translocation unit inserts itself into the outer membrane and creates an opening through which the passenger domain is threaded in a C to N terminal direction. Furthermore, either during or after secretion across the outer membrane, most ATs are cleaved and released into the extracellular space. However, this is not true for all ATs because some remain associated with the outer membrane[22]. This classical model of AT secretion is forever evolving as we learn more about other factors are involved in the secretion of ATs[21]. It is also important to note that the classical model of AT secretion is based off studies on ATs whose passenger domains consist of a right-handed *B*-helical structure because the vast majority of ATs possess this structure. Very little is known about how non- *B*-helical ATs are secreted.

1.2.2 Additional factors that are important for the secretion of ATs

It is now known that the oligomeric protein complex BAM plays an important role in classical AT secretion by assisting in the insertion of the AT translocation unit into the outer

membrane. BamA, a highly conserved component of the BAM complex is also required for AT assembly *in vivo* [23, 24]. More recently, the TAM complex has also been identified as playing a role in the secretion of some ATs. TamA and TamB have been implicated in the secretion of the *Citrobacter rodentium* AT p1121 [25]. However, the TAM complex does not appear to be essential for the secretion of all ATs including EspP and Hbp [26]. It is clear that outer membrane complexes play a role in the secretion of ATs, but much is still unknown about the specific contributions of these complexes.

In addition to the contributions of the outer membrane complexes BAM and TAM, the literature suggests an increasingly important role for periplasmic chaperones in the secretion of classical ATs. In *E. coli*, triple mutants of *skp*, *surA*, and *degP* result in a synthetic lethal phenotype[27]. Furthermore, depletion of SurA in *E. coli* results in reduced levels of the outer membrane proteins LamB and OmpA which suggests that SurA plays a role in the assembly of outer membrane proteins [28]. The importance of periplasmic chaperones for the secretion of ATs has also been directly tested with the AT EspP. It was shown that the secretion of the EspP passenger domain is reduced in *surA, skp,* and *degP* mutants, but the levels of the translocation unit present in the OM were unchanged [29]. Common chaperones and oligomeric complexes are important for the secretion of classical ATs, but it is unknown if uniquely structured ATs require the same or additional factors for secretion.

1.3 Introduction to TcfA

Tracheal colonization factor (TcfA) is one of only a few ATs whose passenger domain is not predicted to possess a right-handed *B*-helical conformation. TcfA is predicted by numerous prediction programs to possess a coil conformation. The amino acid sequence of TcfA is pictured in Figure 1. In total, TcfA is 647 amino acid residues long. The N-terminal first 39

residues make up the signal peptide, and the mature passenger domain is 311 amino acids long. The C-terminal translocation unit is 297 residues long[14]. Very little has been published about the secretion and function of TcfA. It is known that TcfA is present in *Bordetella pertussis*, but is absent in other species of *Bordetella* including *Bordetella bronchiseptica* and *Bordetella parapertussis* [14]. *TcfA* is disrupted by a frameshift mutation in *B. bronchiseptica* and is completely absent from *B. parapertussis* [14]. Interestingly, TcfA possesses a characteristic RGD motif. RGD motifs have been shown to be important for mediating cell attachment to fibronectin, but this interaction has not been shown for TcfA. In an aerosol challenge experiment, it was shown that a *tcfa*-deficient mutant strain of *B. pertussis* colonized the trachea of mice at 10-fold lower levels than wild type [14]. These findings from the two original TcfA papers indicate that TcfA is unique to *B. pertussis*, and appears to play a role in colonization. As a result, the protein was named tracheal colonization factor. Surprisingly, despite the exciting findings of these two early papers, very little has been published about TcfA since that time. In a more recent paper, it was shown that TcfA displays allelic polymorphisms both between strains and within single cultures which could indicate that it is undergoing evolutionary pressure [30]. However, neither this publication nor any other recent publications have elucidated the structure, function or mechanism of secretion of TcfA.

MHIYGNMNRATPCRGAVRALALALLGAGMWTLSPPSAWALKLPSLLTDDELKLVLPTGM
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VRVSRIRHAFKVDDAKGRRVRGQYRGNGVGASLELGKRFTWPGAWYVEPQLEVAAFHAQ
GADYTASNGLRIKDDGTNSMLGRLGLHVGRQFDLGDGRVVQPYMKLSWVQEFDGKGTVR
TNDIRHKVRLDGGRTELAVGVASQLGKHGSLFGSYEYAKGSRQTMPWTFHVGYRYAW

Figure 1. Complete amino acid sequence of TcfA in Tohama 1
The signal peptide is highlighted in blue. The passenger domain is highlighted in red. The translocation unit is highlighted in green. A tcfA antibody was made to the sequence highlighted in yellow. The passenger domain of TcfA is highly proline rich.

1.3.1 The unique characteristics of TcfA

The unique structure of TcfA is highlighted in a recent PSIPRED prediction (Fig. 2). A PSIPRED prediction of the classical β-helical AT BrkA has also been included as a means for comparison (Fig. 3). As you can see, the translocation units of both TcfA and Brka have a similar structure that is characterized by β-strands. However, the passenger domains of the two proteins are markedly different. The entire TcfA passenger domain is predicted to have a coil structure. In contrast, the passenger domain of BrkA consists of mostly β-sheet structure that is common to the vast majority of ATs. The amino acid sequence of the passenger domain of TcfA is unique, and when analyzed previously by our lab it only showed a BLAST hit to the passenger domain of Nbap8 (locus tag: BPP1618) [31]. Nbap8 is predicted to be a coiled AT and a BLAST comparison to TcfA is provided for comparison (Fig. 4). Other than Nbap8, the passenger domain of TcfA has very little sequence similarity to any other AT. Interestingly, Nbap8 is present in the *B. parapertussis* and *B. bronchiseptica* genome, but is absent from the *B. pertussis* genome. Furthermore, in a recent publication TcfA was only shown to cluster with another *B. parapertussis* AT protein TynE (locus tag: BPP1617) [32]. In addition to its unique sequence and structure, TcfA also has several other unique characteristics. The predicted size of the TcfA passenger domain is 34 kDa, but it runs at 60 kDa on SDS page gels[14]. The discrepancy is believed to be due to the high proline content of the TcfA passenger domain (17%) which results in drag on SDS page gels. Additionally, TcfA is relatively resistant to proteolysis by trypsin, thermolysin and proteinaseK despite possessing numerous cleavage sites (D. Oliver, M. Paetzal, unpublished). The resistance to proteolysis indicates that TcfA must be acquiring some significant secondary structure despite being predicted to be relatively unstructured. It is unknown what factors are important for the biogenesis of TcfA.

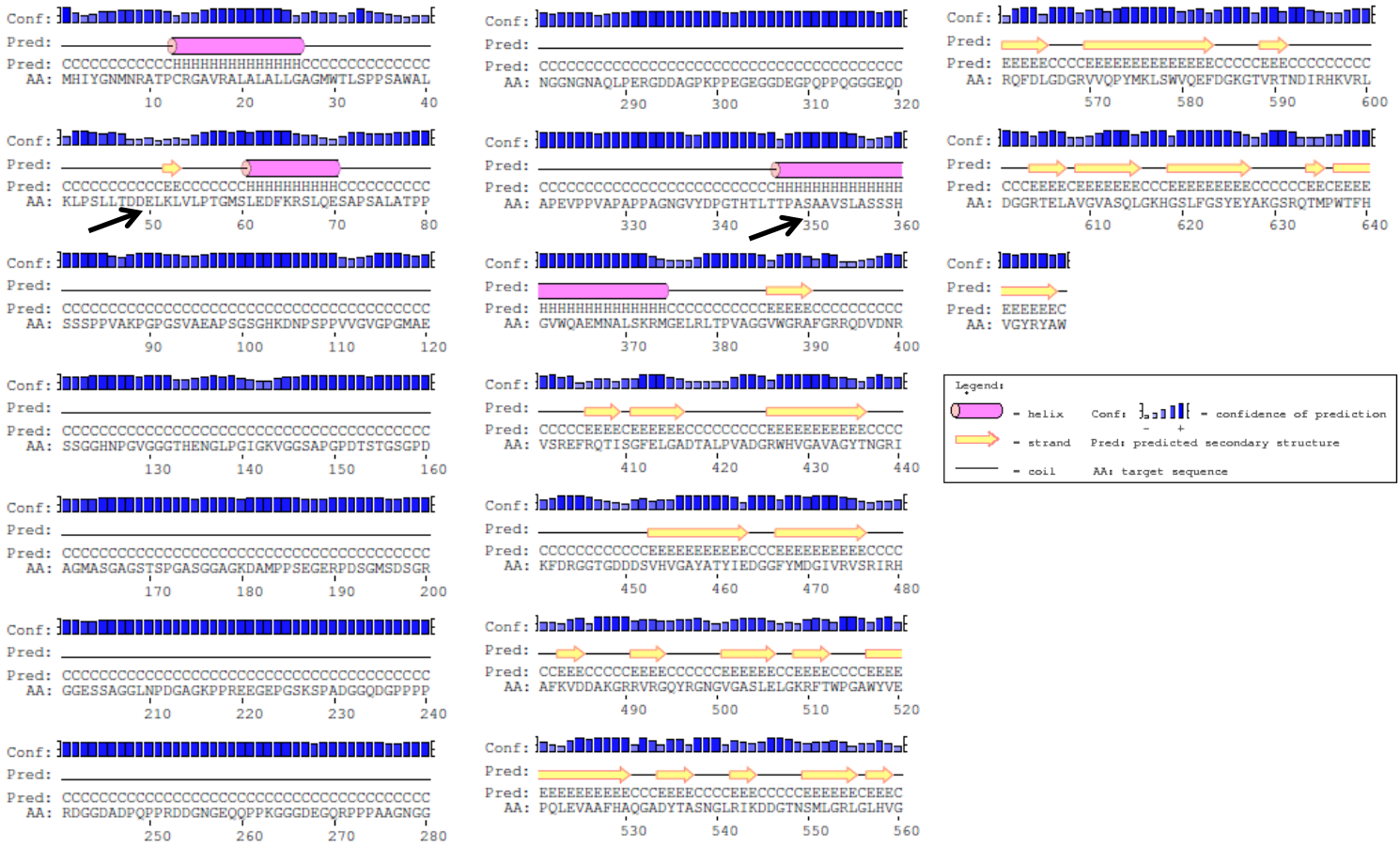


Figure 2. PSIPRED version 3.3 secondary structure prediction of full-length TcfA
The end of the signal peptide and passenger domain are marked with an arrow (residues E^{50} and S^{350} respectively). The legend, including the confidence of prediction, is pictured below the sequence.

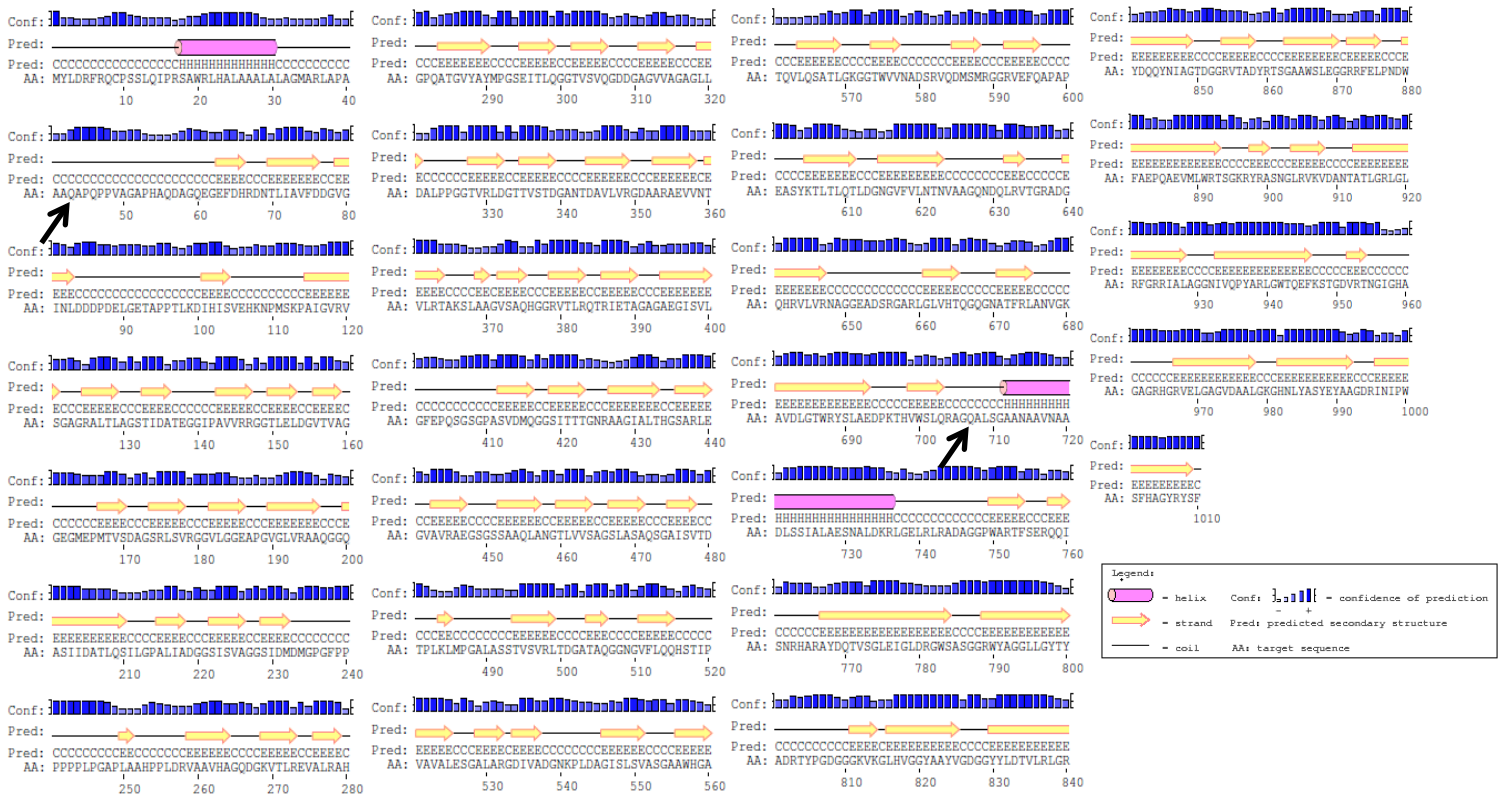
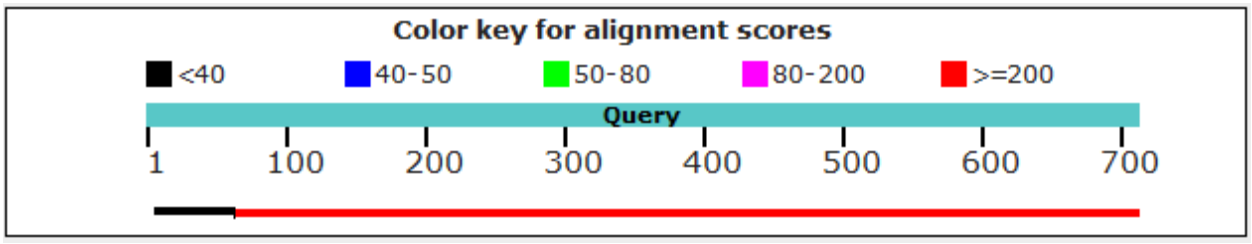


Figure 3. PSIPRED version 3.3 secondary structure prediction of full-length BrkA
The legend, including the confidence of prediction, is pictured below. The end of the signal peptide and passenger domain are marked with an arrow (residues Q^{43} and Q^{707} respectively).



