BIOGENESIS OF BapF, A NOVEL ACYLATED BORDETELLA AUTOTRANSPORTER

by

Peter Vincent Sims

B. Sc., The University of British Columbia 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

in

The Faculty of Graduate Studies
(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

March 2012

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Abstract

Autotransporters are a superfamily of Gram-negative secreted proteins, composed of a C-terminal domain which forms a β-barrel in the outer membrane and plays a significant role in translocation of the N-terminal passenger domain to the cell surface. A subfamily of autotransporters is N-terminally acylated during their biogenesis, a post-translational modification demonstrated to be essential for their function. Considering the autotransporter passenger domain is secreted beyond the outer membrane, how translocation can occur in the presence of N-terminal acyl groups is undetermined. The work in this thesis describes the biogenesis of the Bordetella autotransporter F (BapF) from B. bronchiseptica RB50, which is N-terminally acylated during the initial stages of its secretion to the cell surface. The acyl groups attached to BapF at the Cys$^{28}$ residue were shown to be subsequently removed by signal peptidase 1 cleavage between residues Ala$^{34}$ and Ala$^{35}$, indicating that BapF forms an acylated intermediate in its secretion pathway. Studying the secretion of BapF mutants in which N-terminal acylation was ablated revealed that the passenger domain was still capable of reaching the cell surface; the surface-expressed passenger domain mediated phenotypes of cellular aggregation and an increased rate of culture sedimentation, similar to that observed in E. coli cultures expressing the wild-type BapF protein. However, cells expressing these mutants appear to have damaged outer membranes, potentially due to the observed increase in full-length protein accumulating in the periplasm. Alternatively, E. coli cells expressing a BapF mutant in which signal peptidase 1 cleavage is blocked do not exhibit obvious aggregation and sedimentation phenotypes. Yet, an independent passenger domain is clearly produced. Based on the results presented in this thesis, it can be hypothesized that sequential processing of the BapF signal peptide, producing an acylated intermediate in the secretion pathway, helps to regulate the passage of BapF through the periplasm ultimately permitting surface expression. In addition, bioinformatic and molecular analysis strongly suggest the BapF passenger domain folds into a β-propeller structure, and if proven to do so, BapF will be the first autotransporter reported with this conformation.
Preface

Sections of chapter 3 will be incorporated into: Sims, P., J. John, F. Jean, and R.C. Fernandez. Biogenesis of BapF, a novel Bordetella lipoprotein autotransporter (manuscript in preparation). Chapter 3 is a characterization of the wild-type BapF autotransporter from *B. bronchiseptica* RB50. Creation of the pET30bBapFPass expression plasmid is credited to Emma Kim, who worked as a summer student in the Fernandez lab. Mass spectroscopy was performed in collaboration with Dr. Julius John in Dr. Francois Jean’s lab in the Department of Microbiology and Immunology at the University of British Columbia.

Sections of chapter 4 will be incorporated into: Sims, P., J. John, S. Xu, B. McLeod, F. Jean, and R.C. Fernandez. Influence of signal peptide processing sites in the biogenesis of the Bordetella lipoprotein autotransporter, BapF (manuscript in preparation). Creation of the pBAD24/bapF_{Prn} and pBAD24/bapF^{C28S} expression plasmids is credited to Dr. Brett McLeod who was a postdoctoral fellow in the Fernandez lab. Shengjuan Xu, a graduate student in the Fernandez lab, generated the data for Figure 44. Mass spectroscopy was again performed in collaboration with Dr. Julius John in Dr. Francois Jean’s lab in the Dept. of Microbiology and Immunology at the University of British Columbia.

Biohazard Approval Certificate numbers under which this research was conducted are: H03-0255 and B06-0255 issued by the Biosafety Committee, Office of Research Services at the University of British Columbia.
Chapter 1: Introduction

1.1. The bacterial cell envelope ................................................................. 1

1.2. Secretion across the bacterial inner membrane ........................................... 2

1.2.1. Signal peptides ............................................................................. 3

1.2.2. The Sec system ........................................................................... 3

1.3. Outer membrane protein biogenesis: the β-barrel Assembly Machinery .......... 7

1.3.1. Identification of the BAM complex ..................................................... 7

1.3.2. Composition of the BAM complex ....................................................... 9

1.3.2.1. BamA ...................................................................................... 9

1.3.2.2. BamB-E .................................................................................. 9

1.4. Transit of outer membrane proteins through the periplasm .......................... 10

1.5. Integration of outer membrane proteins into the outer membrane .................. 10

1.6. Lipoprotein biogenesis and localization ..................................................... 12

1.6.1. N-terminal acylation ...................................................................... 13

1.6.2. Lipoprotein localization ................................................................... 15

1.6.2.1. Lipoprotein sorting signals ........................................................... 15

1.6.2.2. The LOL pathway .................................................................... 15

1.7. Secretion systems of Gram-negative bacteria .............................................. 16

1.7.1. Type I secretion ............................................................................ 19

1.7.2. Type II secretion ............................................................................ 19

1.7.3. Type III secretion ............................................................................ 19

1.7.4. Type IV secretion ............................................................................ 20

1.7.5. Type V secretion ............................................................................ 20

1.7.6. Type VI secretion ............................................................................ 21

1.8. Secretion via the Type V monomeric autotransporter pathway .................... 22

1.8.1. General features of monomeric autotransporters ................................ 23

1.8.2. Structure and folding of autotransporter passenger domains .................. 24
1.8.3. Translocation unit structure and outer membrane integration ................................... 25
1.9. Autotransporters with N-terminal acylation ................................................................. 27
1.10. Thesis goals .................................................................................................................. 28