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Sbjct  29   MWTLSP---------PSAWALKLPSLLTDDELKLVLPTGMSLEDFKRSLQESAPSALATP  79

Query  105  QPSAPKN-----GAAGAVGGEHGHSSDSPKVNVPSSEPSVGDASVGGGGGGSNSRHENGD  159
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Sbjct  80   PSSSPPVAKPGPGSVAEAPSGSGHK-DNPSPPVVGVGPGMAESSGGHNPGVGGGTHENGL  138

Query  160  GGVGNGKGGAGGSASGPGAVAGTAPGGAKHSDSGPSAGAPVSPGVAPGPGAGVASGMG--  217
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Sbjct  139  PGIGK----VGGSAPGPDTSTGS----------------------GPDAGMASGAGST  170

Query  218  ---ASGSAGEAHSPPDKKVPAEGGQGKGPGAGTGGAGGSPVPGSGGGGSVPSGAAGGDSP  274
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Sbjct  171  SPGASGGAGKDAMPPS--------EGERPDSG--------MSDSGRGGESSAGGLNPDGA  214

Query  275  PKPPSTGGKPGGGDQPPQKGEGNANGQAPDGGMD-PVPPRDG-DAGAKPPGSGADPDR--  330
             KPP   G+PG              +    DGG D P PPRDG DA  +PP    + ++
Sbjct  215  GKPPREEGEPG-------------SKSPADGGQDGPPPPRDGGDADPQPPRDDGNGEQQP  261

Query  331  PEGGGGDG---------------AVPNLPEAGGN-GPRPPAGND-DAGPRPPQGGDGNDA  373
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Sbjct  262  PKGGGDEGQRPPPAAGNGGNGGNGNAQLPERGDDAGPKPPEGEGGDEGPQPPQGGGEQDA  321

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Query  56   GIDLSPS  62
            G D + S
Sbjct  532  GADYTAS  538
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Figure 4. : BLAST 2 analysis of TcfA and Nbap8

Full-length TcfA from *B. pertussis* Tohama 1 was inputted as the subject sequence. Full-length Nbap8 from *B. parapertussis* was inputted as the query sequence. The two identified alignments are depicted below the graphic summary. The arrow indicates the start of the TcfA translocation unit.

1.4 The secretion of TcfA

ATs represent useful models for determining how proteins cross membranes. As mentioned previously, the passenger domain of TcfA is predicted to have an extremely unique unstructured or coil conformation. Since the vast majority of AT secretion studies have been done on β-helical ATs, very little is known about how non-β-helical ATs such as TcfA are secreted. Since TcfA possesses a prototypical N-terminal signal peptide and C-terminal translocation unit, it is expected to be secreted via the classical model of AT secretion (Fig. 5). In this model of secretion, the N-terminal signal peptide targets the protein for the inner membrane and the sec pathway. The sec pathway facilitates the translocation of the protein across the inner membrane and into the periplasm. Once the protein is in the periplasm, common chaperones such as SurA, Skp and DegP maintain the protein in a secretion competent state and target the protein to the outer membrane for secretion. Additional unique chaperones could also be important for the secretion of a uniquely structured protein like TcfA. The BAM and TAM protein complexes are also believed to play an important role in inserting the translocation unit into the outer membrane. Once the translocation unit is inserted into the outer membrane, it opens a pore through which the passenger domain is threaded from C to N terminus vectorially. The size of the pore and whether or not structured proteins can traverse it requires additional experimentation.

Crystallographic analysis of the translocation unit of NalP has shown that the β barrel pore is about 10 Å in diameter, and therefore too small to accommodate a protein with significant tertiary structure [33]. However, it has been shown that a folded protein domain fused to the N-terminus an AT could be efficiently secreted via the AT pathway [34]. It is clear

that additional work is needed to determine the accuracy of the self-transport hypothesis. Nonetheless, after the passenger domain is translocated into the extracellular space, the protein is either cleaved and released as is the case with TcfA, or remains associated with the outer membrane. However, it is believed that the right-handed β-helical conformation of classical ATs facilitates the vectorial secretion of these proteins so it is unknown if this type of a model is correct for a uniquely structured protein like TcfA.

It has been difficult to definitively determine how the energetics of AT secretion functions. It was originally proposed that segments of the passenger domain passively diffuse across the outer membrane and then fold sequentially. As a result, folding would trap the passenger domain in the extracellular space and provide the driving force for the AT translocation reaction [35]. Since the vast majority of AT passenger domains possess a right-handed β-helical conformation that likely folds in a stepwise fashion, this folding model has gained a degree of acceptance. However, TcfA is one of a few ATs that do not adopt a traditional right-handed β-helical conformation. It is possible that TcfA still folds in a stepwise fashion, but it is also possible that the energetics of AT secretion are more complicated than first thought.

Interestingly, TcfA is relatively resistant to proteolysis despite having numerous cleavage sites. This resistance to proteolysis indicates that TcfA must acquire a significant tertiary structure either during or after its secretion into the extracellular space. If TcfA acquires its structure after secretion then is it maintained in a secretion competent state in the periplasm by chaperone proteins? If so, are common chaperones sufficient for ensuring proper secretion or are there unique chaperones that are important for the secretion of TcfA? If TcfA acquires its structure after secretion, then how is that process mediated? Alternatively, TcfA could acquire its

tertiary structure in the periplasm, but then how could this rigidly structured protein cross the outer membrane? These open questions about the secretion of TcfA still need to be addressed.

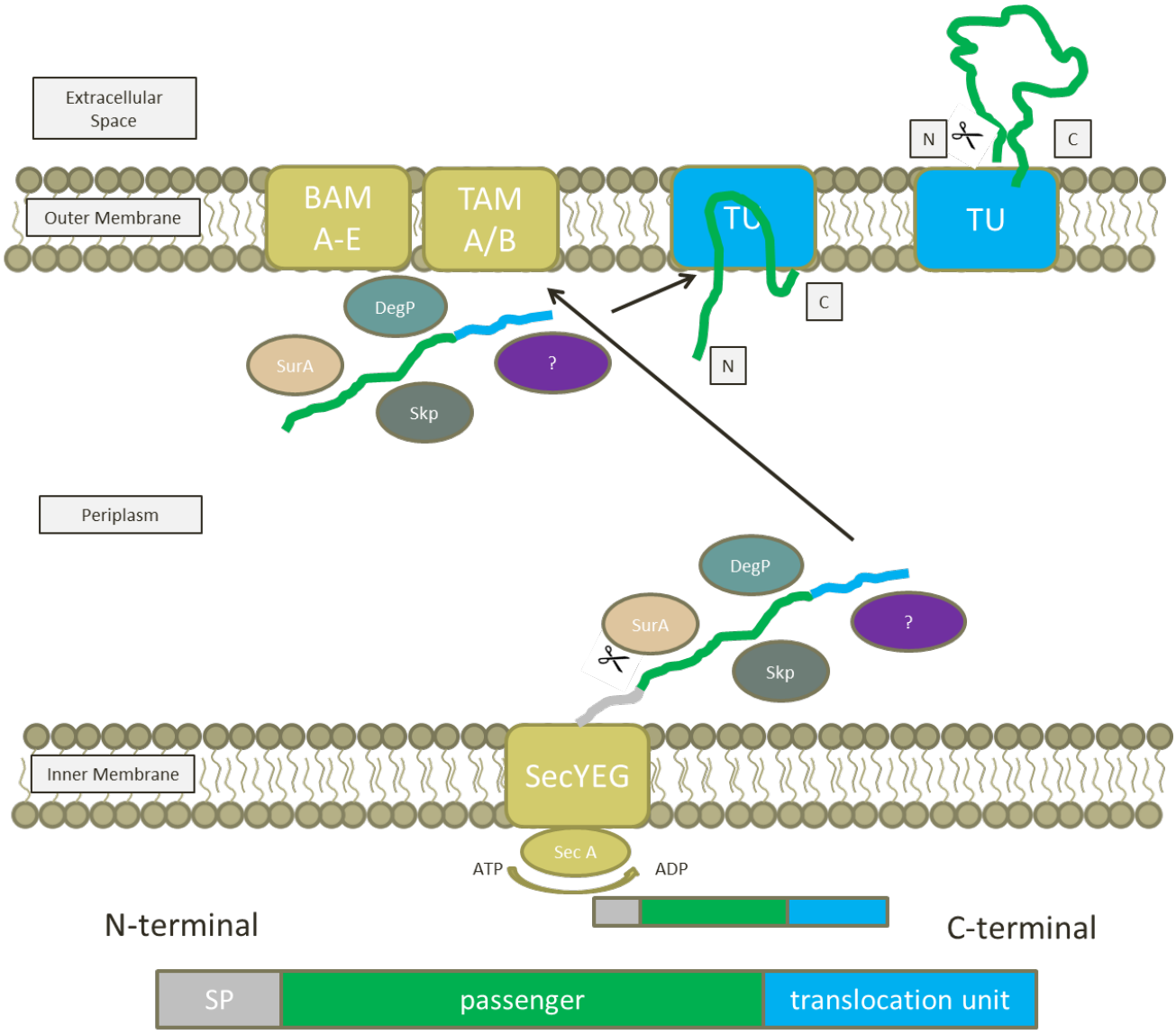


Figure 5. Predicted model for the secretion of TcfA
In this model of secretion, the N-terminal signal peptide targets the protein for the inner membrane and the sec pathway. The sec pathway facilitates the translocation of the protein across the inner membrane and into the periplasm. Once the protein is in the periplasm, common chaperones such as SurA, Skp and DegP maintain the protein in a secretion competent state and target the protein to the outer membrane for secretion. It is also likely that additional unique chaperones are important for the secretion of a uniquely structured protein like TcfA (represented by ? in the model). The BAM and TAM protein complexes are also believed to play an important role in inserting the translocation unit into the outer membrane. Once the translocation unit is inserted into the outer membrane, it opens a pore through which the passenger domain is threaded from C to N terminus vectorially.

1.5 The function of TcfA

The function of TcfA is also not well understood. *B. pertussis* secretes several ATs that are known to be important for pathogenesis. BrkA and Vag8 are important for serum survival, and Pertactin is important for adhesion to epithelial cells[36-38]. However, there are other ATs secreted by *B. pertussis* including tracheal colonization factor (TcfA) for which the role in virulence is not as well-defined. In the original TcfA paper, it was shown that a TcfA-deficient mutant strain of *B. pertussis* colonizes the trachea of mice at 10-fold lower levels than wild type [14]. However, the mechanism responsible for this deficiency has not been elucidated, and little else has been published about the role of TcfA in pathogenesis. However, one common strategy utilized by pathogenic bacteria in order to facilitate its survival in hosts is the manipulation of the host's complement system.

1.6 The complement system

The complement system protects against pathogen invasion. The pathway consists of proteins found in the blood that circulate as inactive precursors. These precursors can be activated by a variety of signals and result in a proteolytic cascade that ultimately leads to the opsonization and lysis of pathogens. Three biochemical pathways makeup the complement system: the classical, lectin and alternative pathways. (Fig. 6)

The classical pathway is activated when C1q binds to antibody attached to antigen, which results in the activation of C1r and C1s. C1r and C1s cleave C4 and C2. Similarly, the lectin pathway is activated when mannose-binding lectin (MBL) comes into contact with pathogen associated molecular patterns (PAMPs). This interaction leads to the activation of MBL-associated serine proteases (MASPs) which like C1r and C1s in the complement system, also cleave C4 and C2. The cleavage products of C4 and C2 make up the C3 convertase of the

classical and lectin pathways. The C3 convertase (C4bC2a) cleaves C3 into C3a and C3b. The resulting C5 convertase of the classical and lectin pathways consists of C4bC2aC3b.

The alternative pathway also results in the formation of a C3 and C5 convertase, but the proteoloytic cascade is significantly different from the classical and lectin pathways. The alternative pathway is initiated when C3 undergoes spontaneous hydrolysis resulting in the initial C3 convertase C3 (H_2O)Bb. The presence of factors B and D result in additional cleavage of C3 and the formation of the C3 convertase: C3bBb. Like in the classical and lectin pathway, the C3 convertase can associate with an additional C3b molecule in order to form the C5 convertase: C3bBbC3b [1].

Although the mechanisms are different, each of the three biochemical pathways of the complement system converges at the generation of the C3 and C5 convertases. These convertases are responsible for the generation of the common effectors of the complement system: anaphylatoxins (C3a, C4a, C5a), the membrane attack complex (MAC) and the principle opsonin C3b. Anaphylatoxins are proinflammatory molecules derived from C4, C3 and C5 cleavage. The anaphylatoxins have a wide range of effects including smooth muscle contraction, histamine release from mast cells and increased vascular permeability. The anaphylatoxins also play a role in mediating the generation of cytotoxic oxygen radicals, inflammation and chemotaxis [39]. The MAC consists of complement components C5b through C9 and forms transmembrane channels that directly lyse target cells [40]. C3b induces the phagocytosis of opsonized targets and facilitates the amplification of complement activation through the alternative pathway [41]. In summary, complement activation results in inflammation, opsonization and direct lysis of pathogens in the blood.

Although the complement system is essential for protecting the human body from pathogenic microorganisms, its activation must be tightly regulated in order to prevent damaging effects on host cells. Complement regulation occurs mostly at two points within the system: at the level of the convertases and during MAC assembly. MAC assembly is inhibited by membrane-bound CD59 or fluid phase vitronectin or S protein [42, 43]. More importantly for my project, host Factor I prevents C4b and C3b fragments from forming active convertases, by cleaving C3b and C4b into inactive fragments [44]. Factor I requires the cofactors CD46, CR1 or Factor H for its proteolytic activity. Factor H has also been shown to facilitate the decay of the alternative pathway C3 convertase via competition, and also by providing a binding platform for the Factor I-mediated proteolysis of C3b [45]. It is known that many pathogenic microorganisms recruit Factor H in order to dampen the effect of the host's complement system. A common site on Factor H via which several of these pathogens bind is also known [46]. *B. pertussis* is one of the pathogens that was identified as binding to Factor H via this common site in domains 19-20. Although it is known that *B. pertussis* binds Factor H, the ligand responsible for the binding has not been determined [47].

1.7 Thesis Objectives

TcfA has a unique sequence and structure for an AT based on BLAST analysis and structural prediction programs. As mentioned previously, TcfA also runs significantly higher than its predicted molecular weight on SDS-PAGE gels which is likely due its high proline content. However, even though it possesses many unique characteristics, TcfA is a BVG-regulated virulence factor that plays an important role in the adherence of *B. pertussis* to the trachea in a mouse model [14]. Surprisingly, despite this significant finding, we know little else

about this protein. My thesis set out to characterize this enigmatic protein, and in the process to increase our knowledge about the autotransporter protein family as a whole.

1.7.1 Characterizing the secretion of TcfA

Initially, I worked to characterize the secretion of TcfA by creating a model system in *E. coli*. I hypothesize that TcfA requires both common and novel accessory factors for its secretion.

1.7.2 Addressing questions about the function of TcfA

I hypothesize that TcfA is the factor in *B. pertussis* that is responsible for the binding of Factor H. In order to test if TcfA is the only factor that binds Factor H in *B. pertussis*, I disrupted *tcfa* in Tohama 1, and tested this mutant in a surface binding assay. I also hypothesize that TcfA contributes to the serum survivability of *B. pertussis*.

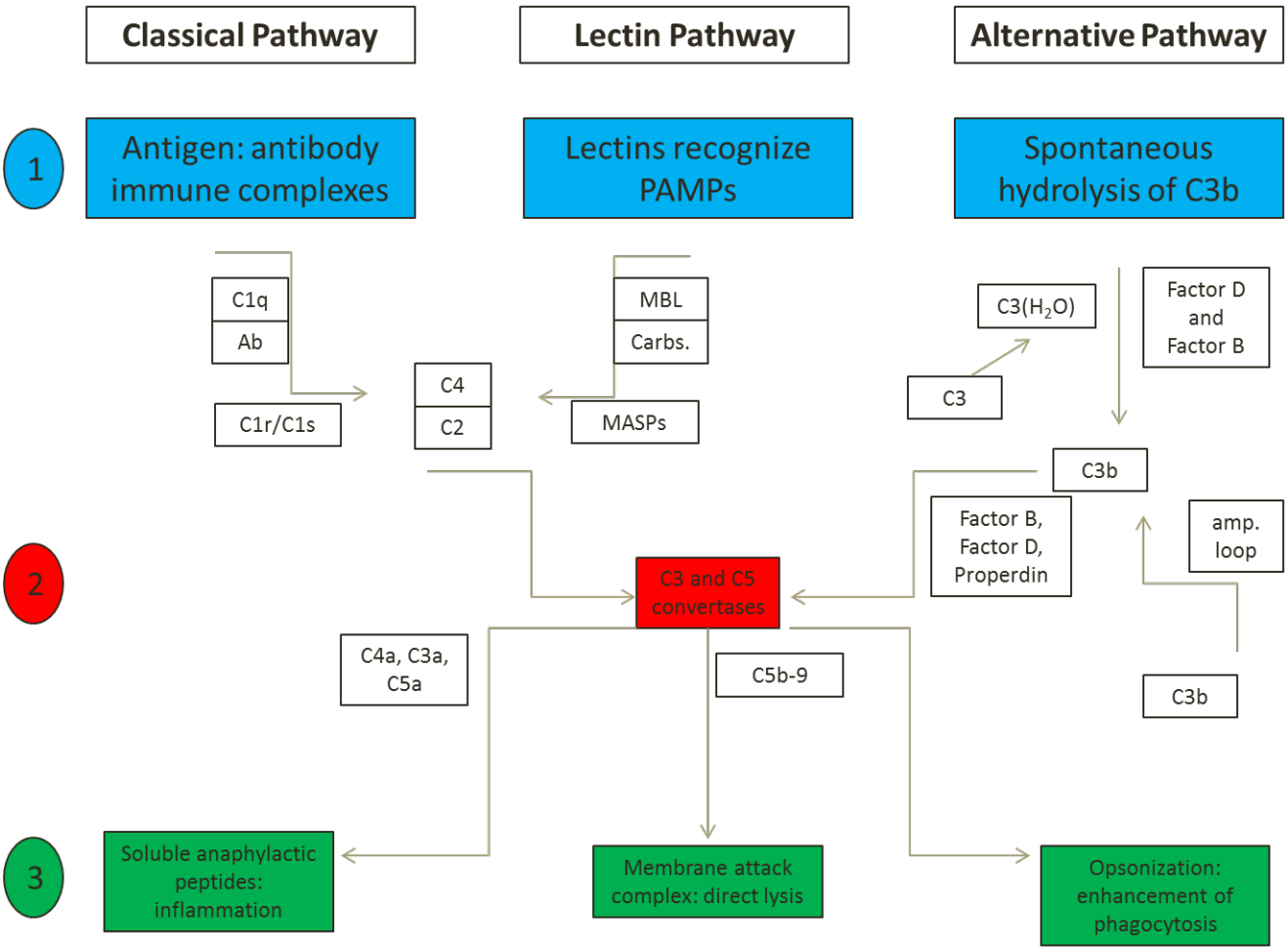


Figure 6. Schematic of the human complement system
1) Consists of the initiation phase for each arm of the pathway 2) Consists of the amplification step for each arm of the pathway which converge at the formation of the C3 and C5 convertases. 3) Consists of the Terminal steps of the pathway including inflammation, direct cell lysis and opsonisation. [1]

Chapter 2: Materials and Methods

2.1 Bacterial strains and growth media

The *E. coli* expression strains BW27783 and BL21 DE3 were grown at 37º on Luria agar or Luria broth with the appropriate antibiotic. The expression strains were generated by transforming pBAD24 constructs into BW27783 and pET28b or pET20b constructs into BL21 DE3. *E. coli* strain DH5α was used for cloning. *B. pertussis* strains BP338 and BP347 were grown on BG agar or broth with the appropriate antibiotic. The BG agar was supplemented with Defibrinated sheeps blood (HS30-100) purchased from Dalynn Biologicals (Calgary, AB). The appropriate antibiotics include gentamicin at a concentration of 20 μg/mL, ampicillin at a concentration of 100 μg/mL, kanamycin at a concentration of 50 μg/mL and nalidixic acid at a concentration of 30 μg/mL. TcfA and Par27 disruptions in *B. pertussis* strain BP338 were generated using the pEG7 suicide vector.

2.2 Plasmids and strains used in this study:

Table 1 lists all of the primers used in this study. Primers were ordered from either Alpha DNA Technologies (Montreal, QC) or Integrated DNA Technologies (Coralville, IA). Table 2 lists all of bacterial strains used in this study. Table 3 lists all of the plasmids used in this study. Sequencing was performed by Genewiz (South Plainfield, NJ).

Table 1. Primers used in this study

Primer Number	Primer Name	Primer Sequence
#1 (517)	tcfA-F	CCAGAATCATGCACATTTACGGAA
#2 (518)	tcfA-R	CCTGGTACCCTACCAGGCGTAGCGATA
#3 (529)	tcfAhis-F	ACGGAATTCACACCACCACCACCACCACCTTAAGCTCCCGTCG
#4 (530)	tcfAhis-R	AGAAAGCTTCTACCAGGCGTAGCGA
#6	Tcf 28-7 fw	TATACATATGCTTAAGCTCCCGTCGACGGAC
#7	Tcf 28-7 rev	TATACTCGAGTTAAGAGGCCGGCGTGGTCAAGGTATG
#8 (611)	Upstream 3' to 5'	GGAATTCTACGAGTACGCCAAGGGCAG
#9 (612)	Second 3' to 5'	CCTTAATTAACCGGAAGACACGACGGCGAA
#10 (737)	BapBTcfA_Flank1FW	GGAATTCCATGCGACACCATCCGCAAAC
#11 (738)	BapBTcfA_Flank1Rev	CCTTAATTAAGGTTCACAACTAGCGAGCCGGA
#12 (684)	Tcf dis forw	GGAATTCCAAACGGTGGCAACGGTGGCA
#13 (685)	Tcf dis rev	CGGGATCCCGCCTCGATGTAGGTAGCGTAA
#14 (794)	Par 27 dis forw	GGAATTCCAACCGCCAGGTGTTCGTGCA
#15 (795)	Par27 dis rev	CGGGATCCCGCTTCTTCAGCTTGGTCACGG
#16 (798)	Par 27 ins forw	CGGGATCCCGATGAAACGCATCGCCATGCT
#17 (799)	Par 27 ins rev	GGGGTACCCCTTACTGGATCTTGGCCTGTT
#18 (613)	First 5' to 3'	CCTTAATTAACCGGAAGACACGACGGCGAA
#19 (614)	Third 3' to 5'	CCTTAATTAACAGATGTCAGTCCTCATGCG
#20 (615)	Second 5' to 3' (with cc)	CCCCTAGGTGGTGCGCGGGCATCGCGT
#21 (616)	Second 5' to 3' (no cc)	CCTAGGTGGTGCGCGGGCATCGCGT
#22 (617)	Downstream 5' to 3'	GCCCAGCGCCTGATGGTCCA

Table 2. Bacterial strains used in this study

Bacterial strain	Relevant characteristics	Source/Reference
E. coli DH5α	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ⁻	Invitrogen
E. coli BW2783	*Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, Δ(araH-araF)570(::FRT), ΔaraEp-532::FRT, φPcp8araE535, rph-1, Δ(rhaD-rhaB)568, hsdR514*	Invitrogen
E. coli BW2783 Δchap	5 strains (ΔdegP, Δskp, ΔsurA, ΔPPiD, ΔPPiA)	Fernandez lab (Shengjuan Xu)
E. coli BL21 DE3	F⁻ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ⁻	Invitrogen
E. coli S17-1		(Simon, Priefer et al. 1983)
B. pertussis Tohama 1 (BP338)	Nalidixic acid resistant derivative of B. pertussis Tohama I wild type strain	A. Weiss
BP338_disTcfA	TcfA disrupted using fragment A^{839}-T^{1370}	this work
BP338_disPar27	Par27 disrupted using fragment A^{187}-G^{594}	this work
BP347	Nalidixic acid and kanamycin resistant bvg-negative phase locked mutant derivative of BP338	(Weiss, Falkow et al. 1983[48])

Table 3. Plasmids used in this study

Plasmid	Description	Vector	Source/Reference
pBAD24 tcfA full*	Full-length TcfA (1944bp)	pBAD24	S. Xu
pET20b tcfA his*	Full-length TcfA lacking the native SP (1846bp)	pET20b	S. Xu
Tcf28-7*	Passenger domain of TcfA (L^{40}-S^{350})	pET28b	D. Oliver
Tcf A^{839}-T^{1370}	Suicide plasmid for disrupting TcfA	pEG7	this work
Par27 A^{187}-G^{594}	Suicide plasmid for disrupting pEG7	pEG7	this work
Par27 full	Full length Par27 from Tohama 1(777bp)	pAM201	this work

* pBAD24 tcfA full and pET20b tcfA his were cloned by Shengjuan Xu of the Fernandez Lab.

Tcf28-7 provided by D. Oliver and M. Paetzal

2.3 General molecular biology protocols

All restriction enzymes and DNA polymerases were purchased from New England BioLabs (Ipswich, MA). Taq DNA polymerase (M0267L) was used for colony screening PCR amplification, and Q5 high-fidelity DNA polymerase (#M0491S) was used for cloning PCR amplification. In general, cloning PCR followed this protocol: 1 cycle for 10 minutes at 95° C, 30 cycles for 45 seconds at 95°C, 30 cycles for 30 seconds at 65°C, 30 cycles for 30 seconds at 72°C and finally 1 cycle for 3 minutes at 72°C. PCR products were run on an agarose gel and the relevant bands were cut out of the gel. The extracted bands were purified using a BioBasic (Markham, ON) Spin Column Gel Extraction Kit. A DNA digestion followed using the corresponding restriction enzymes followed by an additional purification using the same BioBasic Spin Column Gel Extraction Kit. The digested inserts and digested plasmids were ligated overnight at 16°C using T4 DNA ligase. A 45°C heat shock protocol was followed for

transformations. All of the relevant primers used in this study can be found in Table 1, and relevant plasmids are in Table 3.

2.4 Generating mutants in *B. pertussis*

TcfA and Par27 were disrupted in *B. pertussis* Tohama 1 using the pEG7 suicide vector. A diparental mating protocol was performed between *E. coli* S17 expressing the constructed suicide plasmid and WT *B. pertussis* Tohama 1. In short, the two strains were mixed on a mating plate in a 10:1 ratio of *B. pertussis* to *E. coli*. The plate was incubated for 5 hours, and swabs from the plate were removed at 3 and 5 hours. These swabs were streaked on BG Nal/gent plates. After 3 days, the plates were screened for the absence of the gene of interest. The TcfA disruption: primers 684 and 685 were used for amplifying a 559 base pair fragment that spanned between the passenger domain and translocation unit. This fragment was digested using EcoR1 and BamHI and was subsequently ligated into the pEG7 plasmid. The Par27 disruption: primers 794 and 795 were used for amplifying a 408 base pair fragment in the middle of Par27. This fragment was digested using EcoR1 and BamHI and was subsequently ligated into the pEG7 plasmid.

2.5 Expressing Par27 in *E. coli*

Par27 was PCR amplified using primers 798 and 799. The PCR product was digested with BamHI and KpnI and was subsequently ligated into the PAM201 plasmid. The constructed vector was transformed into *E. coli* BW27783. Like the pBAD20 full-length TcfA plasmid, the PAM201 Par27 plasmid is also under the control of an arabinose inducible promoter. Par27 was co-expressed in BW27783 along with TcfA. Par27 was expressed in *E. coli* BW27783 using the PAM201 plasmid.

2.6 Purification of the passenger domain of TcfA

A 100mL culture of BL21 DE3 expressing the tcf28-7 plasmid which encodes the passenger domain of TcfA was prepared. Protein expression was induced with 1mM IPTG for 3 hours. The cells were pelleted by centrifugation @ 5000 rpm for 5 minutes. The cells were resuspended in 8 mL of native binding buffer + protease inhibitor tablet. The cells were lysed using 5 x 10 second bursts with 30 second off using a Sonicator Ultrasonic Processor from Mandel Scientific. Cells were then centrifuged @ 10,000 rpm for 15 minutes. The cell pellets were used for a Nickel affinity column purification following the Invitrogen Ni-NTA Purification system-Native Conditions. Protein was eluted with 100mM-500mM imidazole.