Chapter 2: Materials and methods ....................................................................................... 30
2.1. Bacterial strains, plasmids and primers ............................................................................. 30
2.2. Isolation of genomic DNA from Bordetella bronchiseptica RB50 .................................. 33
2.3. PCR amplification of bapF from B. bronchiseptica RB50 genomic DNA ......................... 33
2.4. Cloning bapF from B. bronchiseptica RB50 into pBS(KS) ............................................. 33
2.5. Subcloning bapF into pET30b and pBAD24 for inducible expression studies .................. 34
  2.5.1. Subcloning bapF into pET30b ....................................................................................... 34
  2.5.2. Subcloning bapF into pBAD24 .................................................................................... 34
2.6. Generating the bapF^{C28S} signal peptide mutant .......................................................... 35
2.7. Generating bapF^{A34-35S} and bapF^{A32S,S33P,A34-35S,A37S} signal peptide mutants .... 35
2.8. Generating the bapF^{Prn} signal peptide mutant ............................................................. 36
2.9. Subcloning the BapF passenger into the pET30b expression vector ................................ 36
2.10. Purification of the BapF passenger domain ................................................................... 37
2.11. Far-UV circular dichroism spectroscopy of refolded BapF passenger ............................ 38
2.12. Raising polyclonal antibodies to the bapF passenger domain ....................................... 38
2.13. SDS-PAGE and immunoblot detection of BapF, BrkA and BamA ................................ 39
2.14. Expression of BapF in E. coli ......................................................................................... 39
2.15. Observation of culture sedimentation differences between BapF clones ....................... 40
2.16. Immunofluorescence microscopy ................................................................................. 40
2.17. N-lauryl sarcosyl fractionation and enrichment of BapF ................................................. 41
2.18. Mass spectroscopy of full length BapF, passenger and β-barrel domains ....................... 42
  2.18.1. In-gel labeling and chymotrypsin digestions ............................................................... 42
  2.18.2. LC-MS/MS and data analysis ..................................................................................... 43
  2.18.3. Identifying the N-terminus of the BapF passenger and translocation unit ................. 44
2.19. Separation of membrane proteins using floatation sucrose gradient fractionation .......... 44
2.20. Protease susceptibility assays ....................................................................................... 45
2.21. Precipitation of protease-released fragments from the cell surface ............................... 46
2.22. Globomycin treatment of E. coli cultures expressing BapF or BrkA ............................... 46

Chapter 3: Characterizing secretion of the novel Bordetella autotransporter BapF ............... 47
3.1. Introduction ...................................................................................................................... 47
3.2. Bioinformatic analysis ..................................................................................................... 50
  3.2.1. Primary sequence analysis .......................................................................................... 50
3.2.2. Secondary structure prediction ................................................................. 54
3.2.3. Tertiary structure prediction ................................................................. 54
3.2.4. Repeats and regions of hydrophobicity identified in the BapF passenger .......... 55
3.3. Expression of BapF in B. bronchiseptica RB50 is regulated by BvgAS ............ 59
3.4. E. coli model system to study BapF biogenesis ........................................... 62
3.5. Phenotype of E. coli cells expressing BapF .................................................. 62
3.6. Molecular analysis of BapF expressed in E. coli .......................................... 64
3.6.1. Enrichment of outer membrane fractions .................................................. 64
3.6.2. Identifying unique bands isolated from E. coli cells expressing BapF ............. 66
3.6.3. Globomycin specifically affects BapF biogenesis ...................................... 66
3.6.4. BapF is localized to the outer membrane .................................................. 72
3.6.5. Surface expression of the BapF passenger domain ...................................... 74
3.6.5.1. Fluorescence microscopy ........................................................................ 74
3.6.5.2. Limited trypsin susceptibility assay ......................................................... 74
3.6.6. Trypsin releases the BapF passenger from the cell surface ......................... 78
3.6.7. In vitro refolded BapF passenger exhibits trypsin resistance ......................... 80
3.7. Conclusions .................................................................................................. 83