2.7 Generating an antibody to TcfA

An antibody was generated to a portion of the TcfA passenger domain. The peptide was determined based on its antigenicity/hydrophilicity and because it is amphipathic. The exact algorithm that was used is proprietary to Genscript (Piscataway, NJ), but it takes multiple factors into account. The chosen peptide is from G^{174} to S^{187}. The antibody was produced in a goat that was pre-screened to ensure it was not colonized with *Bordetella sp.* When we received the antibody, several dilutions were tested in order to determine the optimal dilution to use for future experiments. This experiment was performed using a pBAD24 clone that expressed full-length TcfA including the native signal peptide. The antibody was titrated, and 1:3000 was selected for use in all future experiments.

2.8 Immunoblotting

The day before the experiment, overnight cultures of *E. coli* BW2783 expressing pBAD24 with full-length TcfA were started. Approximately 50 µL of the overnight cultures were used to

inoculate a fresh culture for induction. The cultures were grown for about 2 hours to a 600 nm optical density (OD_{600}) of roughly 0.4. The expression of TcfA was induced with 0.02% arabinose for 3 hours. After the 3 hour induction, 500 µL of the culture was spun down and frozen for SDS-PAGE analysis. Normal western blot protocol was followed using the TcfA antibody and donkey anti-goat HRP-conjugated antibody IgG from Cedarlane (705-035-147 Burlington, ON). For experiments with his-tagged clones, the His-antibody from Santa Cruz Biotechnologies (SC804 Dallas, TX) and the goat anti-rabbit HRP antibody from Cedarlane (111-035-144 Burlington, ON) was used. For experiments using *B. pertussis*, a similar protocol was followed except the bacteria were grown for 3 days on BG agar and then resuspended to the desired OD in SS salts. Normal western blotting protocol was once again followed.

2.9 Protein concentrating

Proteins were concentrated with 20% trichloroacetic acid (TCA). The samples were incubated for 30 minutes on ice, followed by centrifugation at max speed at 4°C for 15 minutes. All of the supernatant was removed, and 300µL of cold acetone was added to each sample. Samples were centrifuged again at max speed for 5 minutes. The supernatant was removed again, and the pellet was allowed to dry. The pellet was then resuspended in disruption buffer for SDS-PAGE and a subsequent western blot.

2.10 Evaluation of Factor H binding to the bacteria

BP338, BP338Δ*tcfa* and BP347 were grown for 3 days prior to the start of the experiment on BG agar plates. Bacteria from the plates were resuspended in 5mL of SS salts. An OD_{600} reading was taken of each suspension. The equivalent of 1mL at OD_{600} =0.25 of each condition was used for the surface binding experiment. 20µL of undiluted normal human serum (NHS) was added

to each sample. The samples were then incubated at 37°C for 15 minutes. The samples were next put on ice for 5 minutes prior to 3 wash steps. After the washes, the pelleted cells were used in normal western blot technique with an anti-Factor H antibody from Quidel (A312 San Diego, CA) in order to determine if the surface binding was successful.

2.11 Serum killing

An aliquot of normal human serum (NHS) was heat-inactivated by incubation for 30 min at 56°C. NHS and heat-inactivated NHS (HI-NHS) were diluted to 10% with Veronal buffer (BioWhittaker). Freshly grown bacteria were harvested from BG-agar in warm SS-salts and the OD600 was adjusted to 0.025. 18 µl of diluted NHS was added to 3 wells per strain on one plate, and 18 µl of diluted HI-NHS was added to 3 wells per strain on the other plate. 2ul of bacteria at OD600 0.025 was added to the 18µl diluted NHS, and in parallel 2µl of each test bacterium was added to the 18µl of diluted HI-NHS. Wells were mixed, and then incubated for 15 minutes at 37°C. Complement activation was stopped by the addition of 80ul PBS with 20 mM EDTA to each sample. Successive 10-fold dilutions were made with warm SS-salts. 90µl of the bacterial dilutions were plated onto BG-agar with the appropriate antibiotic, and colonies were counted after 3 days of growth. 3 replicates were used for each strain, at each dilution. The average of these triplicates was used to calculate percent survival. Percent survival was calculated relative to the control: (average of three replicates when incubated with NHS/ average of three replicates when incubated with HI-NHS). A single factor ANOVA was used to determine statistical significance.

2.12 Evaluation of the surface exposure of TcfA

Western blots were performed using E. coli expressing the full length TcfA plasmid after induction with .02% arabinose for 3 hours or *B. pertussis* strain BP338 grown for 3 days on BG agar and then resuspended in SS salts. SS salts consist of 10.72 g glutamic acid, 0.24 g proline, 2.50 g NaCl, 0.50 g KH_2PO_4, 0.20 g KCl, 0.10 g $MgCl_2 \cdot 6H_2O$, 0.02 g $CaCl_2$, 3.175 g Tris-HCl, 0.59 g Tris base dissolved in 1 liter of distilled deionized water. The *B. pertussis* cultures were adjusted to have the same OD as the *E. coli* cultures. Seven 1mL aliquots of each culture were removed (one for each time point of trypsin incubation: 0, 1, 5, 15, 30, 60, and overnight). The samples were centrifuged and resuspended in 135µL of PBS. Next, 15µL of .1mg/mL trypsin were added to each tube in order to achieve 10µg/mL working concentration. Samples were incubated at 37ºC. Samples were removed and centrifuged samples at each time interval in minutes (1, 5, 15, 30, 60, and overnight). 50µL of 2x disruption buffer was added to each pellet and immediately boiled for 5 minutes to stop digestion. Supernatants were precipitated with 20% TCA. Following boiling, samples were run on an 11% SDS-page gel. An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression.

2.13 Bioinformatic and structural prediction of TcfA

PSIPRED version 3.3 was used for the secondary structure prediction of TcfA and BrkA. PSIPRED incorporates two feed-forward neural networks which perform an analysis on an output obtained from PSI-Blast. PSIPRED incorporates several structure prediction methods in one location. The input file is the amino acid sequence of the protein. The prediction methods included: 1) Transmembrane topology and transmembrane helix prediction 2) Fold recognition 3) Domain recognition 4) Intrinsic disorder prediction

I-TASSER version 4.3 was used for 3D structure prediction of TcfA and BapB. I-TASSER (Iterative Threading ASSEmbly Refinement) is a hierarchical approach to protein structure and function prediction. I-TASSER identifies structural templates from PDB via a multiple threading approach (LOMETS). Function insights of the target are then determined by threading the 3D models through a protein function database. The input file is the amino acid sequence of the protein.

pyDockWEB is a web server for the structural prediction of protein-protein interactions. pyDock was used for predicting an interaction between Factor H and TcfA or BapB. The input is the 3D coordinates of two potentially interacting proteins. pyDockWEB generates the best rigid-body docking orientations using FTDock. The predicted models take into account electrostatics, desolvation energy and limited van der Waals contribution.

BioCyc pathway tools version 13.0 was used for comparative genome analysis. The analysis looked for orthologues of *tcfa* in *Bordetella* species because the protein is unique to *B. pertussis*. BioCyc generated an alignment using a collection of 9389 Pathway/Genome Databases (PGDBs). PeptideCutter on the Swiss Institute of Bioinformatics (SIB) webpage was used to determine potential protease cut sites within TcfA. Artemis genome browser was used for visualizing the genetic layout of *B. pertussis* Tohama 1.

Chapter 3: Characterizing the Secretion of TcfA

3.1 Creating a model system in *E. coli*

My initial efforts to characterize the secretion of TcfA were focused on expressing TcfA in *E. coli.* Previously, our lab has had success expressing *Bordetella* ATs in *E. coli*. Additionally, a lot of tools are already present for asking questions about secretion in *E. coli* including numerous chaperone knockout strains. It is definitely most convenient to use *E.coli* for cloning steps because the follow-up studies are easier to execute. Furthermore, *E. coli* grows significantly faster than *B. pertussis* so creating a model system allows experiments to be completed much quicker. All of these advantages related to throughput are why I initially committed to expressing TcfA in *E. coli.*

Initially, I attempted to purify the passenger domain of TcfA in order to make an antibody to the full protein. I used a his-tagged passenger domain clone expressed using pET28b. Nickel column affinity chromatography was used for the purification (Fig. 7). Unfortunately, additional bands at around 30 kDa and 100 kDa were present in the purification. The band at about 30 kDa has been seen previously and is likely a breakdown product from TcfA. However, the band at around 100 kDa is unlikely to be associated with TcfA. Full-length TcfA runs at about 100 kDa, but this is a clone that consists of only the passenger domain. Due to this contaminant, I was unable to produce a pure elution for antibody production, and an alternative method for antibody production was used. An antibody was manufactured by GenScript to a peptide from position G^{174} to S^{187} of the TcfA passenger domain (Fig. 1).

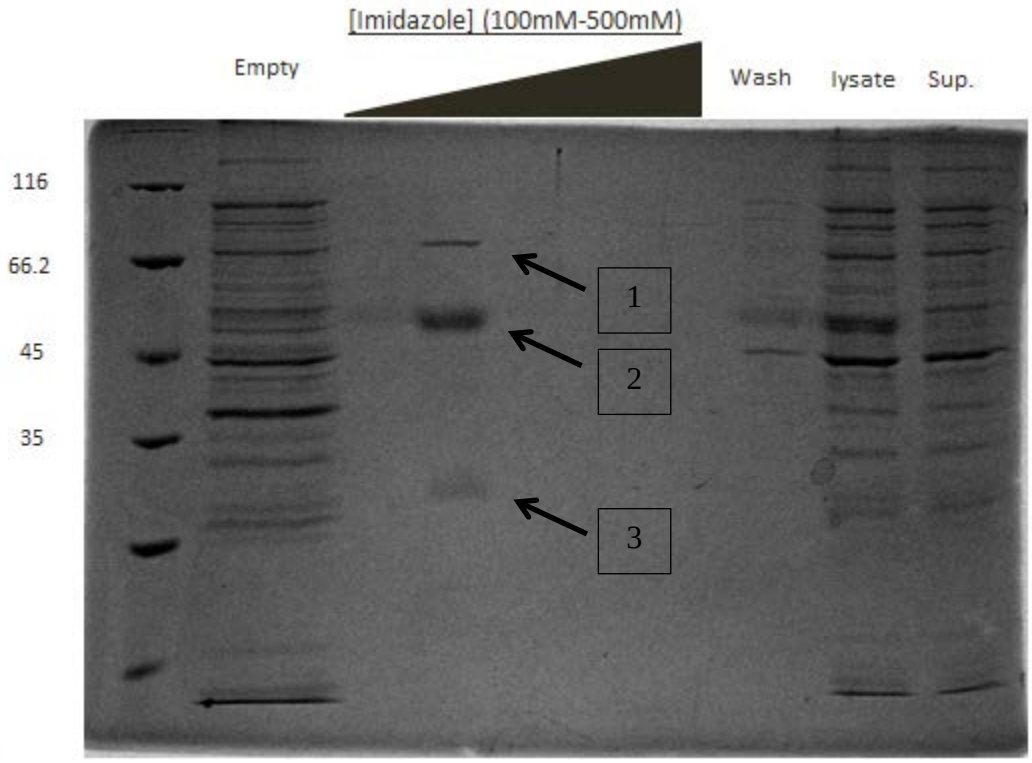


Figure 7. Coomassie stain of nickel column affinity chromatography of pET28b+ TcfAp-His
Empty vector whole-cell lysate was run as a control for western blotting (Empty). Protein was eluted with 100mM-500mM imidazole. Whole cell lysate prior to purification (lysate), the supernatant after running through the column (Sup.) and some of the wash buffer run through the column (Wash) were all run as controls. Molecular weight markers (kDa) are on the left. From top to bottom, the arrows point to: 1) a contaminant that is unlikely to be related to TcfA 2) the desired band for the passenger domain of TcfA 3) a likely breakdown product of TcfA

In normal AT secretion, the protein is targeted to the inner membrane via the signal peptide. After translocation into the periplasm, the signal peptide is cleaved. Subsequently, the protein is translocated to the cell surface and the translocation unit is cleaved. As a result, the mature form of the protein is surface exposed and consists of only the passenger domains. Our understanding of AT secretion allows us to establish several checkpoints that must be met in order to confirm that a model system is working analogously to secretion in *B. pertussis*. First, the protein must be expressed. Secondly, the passenger domain must be abundant as an indication of correct processing. Thirdly, the processed passenger domain must be surface exposed. This is the progression that was followed while trying to create a model system for TcfA in *E. coli*. The native secretion of TcfA in *B. pertussis* is shown in Figure 8. The passenger domain is the functional part of the AT. A western blot using an antibody to the passenger domain should result in the visualization of mostly cleaved passenger domain. The full-length protein is also present because it has not yet undergone processing, but it should not be as abundant as the processed passenger domain. As you can see for the expression of TcfA in *B. pertussis*, the majority of the protein is in the processed passenger domain form at around 60 kDa. The Full-length protein is also present at around 100 kDa (Fig. 8). This is the type of secretion profile that I worked to mimic in *E. coli*. Interestingly, my first attempts to visualize the expression of TcfA using a his-tag in *E. coli* were unsuccessful. Another member of our lab also tried to express and visualize TcfA with a his-tag and was unsuccessful as well. The sequence of the his-tagged clone was confirmed so the complete lack of expression is difficult to explain. I also used the BrkA his-tagged clone as a control so the problem was not with the antibody. I continued trying to get the system to work by manipulating induction conditions up until the point that we had a TcfA antibody produced.

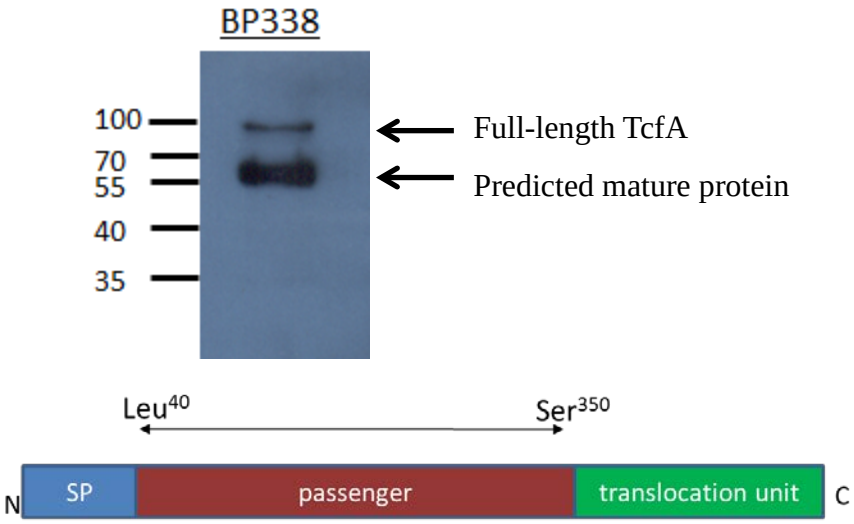


Figure 8. Western Blot of TcfA in Tohama 1
An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

The TcfA antibody allowed for the visualization of TcfA in *E. coli*, but it became clear that the expression in *E. coli* was drastically different from the expression in *B. pertussis*. Since my initial attempts at expressing a his-tagged clone were unsuccessful, I moved to using a full-length TcfA clone that did not include any tags. I tested this pBAD24 clone after inducing for 1 hour, 3 hours and overnight. The largest amount of expression of TcfA was observed after a 3 hour induction with arabinose (Fig. 9). However, although a significant amount of full-length TcfA was produced, markedly less passenger domain was present. I tried to modify the system in order to increase the amount of passenger domain that is produced by manipulating the induction conditions, but it did not have the desired effect.