Chapter 4: The role of signal peptidase 1 and signal peptidase 2 processing in BapF secretion .... 84
4.1. Introduction .................................................................................................... 84
4.2. Disrupting signal peptidase 2 processing results in accumulation of unprocessed BapF .... 84
4.2.1. Generating mutants which block acylation .................................................. 84
4.2.2. Phenotypes induced in E. coli by expression of BapF\text{C28S} and BapF\text{Prn} ...... 86
4.2.3. Detection of BapF\text{C28S} and BapF\text{Prn} in E. coli whole-cell lysates .............. 88
4.2.4. Sucrose gradient fractionation to determine localization of BapF\text{C28S} .......... 90
4.2.5. The BapF\text{C28S} and BapF\text{Prn} passenger domains are surface expressed ...... 92
4.2.6. When outer membrane integrity is disturbed, E. coli cells expressing BapF\text{WT} can produce a similar profile of lower molecular weight fragments as those produced from BapF\text{C28S} and BapF\text{Prn} mutants .......... 95
4.3. Disrupting signal peptidase 1 processing in BapF biogenesis results in lower levels of BapF expression ............................................................................................................. 97
4.3.1. Generating a BapF signal peptidase 1 mutant ............................................. 97
4.3.2. Expression of BapF\text{AA34-35S} in E. coli .................................................... 100
4.3.3. Probing surface expression of the BapF\text{AA34-35S} passenger domain .......... 103
4.3.4. Mass spectroscopy analysis of the BapF\text{AA34-35S} passenger domain reveals signal peptidase 1 processing was not blocked .......................................................... 106
4.3.5. Generating a new BapF signal peptidase 1 mutant ..................................... 107
4.3.6. Expressing BapF\text{A32S,S33P,AA34-35S,A37S} in E. coli ............................... 109
4.3.7. Mass spectroscopy analysis of the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger domain ............... 109

4.3.8. Assessing surface expression of the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger domain............ 112

4.4. Conclusions .............................................................................................................................................. 116

Chapter 5: Discussion ........................................................................................................................................ 118

5.1. Overview of findings from studies describing BapF biogenesis ................................................................. 118

5.2. The effects of removing acylation from the BapF secretion pathway ....................................................... 119

5.3. The effects of blocking signal peptidase 1 cleavage in the BapF secretion pathway .............................. 124

5.4. Biogenesis of the \textit{B. bronchiseptica} RB50 autotransporter BapF .............................................................. 130

References ..................................................................................................................................................... 137
List of Tables

Table 1: Plasmids and strains ................................................................. 31
Table 2: Primers and oligonucleotides .................................................. 32
List of Figures

Figure 1: Structure of the Gram-negative and Gram-positive cell envelope ........................................... 2
Figure 2: Model of translocation by the Sec translocation machinery ......................................................... 6
Figure 3: Schematic representation of outer membrane protein biogenesis in *Escherichia coli* .................. 8
Figure 4: Biosynthesis and outer membrane localization of lipoproteins .................................................. 14
Figure 5: Schematic representation depicting secretion pathways in Gram-negative bacteria .................... 18
Figure 6: Comparison of different autotransporter secretion pathways ..................................................... 22
Figure 7: Domain architecture of monomeric autotransporters ................................................................. 23
Figure 8: Structure of *Bordetella pertussis* pertactin passenger domain .................................................. 25
Figure 9: Structure of an autotransporter translocation unit ....................................................................... 26
Figure 10: Phylogenetic analysis of known and predicted transporter domains from *B. bronchiseptica* auto transporters ................................................................. 49
Figure 11: SignalP and LipoP results for BapF ............................................................................................. 52
Figure 12: Amino acid sequence and stick model of BapF ........................................................................... 53
Figure 13: PSIPRED secondary structure prediction for BapF ................................................................. 56
Figure 14: Phyre prediction of tertiary structure for the BapF passenger domain ........................................ 57
Figure 15: Tango and HHpredI analysis of the BapF passenger domain .................................................... 58
Figure 16: Expression of BapF in *B. bronchiseptica* RB50 under Bvg- conditions .................................... 61
Figure 17: Sedimentation and aggregation of *E. coli* cells expressing BapF ................................................ 63
Figure 18: Observation of BapF in N-aryl sarscino enriched samples derived from *E. coli* BW27783 cultures ................................................................. 65
Figure 19: Titration of globomycin .......................................................................................................... 69
Figure 20: Globomycin inhibition of BapF biogenesis .................................................................................. 70
Figure 21: Globomycin inhibits aggregation and sedimentation phenotypes in BapF-expressing *E. coli* cultures ......................................................................................................... 71
Figure 22: Sucrose-gradient fractionation of membrane fragments from cells expressing BapF ................. 73
Figure 23: Fluorescence microscopy confirms surface expression of the BapF passenger .......................... 76
Figure 24: Trypsin susceptibility assay suggests surface expression of the BapF passenger domain ........ 77
Figure 25: Trypsin-released BapF passenger found in supernatants ......................................................... 79
Figure 26: In vitro trypsin digestion of refolded BapF passenger ............................................................... 82
Figure 27: BapF<sub>C28S</sub> and BapF<sub>Prn</sub> signal peptides and predicted secretion pathway ................. 85
Figure 28: Sedimentation and aggregation phenotypes for BapF<sub>C28S</sub> or BapF<sub>Prn</sub> mutants ................. 87
Figure 29: Western blot detection of BapF<sub>C28S</sub> or BapF<sub>Prn</sub> mutants in *E. coli* BW27783 cells .......... 89
Figure 30: Sucrose gradient fractionation of whole-cell lysate from cells expressing BapF<sub>C28S</sub> ............. 91
Figure 31: Fluorescence microscopy reveals surface expression of BapF<sub>C28S</sub> and BapF<sub>Prn</sub> passenger domains ........................................................................................................ 93
Figure 32: Trypsin susceptibility assay performed on cells expressing BapF<sub>C28S</sub> or BapF<sub>Prn</sub> ............. 94
Figure 33: Trypsin susceptibility assay performed on cells expressing BapF<sub>WT</sub> but with the OM made permeable ................................................................................................................. 96
Figure 34: SignalP and LipoP predictions for the BapF<sub>AA34-35S</sub> signal peptide .................................... 98
Figure 35: The BapF<sub>AA34-35S</sub> signal peptide and predicted secretion pathway .................................... 99
Figure 36: Sedimentation and aggregation phenotype produced in BapF<sub>AA34-35S</sub> expressing cells .......... 101
Figure 37: Expression of BapF<sub>AA34-35S</sub> in *E. coli* BW27783 cells .................................................... 102
Figure 38: Fluorescence microscopy reveals surface expression of the BapF<sub>AA34-35S</sub> passenger ........ 104
Figure 39: Trypsin susceptibility assay performed on *E. coli* cells expressing BapF<sub>AA34-35S</sub> ............... 105
Figure 40: SignalP and LipoP results for BapF<sub>A32S,S33P,AA34-35S,A37S</sub> ........................................... 108
Figure 41: Chymotrypsin cleavage sites within the range of Cys<sup>28</sup>–Lys<sup>60</sup> of the BapF<sub>A32S,S33P,AA34-35S,A37S</sub> passenger ........................................................................................................ 111
Figure 42: Aggregation and sedimentation results for *E. coli* cells expressing BapF<sub>A32S,S33P,AA34-35S,A37S</sub> detected in *E. coli* via Western blot ........................................... 113
Figure 43: Expression of BapF<sub>A32S,S33P,AA34-35S,A37S</sub> detected in *E. coli* via Western blot ...... 114
Figure 44: Fluorescence microscopy suggests less surface expression of the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} mutant compared to the BapF\textsuperscript{WT} clone................................................................. 115
Figure 45: Potential model for secretion of BapF\textsuperscript{Pm} and BapF\textsuperscript{C28S} ................................................................. 123
Figure 46: Potential model for secretion of BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} ................................................................. 129
Figure 47: Potential model for BapF\textsuperscript{WT} secretion.......................................................................................... 136
List of Abbreviations

ABC: ATP-binding cassette
AC: autochaperone
BAM: β-barrel assembly machinery
BG: Bordet-Gengou
BLAST: The Basic Local Alignment Search Tool
BSA: bovine serum albumin
CD: circular dichroism
CID: collision-induced dissociation
COG: Cluster of Orthologous Groups
DIC: differential interference contrast microscopy
ESPR: extended signal peptide region
ETD: electron-transfer dissociation
FITC: fluorescein isothiocyanate
IM: inner membrane
IPTG: isopropyl-β-D-thiogalactopyranoside
LB: Luria broth
LTQ: linear trap quadrupole
Lol: lipoprotein localization
LPS: lipopolysaccharide
MFP: membrane fusion protein
MS: mass spectroscopy
MT: multimeric translocon
OM: outer membrane
OMP: outer membrane protein
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PMSF: phenylmethylsulfonyl fluoride
POTRA: polypeptide transport-associated
PSR: periplasmic spanning region
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec: (secretion) system
SP: signal peptide
SP1: signal peptidase 1
SP2: signal peptidase 2
SRP: signal recognition particle
Tat: twin-arginine translocation
TCA: trichloroacetic acid
TCM: target cell membrane
TF: trigger factor
TPS: two-partner secretion
TU: translocation unit
X-gal: 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
Acknowledgements

I would like to especially thank Dr. Rachel C. Fernandez for her encouragement, thoroughness and commitment. I am very fortunate to have had such a mentor.

Special thanks go to my committee members Dr. J. Thomas Beatty, Dr. Robert E. Hancock and Dr. Frank Duong. I greatly appreciate your efforts to keep me going in the right direction.

I would like to acknowledge Shengjuan Xu, a graduate student currently in the Fernandez lab, for supplying data presented in Figure 44.

I would also like to thank the Michael Smith Foundation for Health Research, the Canadian Institutes for Health Research, the Natural Sciences and Engineering Research Council of Canada and the University of British Columbia for their gracious funding over the years.