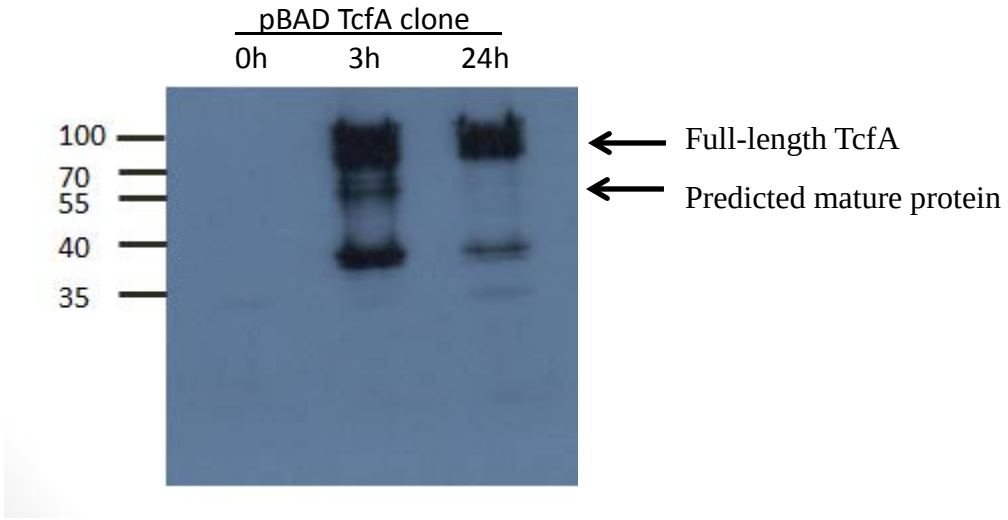


Figure 9. Western blot of whole cell lysates of *E. coli* expressing full-length TcfA
0h, 3h, 24h: refers to the induction time. The expression of TcfA was induced with 0.02% arabinose for 3 hours or 24 hours. Arrows indicate the expected location of full-length TcfA and the predicted mature protein (cleaved passenger domain). An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

One possible explanation for why the TcfA passenger domain is not abundant in the western blots is that I was only performing analysis on the cell lysate. It is possible that the majority of the passenger domain of TcfA is actually secreted into the supernatant. In order to concentrate any protein released into the media, I did a TCA preparation of the LB broth from one of the pBAD24 TcfA clone inductions and compared the results to the cell lysate fraction from the same experiment. Multiple concentrations of arabinose were used for induction in this experiment because at the time induction conditions were still being optimized. A significant amount of full-length TcfA is present in the cell lysate fraction for all of the induction conditions, and a very small amount of the passenger domain is present. In contrast, there is basically no TcfA present in either form in the supernatant (Fig. 10). The background bands in the supernatant fractions are likely due to the fact that the supernatant was not filtered prior to precipitation with TCA. The low levels of passenger domain in the cell lysate fraction, and the complete absence in the supernatant, indicate that TcfA is not undergoing its normal biogenesis in *E. coli*.

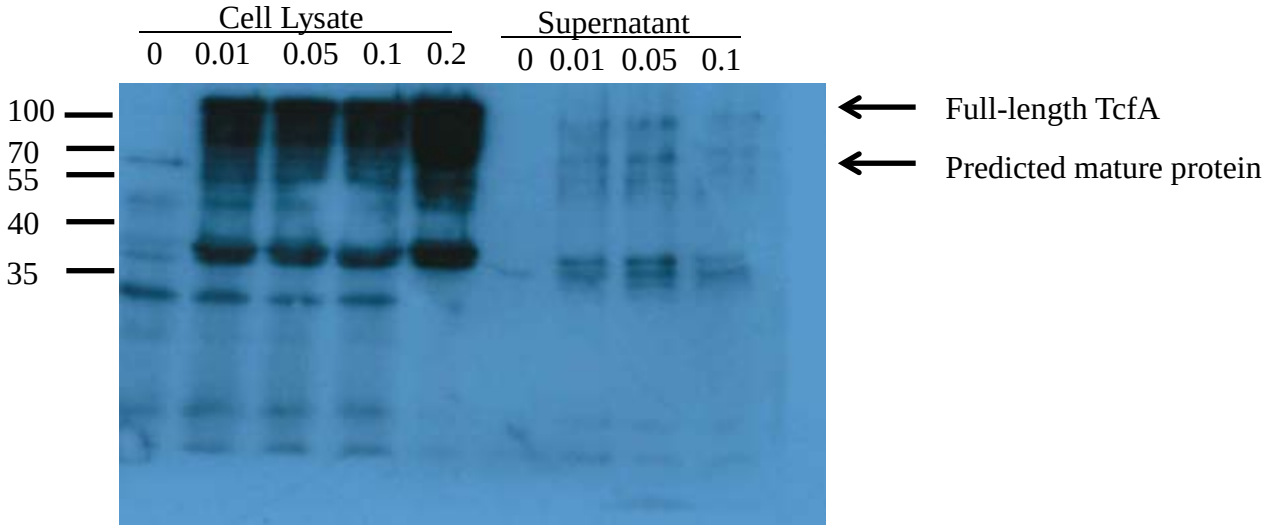


Figure 10. Western blot of whole cell lysates and TCA precipitated supernatants of *E. coli*
The *E. coli* clone expressing the full length TcfA plasmid was induced with varying concentrations of arabinose. 0, 0.01, 0.05, 0.1 and 0.2 refers to the arabinose percentage that was used for each 3 hour induction. The supernatant was precipitated with TCA prior to the western blot. The 0.2% arabinose induced supernatant was not precipitated. Arrows indicate the expected location of full-length TcfA and the predicted mature protein (cleaved passenger domain). An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

The TcfA antibody was also used to visualize the expression of TcfA in *B. pertussis* strain Tohama 1. *B. pertussis* was grown for 3 days on a BG agar plate, and on the 3rd day the bacteria was resuspended in SS salts. The suspension was adjusted to an OD of 0.15, and was then used in a western blot. In stark contrast to the expression of TcfA in the *E. coli* system, in *B. pertussis* the majority of TcfA was present in the processed passenger domain form (Fig. 8). These expected results in *B. pertussis* confirmed that the expression of TcfA in *E. coli* is clearly different than its expression in *B. pertussis*.

In addition to the majority of an AT being in the cleaved passenger domain form, another indication of normal biogenesis is that the passenger domain is surface exposed. If the passenger domain is present but not surface exposed, then it is likely accumulating in an unfolded form in the periplasm rather than undergoing its normal biogenesis. The surface exposure of TcfA was further tested in a trypsin experiment using both *B. pertussis* and *E. coli* expressing the full-length TcfA plasmid (Fig. 11). The blots were exposed for 3 minutes, and a 30% brightness increase was applied to all blots in order to make visualization easier. *B. pertussis* blots are on the left side, and *E. coli* blots are on the right side. Prior to the addition of trypsin, the vast majority of TcfA expressed in *B. pertussis* is in the cleaved passenger domain form (Fig. 10a, T=0). Even at a high concentration of trypsin (10µg/ml final), the passenger domain in the cell lysate fraction is fairly resistant to proteolysis (Fig. 11a, T=1 and 5). However, by 15 minutes the majority of the passenger domain is degraded (Fig. 11a, T=15 to overnight (ON)). In contrast, TcfA expressed in *E. coli* is almost exclusively in the full-length form (Fig. 11b, T=0-60). The full-length protein is not degraded by the high concentration of trypsin even after 60 minutes, indicating that it is likely not surface exposed (Fig. 11b, T=60). As expected, the mature passenger domain of TcfA is present in the supernatant of the *B. pertussis* culture (Fig.

11c, T=0). Full-length TcfA is absent from the *B. pertussis* supernatant which is expected since all of the protein on the surface should be in the cleaved mature form (Fig. 11c, top arrow). In stark contrast, the passenger domain of TcfA is not present in the supernatant of the *E. coli* cultures. Only a faint band representing the full-length TcfA protein is present in the *E. coli* supernatants (Fig. 11d, T=0). It is surprising that the full-length protein is present in the supernatant, and could be an artifact due to lysed cells leaking into the supernatant since these samples were not filtered. Clearly, the expression of TcfA in *E. coli* is vastly different from its natural expression in *B. pertussis.*

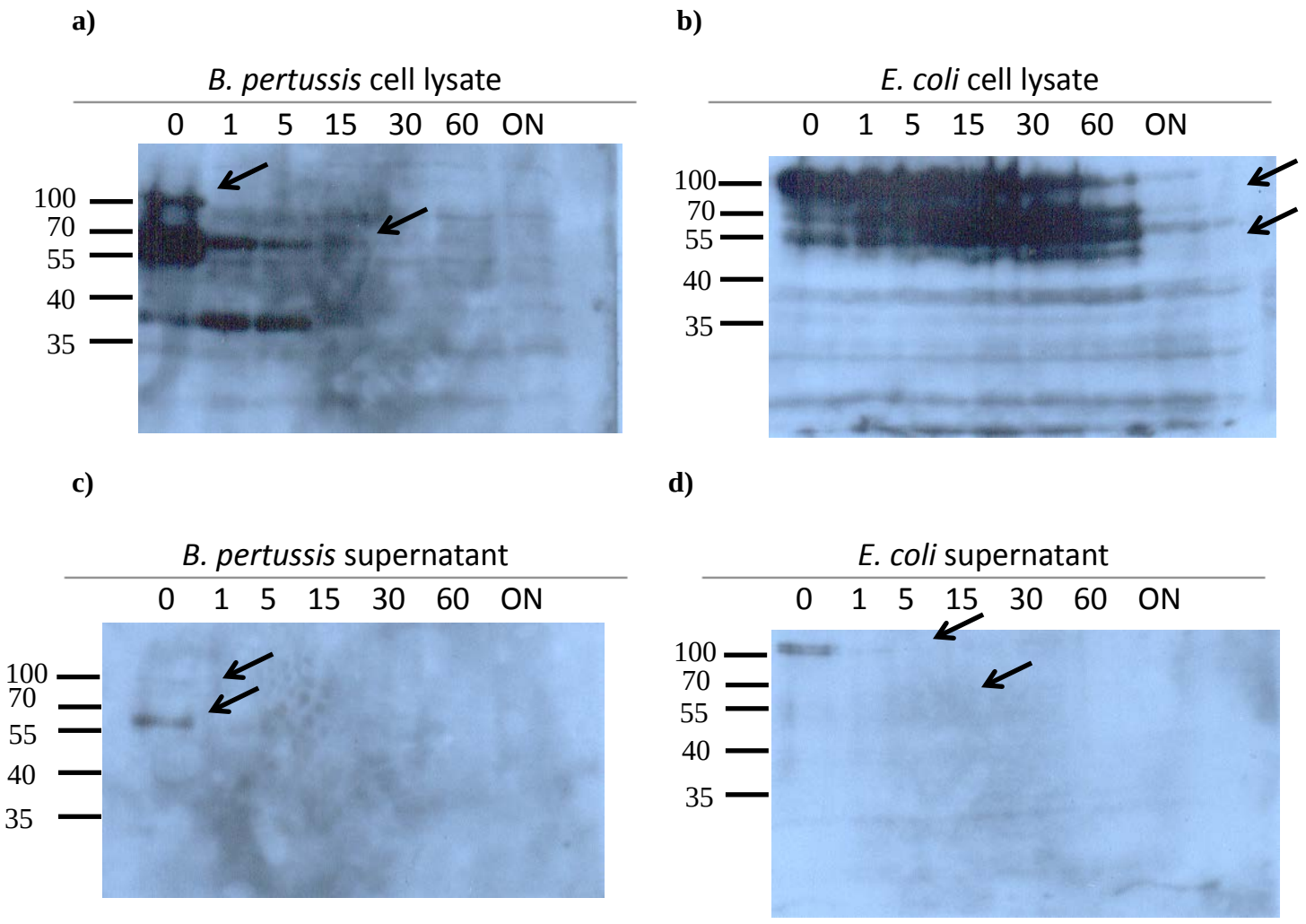


Figure 11. Trypsin surface exposure experiments in *E. coli* or *B. pertussis*
Western blots were performed using *E. coli* expressing the full length TcfA plasmid after induction with .02% arabinose (on the right) and *B. pertussis* wild type strain BP338 (on the left). Both whole cell lysates and TCA precipitated supernatants were tested. Supernatant refers to the fraction that is not pelleted after centrifugation. 15µL of .1mg/mL trypsin was added to each aliquot to achieve 10µg/mL working concentration. 1, 5, 15, 30, 60, and overnight (ON) refers to the incubation time with trypsin prior to centrifugation. The top arrow in each blot refers to the predicted location of full-length TcfA, and the bottom arrow refers to the predicted location of the predicted mature protein (passenger domain) of TcfA. An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left of each blot. A 30% brightness increase was applied to all blots in order to make visualization easier.

3.2 The role of the Par27 chaperone in the secretion of TcfA

The inability to successfully express full-length TcfA in *E. coli* presented an opportunity to figure out what the special secretion requirements are for TcfA. One possible cause of the difference in expression in *E. coli* versus *B. pertussis* is that a unique *Bordetella* chaperone is essential for the expression of TcfA. It is known that chaperones play an important role in the secretion of ATs, and it is plausible that *B. pertussis* possesses some unique chaperones to aid in the secretion of proteins. Furthermore, the unique predicted structure of TcfA could indicate a need for some additional secretion factors. I turned to the literature and found a paper that identified a novel chaperone in *B. pertussis* [49]. In the paper, the authors identified the chaperone Par27. Par27 is a PPIase. PPIases are important for interconverting between the cis and trans forms of the amino acid proline. A protein BLAST analysis of Par27 shows that only *Bordetella* species and *Achromobacter* species possess proteins with greater than 65% identity to the Par27 of *B. pertussis*. *B. parapertussis* and *B. bronchiseptica* have copies of Par27 that are identical to Par27 of *B. pertussis*.