Lastly, I would like to truly thank all those friends I made throughout the years in the Department of Microbiology and Immunology; the laughs we shared during my time spent there made the experience so much more memorable.
Dedication

For all those people who believed I could do it,

And for my daughter Maya, who sees all that is good in me.
1. Introduction

1.1. The bacterial cell envelope

All living cells are bound by a biological membrane, composed mainly of a symmetrical phospholipid bilayer. In the case of bacteria this membrane (cytoplasmic membrane) is the innermost constituent of the cell envelope. Christian Gram (1884) developed a method of differentiating between two major types of bacteria based on the ability to retain a crystal violet-iodine dye complex. Bacteria unable to retain the stain were termed “Gram-negative” and those retaining the stain “Gram-positive”, with the major difference between them being composition of the cell envelope.

Gram-positive bacteria are enveloped by a cytoplasmic membrane (inner membrane, IM) followed by a cross-linked multi-layered structure called peptidoglycan. Common structures observed in the Gram-positive envelope are integral membrane proteins, teichoic acids (either anchored to the inner membrane via lipid groups, or anchored to the peptidoglycan wall) and proteins covalently attached to the peptidoglycan (Figure 1) (157).

In the case of Gram-negative cells, the peptidoglycan layer is substantially thinner and is encased within an outer membrane (OM). The outer membrane is asymmetrical, composed of a phospholipid inner leaflet and lipopolysaccharide (LPS) outer leaflet. Proteins can be integrated into either inner or outer membrane, performing a myriad of functions such as signal transduction, post-translational modifications and secretion (Figure 1) (157). Both Gram-positive and Gram-negative bacteria may possess an S-layer, a structure external to the outer membrane which is composed of a singular protein array encompassing the entire bacterium. Bacterial cells may also possess a capsule, a structure external to the outer membrane and composed of a thick polysaccharide layer.
1.2. Secretion across the bacterial inner membrane

Constructing the bacterial cell envelope requires targeting and translocation of proteins across the inner membrane. The Sec (secretion) system is a protein machine designed to translocate other proteins across the inner membrane. There exist other methods for translocation to the periplasm; therefore, secretion can either be Sec-dependent, or Sec-independent. Proteins secreted in a Sec-dependent manner require an N-terminal signal peptide (leader sequence, signal sequence) which is recognized by chaperones and the Sec machinery, permitting targeting of the secretory protein to the inner membrane. The protein is then translocated in an unfolded conformation into the periplasm (33).

Where the Sec machinery will translocate unfolded protein to the periplasm, the Twin-arginine translocation (Tat) system has the ability to translocate folded and even oligomeric proteins. The Tat system is an example of Sec-independent secretion, and requires an N-
terminal twin arginine motif for targeting to the inner membrane and translocation to the periplasm (134).

1.2.1. Signal peptides

Sec-dependent secretory proteins are synthesized with an N-terminal signal peptide, comprised of a tripartite structure of hydrophilic (N-domain), central hydrophobic (H-domain) and cleavage (C-domain) regions. Very little sequence conservation is observed amongst bacterial signal peptides, yet they can be conveniently predicted using algorithms such as SignalP 3.0 (124). Following translocation of the secretory protein, the signal peptide is removed by the activity of a membrane-bound signal peptidase 1 enzyme (SP1 or Lep in *E. coli*), by virtue of a Lys-Ser catalytic diad which mediates cleavage (121). Translocated protein is then released into the periplasm to follow its pathway of secretion.

1.2.2. The Sec system

Translocation across the bacterial inner membrane is mediated primarily by the Sec translocation machinery and various chaperones (Figure 2). Proteins destined for membrane insertion, or translocation, contain hydrophobic stretches of amino acids (such as the H-domain of signal peptides) which must be protected by cytoplasmic chaperones once the translated protein emerges from the ribosome. One such chaperone is trigger factor (TF) which binds L23 of the ribosomal unit, and interacts with emergent polypeptides, and notably hydrophobic stretches in the nascent polypeptide chain (71, 83, 142). Sequestering of hydrophobic regions of the nascent chain by TF blocks premature folding. TF is generally associated with biogenesis of integral inner membrane proteins, not that of secreted proteins (90), and releases polypeptides containing a cleavable signal peptide to the secretion-dedicated chaperone SecB. SecB is mainly found in α-, β- and γ-proteobacteria (179) and
functions as a homotetramer organized as a dimer of dimers (135). A secretion-
competent form of the protein is produced by interaction with SecB, which is then
delivered to the ATP-dependent peripheral membrane motor protein SecA.

SecA interacts with all members involved in Sec-dependent translocation, and
delivers unfolded preprotein to the SecYEG protein-conducting channel. SecYEG is a
highly conserved protein complex found in both bacterial inner membranes and
eukaryotic structures such as the endoplasmic reticulum and thylakoid membrane of
chloroplasts (128). In most crystal structures obtained of SecA, it appears as a dimer
organized into an antiparallel conformation (18, 199). The stable SecYEG
heterotrimeric complex contains a central pore occupied by a distorted alpha-helix,
termed the plug, originating from the first periplasmic loop of SecY folding back into
the central pore. The plug is displaced upon initiation of protein translocation (98)
creating a continuous aqueous channel large enough to facilitate passage of
polypeptides containing alpha-helical secondary structure (178). The SecYEG
channel can also translocate larger proteins and even proteins conjugated to
fluorescent markers, with data suggesting that translocation is facilitated by a single
SecYEG monomer (72, 73). The SecYEG channel can also interact with other
complexes such as SecDF-YajC, which in itself is not required for protein
translocation (193), but may play a role in secretion following SecYEG-mediated
translocation (109).

In contrast to proteins destined to be secreted past the inner membrane, integral inner
membrane proteins generally possess a signal peptide with a very hydrophobic H-
region (35). Translocation of integral inner membrane proteins proceeds in a co-
translational mechanism utilizing GTP hydrolysis and the Signal Recognition Particle
(SRP) chaperone in place of SecB (35). The sole bacterial receptor for SRP is FtsY
(11), which relays the secretory protein to SecA. TF is still capable of recognizing the hydrophobic segments localized near the N-terminus of the secreted protein; however, SRP has increased affinity for amino acid segments with greater hydrophobicity. Therefore, proteins containing a more hydrophobic H-domain region are recognized by SRP, instead of SecB, for membrane integration.

In some cases, integral inner membrane proteins are inserted via YidC, independent of the Sec machinery. These proteins have some common properties such as short periplasmic regions, and relatively small size (17, 143). Larger integral membrane proteins have also been shown to utilize YidC in conjunction with the Sec machinery (112, 192).
Figure 2: Model of translocation by the Sec translocation machinery
The Sec translocase machine located in the bacterial inner membrane (IM), comprised of a peripheral motor domain SecA (blue), the protein-conducting channel, SecYEG (yellow), accessory proteins SecDF-YajC (orange) and YidC (green). Signal peptidase1 (SP1) cleaves and removes the signal peptide from proteins as they enter the periplasm. Secretory proteins (black ribbons) can be post-translationally targeted to the SecYEG translocase via SecB (dark yellow). Membrane proteins utilize SRP (olive) for cotranslational translocation via the Sec translocase and the SRP-receptor FtsY (blue-green). Some membrane proteins can be directly inserted into the cytoplasmic membrane via YidC (light green).

1.3. Outer membrane protein biogenesis: the β-barrel Assembly Machinery

Integral inner membrane proteins demonstrate an α-helix conformation, for the Sec machinery is responsible for the lateral diffusion of these proteins into the inner membrane. Outer membrane proteins are mostly β-barrels, which are not observed in the inner membrane for the Sec machinery translocates them directly through the membrane to the periplasmic space. Therefore, proteins destined to be exported out of the Gram-negative cell, translocation across the inner membrane via the Sec machinery may only be the first step of this journey. The next significant barrier to overcome is the outer membrane. Specialized protein machines are harbored within the outer membrane, one of which being dedicated to integration and assembly of outer membrane proteins (OMPs).

1.3.1. Identification of the BAM complex

Of great significance was discovery of the *Neisseria meningitidis* Omp85 outer membrane protein; a discovery which ushered in a new understanding of outer membrane protein biogenesis. Omp85 was observed to function in a protein complex, facilitating β-barrel folding and insertion into the outer membrane (186). Soon after discovery of Omp85, the *E. coli* orthologue YaeT was also identified, and demonstrated similar function to Omp85 (191).

Since identification of Omp85/YaeT, and elucidation of their role in outer membrane protein biogenesis, homologues have been identified in numerous Gram negative bacteria. Omp85/YaeT is now understood to be a component of a protein complex formed by up to 5 members, with the entire complex potentially forming oligomers (133). The complex is currently referred to as the β-barrel Assembly Machinery (BAM), consisting of BamA (Omp85/YaeT) and accessory lipoproteins BamB-E which assist in folding and insertion (81) (Figure 3).
Figure 3: Schematic representation of outer membrane protein biogenesis in *Escherichia coli*

Translocation of unfolded outer membrane proteins via the Sec translocon permits entrance to the periplasm, where misfolding is deterred by association of chaperones SurA or Skp/DegP. Misfolded outer membrane proteins are targeted for degradation by DegP. OMP/chaperone complexes dock with BamA POTRA domains, initiating assembly and integration of outer membrane proteins into the OM. LPS = lipopolysaccharide; OM, outer membrane; IM, inner membrane. Bam complex components are represented by letter designations: B = BamB; D, BamD; E, BamE; C, BamC. BamA POTRA domains depicted as blue circles.

1.3.2. Composition of the BAM complex

1.3.2.1. BamA

Homologues of BamA are found in all Gram-negative bacteria, consistent with its essential role in outer membrane protein biogenesis. The structure of BamA reveals domain architecture with the carboxy terminal β-barrel domain integrated into the outer membrane and five POTRA (polypeptide transport-associated) domains P1-5 oriented toward the periplasm (77) (Figure 3). POTRA domains have little sequence identity, yet fold into the same conformation: a three-stranded β-sheet with two antiparallel α-helices overlaid.