TcfA is abnormally proline rich, and the passenger domain is 17% prolines. As a result, it seems plausible that a unique PPIase could be important for its secretion. The original paper showed that Par27 exhibits both PPIase and chaperone activity *in vitro*. Most of the work in the paper focused on determining the effect of Par27 on the aggregation and proteolysis of an FHA derivative. However, the paper also provided additional evidence to suggest a role of Par27 in the secretion of TcfA. In an overlay assay, Par27 selectively bound a few *B. pertussis* proteins including the major outer membrane porin of B. pertussis (MOMP), SphB1-*Beta*, OmpA, the BrkA translocation unit and the TcfA translocation unit[49]. The unique structure and high proline content of TcfA coupled with the fact that the TcfA β-domain bound to Par27 suggests

that Par27 is a unique *B. pertussis* factor that is important for the secretion of TcfA. I decided to directly test this theory in both *B. pertussis* and the *E. coli* model of secretion.

Two complementary approaches were used to determine the importance of Par27 for the secretion of TcfA. First, Par27 was co-expressed with TcfA in *E. coli*. The proteins were expressed on different plasmids, but both were under the control of arabinose-inducible promoters. The presence of Par27 was confirmed with a coomassie brilliant blue stain (Fig. 12). A unique band is present at around 30kDa (Par27 is 27kDa) in the fraction where both TcfA and Par27 are expressed. As in previous *E. coli* experiments, the plasmids were induced for 1-3 hours and then the bacteria were spun down and lysed for western blot detection. The amount of full-length TcfA produced when Par27 is co-expressed is increased relative to what we have seen in previous experiments in *E. coli* (Fig. 13). This increase was consistent across several experiments. However, the presence of Par27 did not affect the amount of TcfA passenger domain produced. These results indicate that the presence of Par27 increases the relative amount of TcfA, but does not abrogate the dysregulated secretion in *E. coli*.

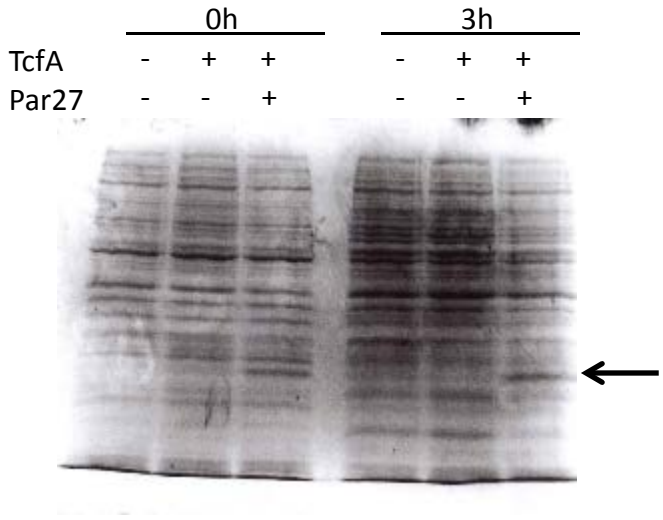


Figure 12: Coomassie stain of whole cell lysates of *E. coli* expressing full-length TcfA
+ or -: refers to whether or not the full-length TcfA pBAD24 plasmid and the par27 pAM201 plasmid are expressed for each sample. (-,- means samples of BW27783 expressing the empty pBad24 plasmid)
0h, 3h: refers to the induction time with 0.02% arabinose
An arrow points to the unique band in the TcfA and Par27 induced sample. The arrow on the right indicates the unique band at the size of Par27 (27kDa).

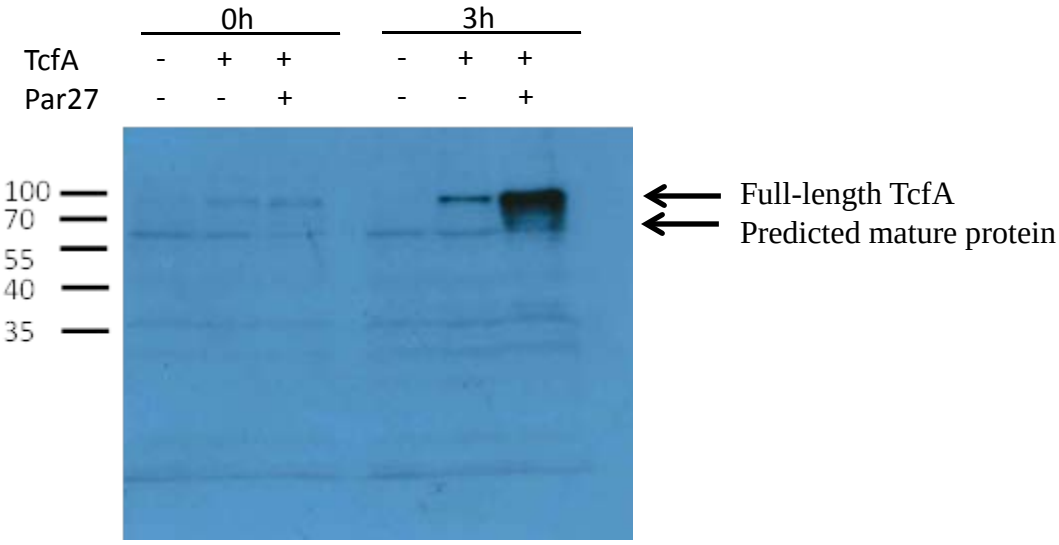


Figure 13: Western blot of whole cell lysates of *E. coli* expressing full-length TcfA and Par27
+ or -: refers to whether or not the full-length TcfA pBAD24 plasmid and the par27 pAM201 plasmid are expressed for each sample. (-,- means samples of BW27783 expressing the empty pBad24 plasmid). 0h, 3h: refers to the induction time with 0.02% arabinose. Arrows indicate the expected location of full-length TcfA and the predicted mature protein (cleaved passenger domain). An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

The effect that co-expressing Par27 with TcfA in *E. coli* has on TcfA expression makes sense in the context of additional work that was completed using chaperone knockout mutant strains in *E. coli*. While trying to troubleshoot the *E. coli* system, I also tested the secretion of TcfA in several chaperone knockout strains that another member of our lab had generated. I tested the expression of TcfA in SurA, Skp, DegP, PPiA and PPiD knockout strains. SurA, Skp and DegP are well characterized periplasmic chaperones in *E. coli*. PPiA and PPiD are PPIases that likely play roles in the folding of proteins in the periplasm. The results generated using the knockout strains were somewhat variable, likely a result of the integrity of the periplasmic space being compromised due to the absence of a specific chaperone. However, in general, the induction of TcfA in the chaperone knockout strains produced less full-length TcfA than in the WT BW27783 strain (Fig. 14). Since the secretion of TcfA is so dysfunctional in *E. coli* to start with, it is hard to make much of these results with some of the common chaperones. However, co-expressing Par27 with TcfA in *E. coli* had the opposite effect as disrupting these characterized chaperones in *E. coli*. Due to these complementary results, both unique and common chaperones are likely to be important for the secretion of TcfA.

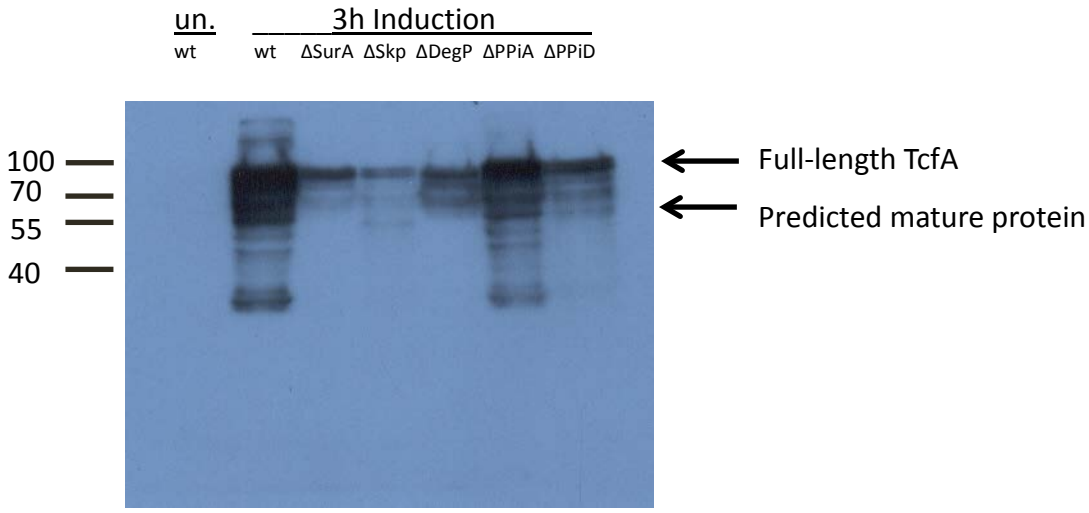


Figure 14: Western blot of whole cell lysates of chaperone knockout strains of *E. coli*
wt: wild type of *E. coli* strain BW27783 expressing the full-length pBAD TcfA clone. Δchaperone: chaperone knockouts strains of BW2778. un.: uninduced with arabinose. 3h induction: induced with 0.02% arabinose. Arrows indicate the expected location of full-length TcfA and the predicted mature protein (cleaved passenger domain). An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

In addition to putting Par27 into *E. coli*, I also disrupted Par27 in *B. pertussis*. Par27 was disrupted using the pEG7 suicide plasmid. The production of TcfA in wild type *B. pertussis* Tohama 1 was compared to the production of TcfA in the Par27 knockout. Although the results were not as pronounced as in *E. coli*, the Par27 knockout strain produced less TcfA than wild type Tohama 1 (Fig. 15). The coomassie stain showed that equivalent amounts of protein was loaded for WT and the Par27 knockout strain. However, disrupting Par27 in *B. pertussis* does not result in the kind of dysregulated secretion that is seen when TcfA is expressed in *E. coli*. It is clear that Par27 plays an important role in the secretion of TcfA, but it alone is not enough to restore the normal secretion of TcfA in *E. coli*.

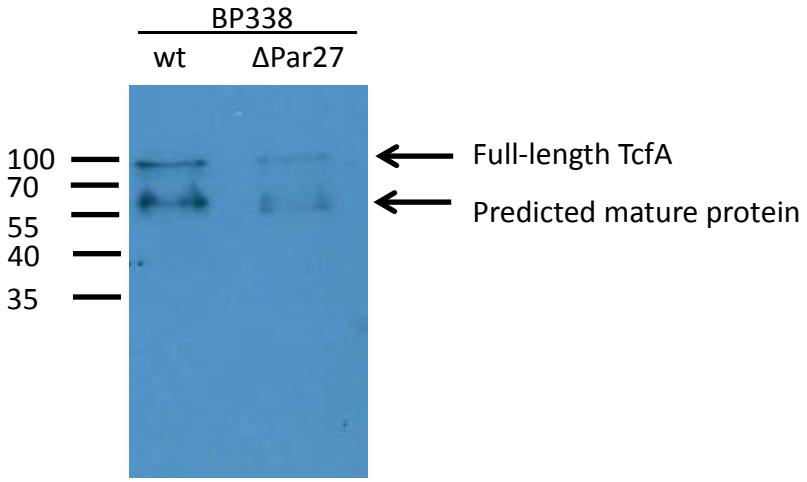


Figure 15: Western blot of whole cell lysates of *B. pertussis* using the TcfA antibody
wt: wild type *B. pertussis* strain BP338. ΔPar27: BP338 in which *par27* is disrupted. Arrows indicate the expected location of full-length TcfA and the predicted mature protein (cleaved passenger domain). An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

Since Par27 is important for the secretion of TcfA, I also wanted to determine if Par27 plays a similar role for the classically structured β-helical AT BrkA. In addition to its classical structure, BrkA also has a more common proline content of 4.6% compared to the 17% for TcfA. This experiment gives insight into whether Par27 plays a general role in AT secretion in *B. pertussis* or a more specialized role for specific ATs or possibly just TcfA. A western blot was performed using the WT Tohama 1 strain and the ΔPar27 Tohama 1 strain. An antibody generated to the passenger domain of BrkA was used for protein detection. The WT and ΔPar27 strains produced a similar amount of BrkA (Fig. 16). The coomassie stain again showed that equivalent amount of protein was loaded for WT and the Par27 knockout strain.

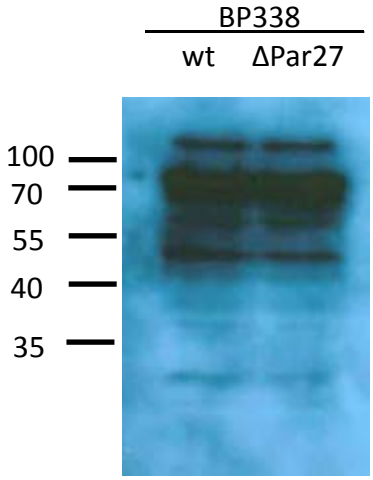


Figure 16. Western blot of whole cell lysates of *B. pertussis* using the BrkA antibody
wt: wild type *B. pertussis* strain BP338. ΔPar27: BP338 in which *par27* is disrupted. An antibody generated to the entire passenger domain of BrkA was used to detect expression. Molecular weight markers (kDa) are on the left. A 30% brightness increase was applied to the blot in order to make visualization easier.

Chapter 4: Determining the function of TcfA

4.1 Evidence that TcfA may bind Factor H

It is known that *B. pertussis* binds Factor H, but the ligand responsible for the binding has not been identified [47]. It has also been shown that residues in domain 19-20 of Factor H have been shown to be important for the ability of several microbes, including *B. pertussis,* to bind Factor H [46]. Due to these findings, I used the program pyDock which predicts protein-protein interactions in order to determine if TcfA could likely be the ligand that binds Factor H. An I-TASSER predicted model of the passenger domain of TcfA was used for the pyDock modeling because the crystal structure has not been solved. The pyDock model with the highest confidence factor of the predicted interaction between the I-TASSER model of TcfA and the crystal structure of domains 19-20 of human Factor H is pictured (Fig. 17). Figure 17 highlights the predicted interface between the passenger domain of TcfA (top) and Factor H (bottom). The three residues in Factor H that have been shown to be important for the ability of *B. pertussis* to bind Factor H are orange in the model [46]. According to the model, these three important residues appear to be present at the interface between TcfA and Factor H which suggests that an interaction is likely. However, the pyDock results need to be interpreted carefully because it is a model of a model.