1.3.2.2. BamB-E

The BAM complex includes BamA and four accessory lipoproteins BamB-E, which interact with BamA via the POTRA groups (77). Only BamA and BamD are essential for cell viability (77). Crystal structures of BamB reveal a β-propeller conformation which directly interacts with BamA (2, 49, 187) through β-augmentation interactions with the BamA POTRA domain P3 (110). The structure of BamC remains partially unresolved, with the first 70-100 residues disordered (2, 188). BamC structures reveal two helix grip domains, yet the BamC function or protein associations are not clear. Crystal structures of BamD (2, 144) reveal a superhelical structure composed of tetratricopeptide repeats. Other tetratricopeptide repeat containing proteins perform a scaffold function, and it is suggested that BamD may perform a similar function by both interacting with the C-terminus of secretion-competent outer membrane proteins, and to other members of the BAM complex via its own C-terminal α-helix domain (99).

BamE folds as two α-helices packed against a three-stranded antiparallel β-sheet (76), and may function in protein-protein or protein-membrane interactions. BamE can interact with BamD, and the anionic outer membrane constituent
phosphatidyl glycerol, possibly aiding in localization of the BAM complex to phosphatidyl glycerol rich regions in the outer membrane (79).

1.4. Transit of outer membrane proteins through the periplasm

The process of outer membrane protein insertion differs greatly from that of integral inner membrane proteins. The dominant conformation for inner membrane proteins is the $\alpha$-helix, whereas $\beta$-barrel conformation is dominant for outer membrane proteins. The $\beta$-barrel structure is oriented with hydrophilic residues facing the aqueous channel and hydrophobic residues on the exterior surface interacting with the outer membrane lipid environment.

Considering the amphipathic properties of $\beta$-barrels, they must be protected from aggregation during their transit through the periplasm. Indeed, once the unfolded polypeptide has been released by the Sec translocation machinery it is bound by chaperones to guard against premature folding and aggregation which would otherwise accumulate to toxic levels threatening cell viability. SurA handles the majority of the chaperoning, with an alternate pathway involving Skp/DegP (185) (Figure 3). Even though SurA mediates passage of the majority of outer membrane proteins through the periplasm, Skp does play a role in the initial stages of outer membrane protein secretion (45, 150). SurA, however, is not an essential protein, and its absence can be compensated for by other chaperones, such as FkpA, PpiA and PpiD (183); these chaperones are not essential for survival and may instead function under conditions of cellular stress where outer membrane homeostasis is critical (70, 131, 146). SurA, DegP and Skp are also involved in targeting secretion-competent outer membrane proteins to the BAM machinery.

1.5. Integration of outer membrane proteins into the outer membrane

Crystal structures of BamA POTRA domains P1-4 and a fragment of P5 revealed that the fragment of P5 interacted with the $\beta$2-sheet of P3 of another monomer via $\beta$-augmentation
Also, outer membrane protein substrates require a β-strand conformation for interaction with POTRA domains (80), suggesting β-augmentation is the method by which secretion-competent outer membrane proteins interact with BamA POTRA domains. In the POTRA domain crystal structures, β-augmentation was observed to occur in either parallel or antiparallel conformations (40, 77); considering β-hairpin and ultimately β-barrel structures are formed during the folding process, POTRA domains may bind β-structures in alternating orientations facilitating correct insertion of outer membrane proteins.

The POTRA domains of BamA are oriented toward the periplasm; though POTRA domains initiate the insertion process, outer membrane proteins must eventually be inserted into the membrane, suggesting the β-barrel of BamA functions in the later stages of the mechanism. Therefore, insertion and assembly of outer membrane proteins into the outer membrane would be a multi-step process. Isolation of intermediates, along with data derived from mutants, would be useful to elucidate the role of each component of the BAM complex. However, due to the essentiality and efficiency of the process, information from intermediates and mutants has been challenging to obtain.

The first stage of outer membrane protein insertion and assembly would be recognition of the secretion-competent substrate, which is suggested to be mediated by the C-terminus of the outer membrane protein β-barrel. Delivery of some outer membrane proteins to the POTRA domains is mediated by SurA (44), and interaction between BamA and SurA has been observed (159, 187), with data suggesting that specific interaction between BamA POTRA 1 and SurA initiates interaction of outer membrane proteins with BamA (10, 77). This recognition is also observed to be greatly influenced by the presence of terminal aromatic residues present not only in the outer membrane protein’s C-terminal β-strand, but other β-strands within the structure (133, 165). Completion of β-barrel assembly then
results in aromatic residues from successive β-strands creating a ring on either side of the membrane (151, 190).

Later stages of outer membrane protein insertion and assembly have been the most poorly understood, yet recent discoveries illuminated how some BAM components interact with outer membrane proteins during the process. Recognition of unfolded outer membrane proteins by BamA POTRA domains (facilitated by BamB) can initiate β-strand conformation, followed by movement within the POTRA domains between P2 and P3, providing a “hinge” mechanism by which β-hairpins can be formed (40).