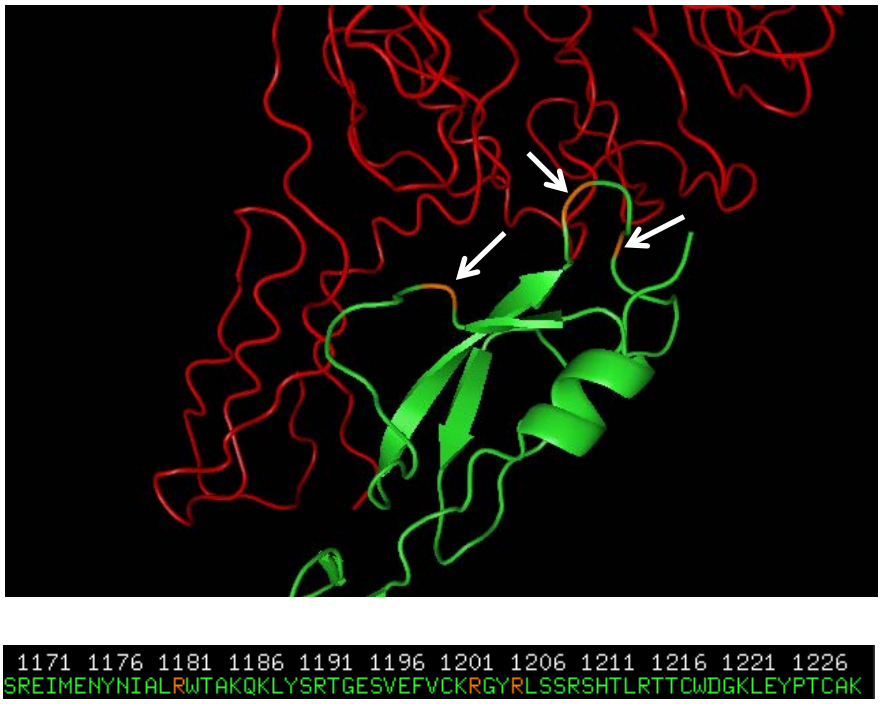


Figure 17: pyDock predicted interactions between TcfA and Factor H
Factor H domains 19-20 are in green. TcfA is in red. Arrows point to three residues (in orange) that were previously identified as being important for the ability of *B. pertussis* to bind Factor H (12). The N terminal portion of TcfA is on the bottom, and the C-terminal portion is above it

4.2 Determining if TcfA is essential for the ability of *B. pertussis* to bind Factor H

TcfA was disrupted in *B. pertussis* using the pEG7 suicide plasmid. A western blot using the anti-tcfA antibody confirmed the TcfA deletion (Fig. 18). The *B. pertussis* TcfA knockout reproducibly did not produce any full-length or passenger domain of TcfA. The mutant was then tested in a Factor H surface binding assay. *B. pertussis* Tohama 1, the BVG-negative derivative BP347 and *B. pertussis* Δ*tcfa* were used in the experiment. The three strains of *B. pertussis* were grown on plates for three days prior to the initiation of the experiment. On the third day, the bacteria were re-suspended in SS-Salts, and the ODs were adjusted to equivalent CFU amounts for the experiment. Each aliquot of bacteria was incubated with HI-NHS which provides a source of Factor H. After the incubation, a western blot was performed using anti-Factor H antibody. The molecular weight of Factor H is 155 kDa. As expected, Factor H did not bind to the BVG-negative BP347. However, Factor H still bound the *B. pertussis* Δ*tcfa* strain (Fig. 19). Clone #22 was used for all future experiments. These results indicate that either TcfA does not bind Factor H, or that multiple factors including TcfA bind Factor H.

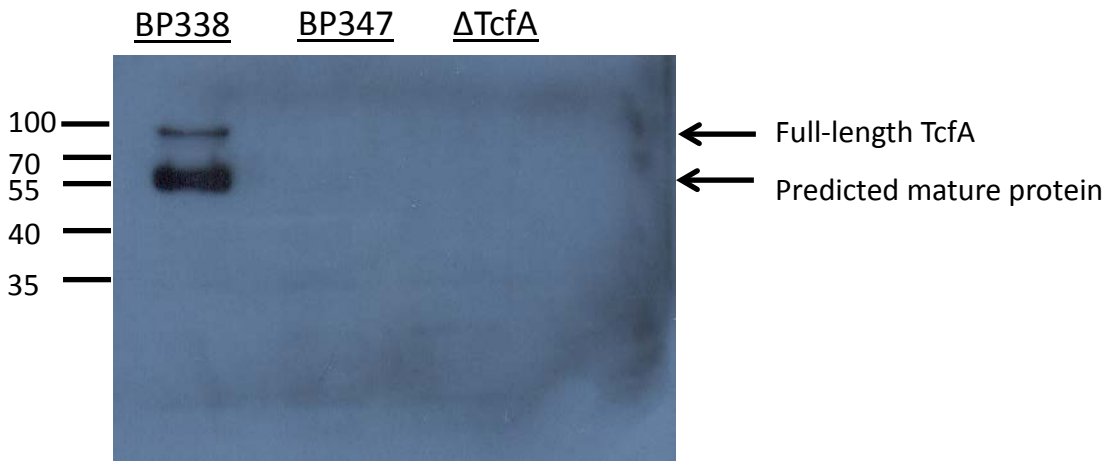


Figure 18. Western blot of whole cell lysates of wt *B. pertussis* and mutants

This figure is an expansion of the same blot as Figure 6, but it includes the results BP347 as well as ΔTcfA. An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. BP338 is a wild type strain of *B. pertussis.* BP347 is a BVG-negative derivative of BP338 which does not produce the protein virulence factors. ΔTcfA is a strain of BP338 in which *tcfa* is disrupted by a suicide plasmid. Western blots were performed on each strain following growth for 3 days on BG agar plates. Arrows indicate the expected location of full-length TcfA and the predicted mature protein (cleaved passenger domain). An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

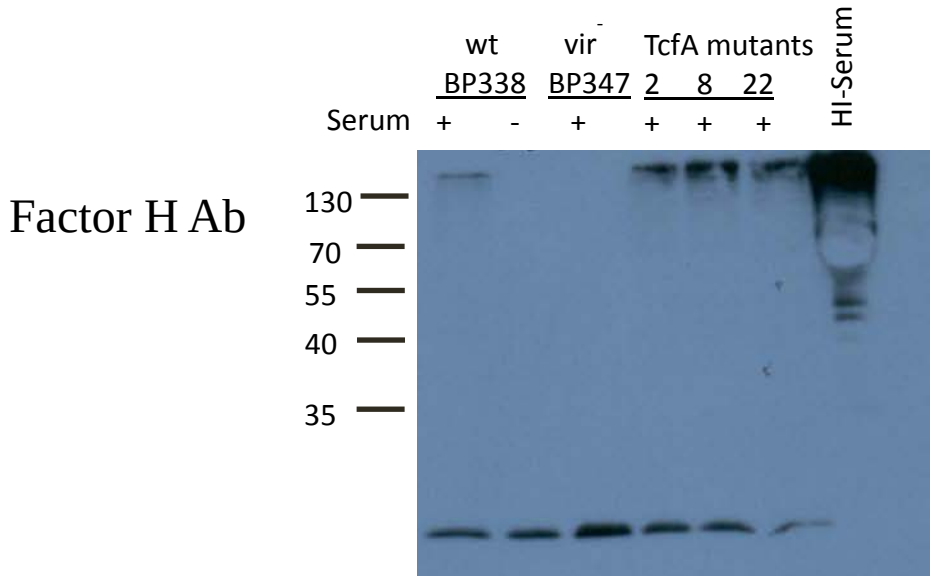


Figure 19: Factor H surface binding assay

BP338 is a wild type strain of *B. pertussis*. BP347 is a BVG-negative derivative of BP338 which does not produce the protein virulence factors. The TcfA mutants are three different transconjugants from the pEG7 *tcfA* disruption. HI-Serum is the serum control lane. +/- indicates whether or not heat-inactivated NHS was added to the bacteria. An antibody generated to a portion of the TcfA passenger domain was used to detect TcfA expression. Molecular weight markers (kDa) are on the left.

4.3 What could be the additional factor in *B. pertussis* that binds Factor H?

Directly upstream of TcfA in *B. pertussis* Tohama 1 is another AT that is predicted to have a coil structure: BapB. The layout of TcfA and BapB in Artemis is shown in Figure 20. Interestingly, approximately 200 base pairs of the translocation unit of BapB are identical to the translocation unit of TcfA. Furthermore, BapB in *B. parapertussis* is the best match to TcfA in *B. pertussis*. Since *B. parapertussis* binds Factor H but lacks TcfA, there must be an additional factor that is responsible for the binding in this species. BapB is also listed as an orthologous protein to TcfA in several species of *Bordetella* where TcfA is missing. The similarities in structure between TcfA and BapB suggest that they may play a similar functional role as well.

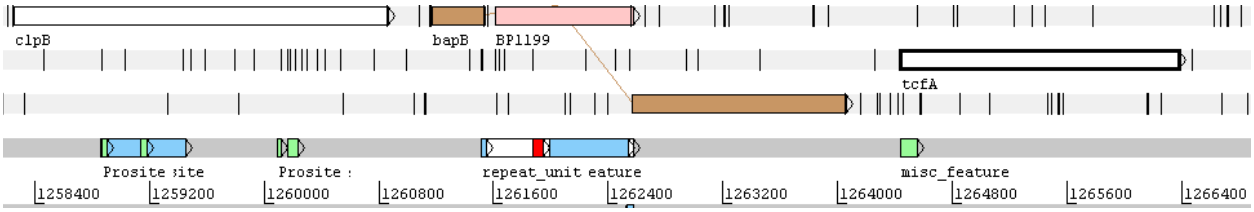


Figure 20. The genetic layout of *tcfa* and *bapb* in *B. pertussis* in Artemis *bapb* is in brown and the transposase is shown in pink. *Tcfa* is directly downstream of *bapb*.

There are many indications that BapB is the additional factor that binds Factor H in *B. pertussis*. However, in *B. pertussis* Tohama 1, BapB is annotated as being disrupted by a transposase. The genetic layout of *bapb* and *tcfa* in Tohama 1 is shown in Figure 20. This disruption by a transposase was confirmed by PCR using a forward primer at the start of *bapb* and a reverse primer at the end of *bapb*. The resulting product corresponded to the cumulative size of *bapb* and the transposable element, but it does not mean that BapB does not bind Factor H. Although unlikely, it is possible that a large fusion protein is created that retains its functionality. In order to address this possibility, I tried to create a *tcfa/bapb* clean deletion that could be tested for the ability to bind FactorH.

I was unable to generate the *bapb/tcfa* clean deletion in *B. pertussis*. The transposase present in *bapb* could have caused problems during cloning and the associated diparental mating protocol. Furthermore, the roughly 300 base pair identical sequence in the translocation unit of *tcfa* and *bapb* could have also caused problems. I tried to create the clean deletion mutant several times, but could never get the desired final product. An additional strategy could be to try to disrupt *bapb* in *B. parapertussis* where the full protein is intact.

4.4 Determining if TcfA plays a role in serum killing

Previously in a different strain of *B.* pertussis, TcfA was disrupted using a transposon mutant. However, there are a lot of potential problems with transposon mutagenesis, including downstream and non-specific effects. *B. pertussis* strain 18323 which was used for this previous mutation has also been shown to be an outlier of the *B. pertussis* strains. It has been described as being an intermediate in the evolution from *B. pertussis* to *B. parapertussis* and *B. bronchiseptica*, and has been shown to have other abnormalities including producing less

pertussis toxin than other *B. pertussis* strains[50]. Interestingly, in this strain of *B. pertussis*, deleting *tcfa* did not have an effect on serum killing [37]. I wanted to determine if similar results would be observed for the mutant that I generated: BP338Δ*tcfa*. BP338Δ*tcfa* was tested against WT BP338 and BP347 which does not produce the protein virulence factors. In the experiment, the three strains are incubated with either normal human serum (NHS) or heat-inactivated normal human serum (HI-NHS). The HI-NHS should not effectively kill *B. pertussis*. As a result, a percent survival can be calculated by comparing the CFU of the NHS groups to the HI-NHS groups. WT BP338 had a percent survival of 81%, BP347 had a percent survival of 59% and BP338Δ*tcfa* had a percent survival of 7% (Fig. 21). The results for BP338Δ*tcfa* were not statistically different from the results for WT BP338 by 1 way ANOVA. TcfA may play a small role in the serum survival of *B. pertussis*, but it does not seem to be the principle function of the protein.

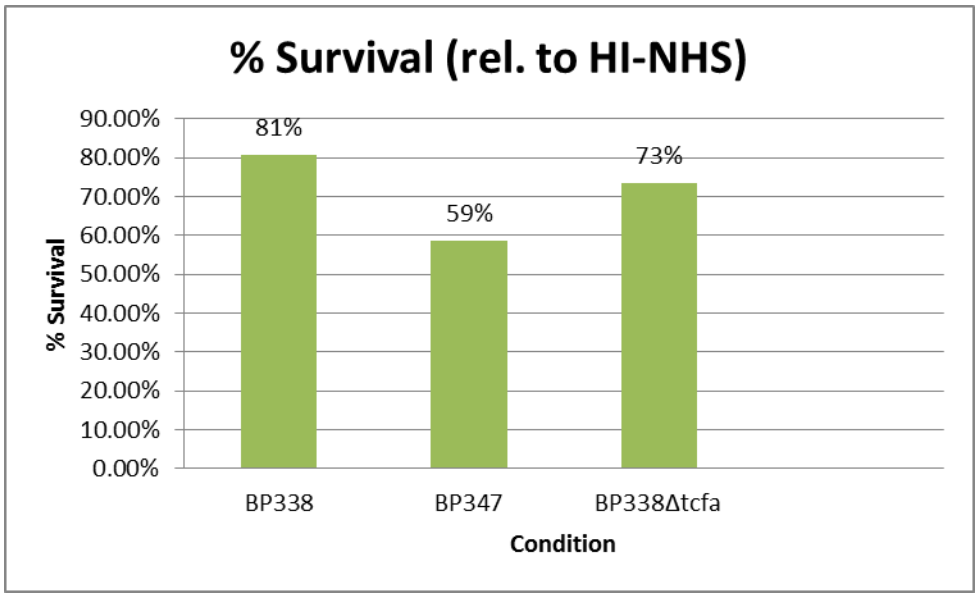


Figure 21: Results of Serum Killing Assay

BP338 is a wild type strain of *B. pertussis.* BP347 is a BVG-negative derivative of BP338 which does not produce the protein virulence factors. BP338ΔTcfA is a strain of BP338 in which *tcfa* is disrupted by a suicide plasmid. % Survival is calculated by comparing the CFU of the NHS treated group to the HI-NHS group (for each: BP338, BP347, BP338Δ*tcfa*). The percentages reflect an average of three replicates.

Chapter 5: Discussion

5.1 Overview

TcfA is an elusive protein. It has a predicted structure that is much different from the vast majority of ATs. TcfA is also only naturally expressed in *B. pertussis* and possesses a unique amino acid sequence [32]. Our lab and others have had success expressing *B. pertussis* ATs in *E. coli*, but TcfA simply cannot undergo its normal biogenesis in *E. coli*. Furthermore, despite being identified as a potential adhesin in a paper from 1995, TcfA's specific contribution to *B. pertussis* pathogenesis continues to be difficult to determine. [14] This study worked to characterize the secretion of TcfA and to increase our understanding of the potential contribution of TcfA to *B. pertussis* pathogenesis. The study answered some questions, and raised many additional questions.

5.2 TcfA has unique requirements for expression

A significant amount of time was committed towards trying to express TcfA in *E. coli*. The length of induction time, concentration of inducing agent and induction temperature were just some of the experimental conditions that were optimized. However, despite these efforts, I was unable to mimic the expression of TcfA in *B. pertussis*. Moving to the native host was useful, and suggests that *Bordetella* secretion should be studied in *Bordetella*. The addition of the unique *B. pertussis* chaperone Par27 to the *E. coli* system resulted in increased expression of TcfA, but Par27 alone was not sufficient to rescue the biogenesis of TcfA in *E. coli*. It is likely that Par27 is one of a few *B. pertussis*-specific factors that are important for the secretion of TcfA. It remains to be determined if there are other unique *B. pertussis* chaperones that are

important for secretion, as well as what the contribution could be of secretion machinery such as TAM and BAM[25].

5.2.1 Future directions: secretion studies

Many questions about the secretion of TcfA still remain unanswered. Although I tested the secretion of TcfA in the *E. coli* chaperone knockouts, since TcfA does not undergo its normal biogenesis in *E. coli* it is difficult to draw conclusions from the results. Furthermore, there are a lot of unanswered questions about how TcfA acquires its unique structure either during or after secretion. TcfA is not predicted to have a characteristic autochaperone region, but it is still possible that the C-terminus region of the passenger domain acts as scaffold upon which folding is initiated [51, 52]. It would be useful to test this hypothesis by complementing BP338ΔTcfA with variants of TcfA with deletions in portions of the C-terminus and then evaluating secretion by western blot.

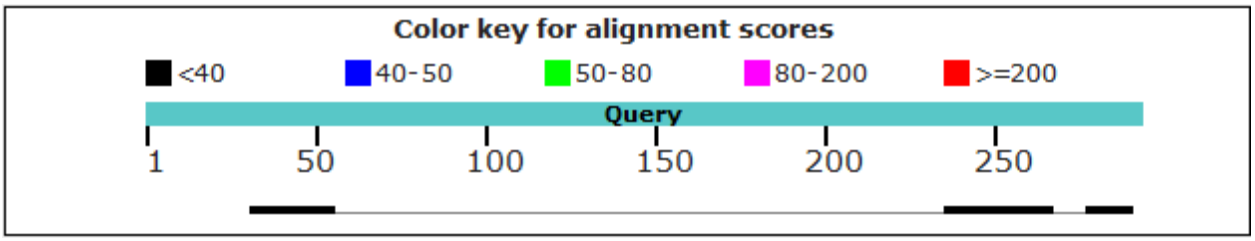
The promising results that were generated by disrupting Par27 in *B. pertussis* begs the question of whether or not other accessory factors are important for the secretion of TcfA in *B. pertussis*. A logical next approach would be to test the secretion of TcfA in *B. pertussis* knockout strains of some of the well-characterized chaperones. Another group tried to generate a SurA knockout strain of *B. pertussis*, but it was deemed essential [49]. However, other well-characterized chaperones such as DegP have been successfully knocked out in *B. pertussis*[53]. It would be valuable to obtain or generate additional chaperone knockout strains and testing for the effect on TcfA secretion. Additionally, an *in vivo* cross-linking approach or overlay assay could be used to identify other accessory factors that are important for the secretion of TcfA. If accessory factors that are unique to *B. pertussis* are identified, then these factors could be expressed in *E. coli* in a similar strategy to what was used for testing for the importance of Par27.

An additional strategy would be to use a bioinformatic approach. PSORT is a computer program for the prediction of protein localization. A PSORT comparison of the periplasmic proteins in *E. coli* and *B. pertussis* could help with the identification of additional unique *B. pertussis* chaperones that could be important for the secretion of TcfA.

TcfA appears to require both common and unique chaperones for its secretion, but it is unknown what other factors are important for the secretion of this unique AT. Further studies are required to determine if additional factors such as BAM and TAM are important. In one study, it was shown that the BAM complex and SurA are both necessary and sufficient for promoting the assembly of the *E. coli* O157:H7 autotransporter EspP in vitro[54]. In this study, AT assembly was reconstituted *in vitro* using purified components that were known to be important for secretion. It would be significantly more difficult to perform a study like this for TcfA because there are clearly more factors involved, but a system like this would allow for the importance of assembly machinery to be evaluated. Furthermore, it has been shown that the BAM complex of *E. coli* and *Neisseria* have some important differences including the fact that the *E. coli* BamD cannot replace the *Neisseria* BamD. The *Neisseria* BAM complex does not form when the *E. coli* BamD is substituted in the complex [55]. Although the *E. coli* BAM complex works for the secretion of some *B. pertussis* proteins such as BrkA, it is possible that other proteins such as TcfA require the native assembly machinery. *E. coli* is the most commonly used model system for the secretion of proteins from virulent microbes, but the previously mentioned results with *Neisseria* mean that TcfA would not be the first protein to require secretion machinery that is unique to its native host[55].

5.3 What else could be binding Factor H in *B. pertussis*?

Evidence from modeling suggests that TcfA binds Factor H *in vitro*. However, work done for this study proved that if TcfA binds Factor H to the surface of the bacteria, it cannot be the only factor that is responsible for the binding. Logically, the question becomes, what is the additional factor that binds Factor H in *B. pertussis*? In the results section I outlined the reasons why BapB could very well be the additional factor that binds Factor H in *B. pertussis*. Some of the reasons include the fact that BapB has significant sequence similarity to the translocation unit of TcfA, is also listed as a coiled AT and is listed as an orthologous protein to TcfA in other species of *Bordetella* where *tcfa* is not expressed. Since the translocation units of BapB and TcfA are virtually identical, I also did a BLAST analysis in order to determine the similarities between signal peptides and passenger domains of the two proteins (Fig. 22). Three alignments were identified, but they are for small regions and do not have high percent identity.



TcfA

Sequence ID: Query_102529 Length: 350 Number of Matches: 3

Range 1: 62 to 89 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
15.0 bits(27)	3.0	Compositional matrix adjust.	11/32(34%)	21/32(65%)	4/32(12%)

```
Query  234  QEYRKDLDKWLAPPAKSGPPSAPPEAPPQAQP  265
            +++++ L +  AP A + PPS+   +PP A+P
Sbjct  62   EDFKRSL-QESAPSALATPPSS---SPPVAKP  89
```

Range 2: 77 to 90 Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Method	Identities	Positives	Gaps
14.2 bits(25)	4.7	Compositional matrix adjust.	6/14(43%)	8/14(57%)	0/14(0%)

```
Query  275  AEPPAARPPAVPPA  288
            A PP++ PP   P
Sbjct  77   ATPPSSSPPVAKPG  90
```

Range 3: 163 to 183 Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Method	Identities	Positives	Gaps
13.9 bits(24)	6.4	Compositional matrix adjust.	9/25(36%)	14/25(56%)	4/25(16%)

```
Query  31   LAALAGSGAICAAPAWATGAGLDDL  55
            +A+ AGS     +P  + GAG D +
Sbjct  163  MASGAGS----TSPGASGGAGKDAM  183
```

Figure 22: BLAST 2 analysis of the passenger domains of TcfA and BapB
The signal peptide and passenger domain of TcfA from *B. pertussis* Tohama 1 was inputted as the subjected sequence. The signal peptide and passenger domain of BapB from *B. parapertussis* Bpp5 was inputted as the query sequence. The three identified alignments are depicted below the graphic summary.

Several strategies could be used in order to determine if BapB is the additional factor that binds Factor H. One strategy is to create a clean deletion of both *tcfa* and *bapb* in *B. pertussis* and test this mutant for the ability to bind Factor H. I have not had success generating clean deletions of TcfA in *B. pertussis*, but it should be a viable strategy. An additional strategy would be to disrupt *bapb* in *B. parapertussis* and then test this mutant for the ability to bind Factor H. The logic behind this strategy is that it is known that *B. parapertussis* binds Factor H despite not expressing TcfA. If *B. parapertussisΔbapB* does not bind Factor H, then it is likely that BapB is also the additional factor in *B. pertussis* that binds Factor H. If BapB does not prove to be the additional factor that binds Factor H, then some other techniques could be used in order to identify other candidate proteins. One such strategy would be to use what is known as a Far-Western or gel-overlay strategy in combination with mass spectrometry. This strategy was used for identifying Vag8 as the factor in *B. pertussis* that bind C1 inhibitor[36]. In brief, whole cell lysates of *B. pertussis* are subjected to SDS-PAGE and then the separated proteins are blotted on a membrane. The proteins are renatured, and then overlaid with Normal Human Serum. Standard immunoblot procedure would follow using an anti-Factor H antibody. Mass spectrometry and bioinformatic analysis would hopefully allow for the determination of any proteins that associate with Factor H. Furthermore, pull down assays could also be used as another means of identification.

Modeling done using the program pyDock suggested that TcfA and Factor H interact (Fig. 17). In order to determine if pyDock modeling would also suggest an interaction between BapB and Factor H, the same modeling parameters were used except BapB was inputted in place of TcfA (Fig. 23). The three residues that were previously identified as being important for the ability of B. pertussis to bind Factor H do not appear to associate as closely with BapB as they

did with TcfA. BapB could interact with Factor H in a different way than TcfA, or could simply not interact at all. It also must be noted that these are once again predictions of predictions since the structure of BapB has not been solved.

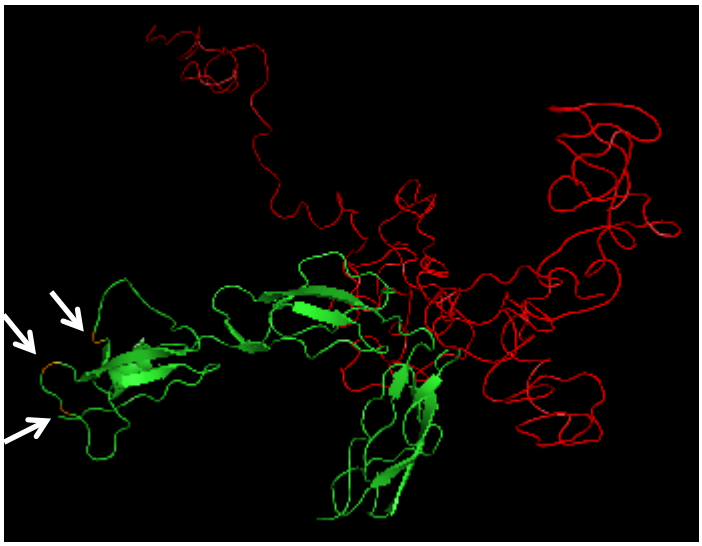


Figure 23: pyDock predicted interactions between BapB and Factor H
Factor H domains 19-20 are in green. The passenger domain of BapB is in red. Three residues that were previously identified as being important for the ability of *B. pertussis* to bind Factor H are in orange (12). The N terminal portion of BapB is on the left, and the C-terminal portion is to the right of it.

5.4 What exactly is the role of TcfA in *B. pertussis* pathogenesis?

Regardless of whether BapB is the additional factor that binds Factor H, the specific role of TcfA in *B. pertussis* pathogenesis still needs to be elucidated. One possibility is that TcfA does not actually bind Factor H *in vivo*. It is also possible that TcfA does bind Factor H, but that the binding is not actually beneficial to *B. pertussis*. A study using *C. albicans* showed that binding of Factor H by the yeast actually facilitated the killing of *C. albicans* by neutrophils via recognition by complement receptor 3[56]. It is possible that a similar phenomenon could occur for a microbe such as *B. pertussis*. If so, then this could be an explanation as to why disrupting *tcfa* in *B. pertussis* has no effect on the ability of *B. pertussis* to survive in the lung in a mouse model, despite being important for survival in the trachea [14]. It could be that the positive effect of recruiting Factor H is balanced out by the negative effect of increased detection and killing due to increased recognition by macrophages. Strains of *B. pertussis* that do not express TcfA have also emerged. It is possible that not expressing TcfA may confer an advantage during certain stages of transmission and infection [57].

TcfA may play a small role in serum survival, but it seems unlikely that it is the main function of the protein. It is also possible that TcfA has a specific role as an adhesin that remains to be elucidated. Recently, the non-human primate model of pertussis has been shown to be an excellent clinical model [58]. This model is likely the most useful model for investigating pertussis pathogenesis as well as for evaluating current and future vaccines. As a result, the non-human primate model of pertussis could be useful for addressing questions about the role of TcfA in colonization.

One question that this study raises is: what is the benefit or cost of a microbe potentially having multiple factors that bind Factor H to the surface of the bacteria? One possible answer is

that it is a case of host/pathogen evolution. If the ability for *B. pertussis* to bind Factor H is essential for virulence, then *B. pertussis* might have evolved to have multiple factors capable of facilitating the binding. Since clinical isolates of *B. pertussis* that do not produce TcfA have been identified, it is possible that TcfA is under immune surveillance. Possessing multiple factors to do the same job would allow *B. pertussi*s to lose TcfA without losing an important virulence function. Further analysis of the genetic makeup of clinical isolates of *B. pertussis* would allow this theory to be evaluated. As mentioned previously, it is also possible that the main function of TcfA is not complement evasion after all but rather a specific role as an adhesin. If this is the case, then the specific role as well as its contribution to the overall ability of *B. pertussis* to bind to epithelial cells would need to be deciphered.

5.5 Conclusion

TcfA is an elusive protein with unique secretion requirements that make it difficult to study. Attempts to generate a model system in *E. coli* were unsuccessful, and highlight the importance of studying protein secretion in *B. pertussis*. This thesis identified that the secretion of TcfA is aided by *B. pertussis* specific secretion factors. The contribution of the *B. pertussis* chaperone Par27 was assessed both in the flawed *E. coli* model system as well as in the native host. It is likely that Par27 is one of several *B. pertussis* secretion factors that are important for the secretion of TcfA. Additional research is needed in order to assess the importance of the additional factors. Since ATs are good models for protein secretion, it is possible that our increased knowledge about the special requirements for the secretion of TcfA could be useful for understanding the secretion of other proteins as well. Furthermore, it is difficult to determine the specific contribution of TcfA to *B. pertussis* pathogenesis. This thesis determined that TcfA is not the sole factor responsible for the binding of Factor H to the surface of *B. pertussis* Tohama

1. Preliminary work was done in order to identify BapB as a potential additional factor that Binds Factor H. However, additional work is needed in order to definitively determine if TcfA binds Factor H *in vivo*, and if not, what else is responsible for the binding. This thesis answered many questions, and raised many more.

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