The function of BamD is suspected to be downstream of BamA activity, possibly playing a role in release of the folded β-barrel from the BAM complex. BamC and E play a stabilizing role in the BAM complex (77, 99). Sequential insertion of β-strands with support from BamA, B and D can produce the assembled β-barrel. Indeed, the BamA, B and D components have been observed to bind different locations around the β-barrel during the secretion process (55).

1.6. Lipoprotein biogenesis and localization

Another resident of the outer membrane are lipoproteins, which are anchored by virtue of their hydrophobic acyl chains. A variety of essential functions are performed by lipoproteins, including cell integrity, signal transduction, motility, transport and outer membrane protein assembly. Acylation of proteins and their localization in the cell envelope is highly regulated, and disruption of this process is often lethal. Lipoprotein biogenesis initiates in the bacterial cytoplasm, with translocation into the periplasm mediated by the signal peptide of the lipoprotein and the Sec translocon. Following translocation, N-terminal acylation ensues on the periplasmic side of the inner membrane, and requires a specific motif harboring a cysteine residue as the site of lipid attachment. Newly formed lipoproteins
can then be sorted and anchored to the periplasmic side of the inner or outer membrane via the Lol pathway (175).

1.6.1. N-terminal acylation

Proteins destined for N-terminal acylation require both a signal peptide and lipobox motif [LVI][ASTVI][GAS]C. The signal peptide mediates translocation across the inner membrane via the Sec machinery, yet the C-region of the signal peptide is often missing, blocking release of the protein from the inner membrane by signal peptidase 1. The lipobox is found C-terminal to the H-region, and once exposed to the periplasmic environment becomes the target for acylation. The lipobox is recognized by enzymes anchored to the periplasmic side of the inner membrane. In *Escherichia coli*, the first enzyme phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt) adds a diacylglycerol via thioether linkage to the cysteine residue. This is followed by the activity of prolipoprotein signal peptidase (LspA or signal peptidase 2) which cleaves and removes the signal peptide leaving a free N-terminal cysteine residue. Finally, phospholipid/apolipoprotein transacylase (Lnt) aminoacylates the N-terminal cysteine (51, 102, 145) yielding the final product N-acyl diacylglyceryl-cysteine (Figure 4). A similar modification pathway is also found in Gram-positive bacteria (29, 52), solidifying the importance of N-terminal acylation in bacterial homeostasis. However, Gram-positive bacteria generally lack the amino-acyl group (174) due to the absence of Lnt, with some exceptions where amino-acylation occurs in the absence of Lnt homologues (86) and also where Lnt homologues are present yet their function remains unknown (184).
**Figure 4: Biosynthesis and outer membrane localization of lipoproteins**

Secretion of lipoproteins begins with translocation of unfolded protein across the IM by virtue of an N-terminal signal peptide (SP) and Sec translocon. Recognition of the lipobox by Lgt results in addition of diacylglycerol (DAG) to the sulfhydryl group of the cysteine residue. Diacylglyceryl-cysteine is a substrate for Lsp (signal peptidase 2), resulting in proteolytic cleavage and exposure of the cysteine amino-terminus. Lnt can now amino-acylate the free N-terminal cysteine, yielding N-acyl diacylglyceryl-cysteine.

1. Lipoproteins destined for OM integration are recognized by LolCDE, and released from the IM (using energy from ATP hydrolysis) to the periplasmic chaperone LolA. LolB receives lipoprotein from LolA for subsequent integration into the periplasmic side of the OM.
2. Lipoproteins retained in the inner membrane avoid the Lol pathway. LPS = lipopolysaccharide; OM, outer membrane; IM, inner membrane; C, C-terminus; N, N-terminus.

1.6.2. Lipoprotein localization

Inner membrane localized lipoproteins maintain their lipid groups embedded in the hydrophobic environment of the inner membrane. Lipoproteins destined for the outer membrane must therefore overcome the significant energy threshold of removing hydrophobic lipid groups from the inner membrane, a threshold overcome by hydrolysis of ATP. Once released from the inner membrane, the hydrophobic lipid groups must be sequestered away from the hydrophilic environment of the periplasm, until outer membrane integration is completed. The mechanism by which this happens is outlined below.

1.6.2.1. Lipoprotein sorting signals

Localization of lipoproteins to either the inner or outer membrane is mediated by amino acids directly following the N-acyl diacylglyceryl-cysteine (154, 196). Initially, it was understood that if the residue at position +2 is aspartate the lipoprotein is retained in the inner membrane; all other residues at the +2 position mark the lipoprotein to be released from the inner membrane for transit to the outer membrane. Later, data revealed the “+2 rule” was not always obeyed (158), and residues at positions +3 and +4 can also influence lipoprotein localization (41, 171).

1.6.2.2. The LOL pathway

Sorting and outer membrane localization of lipoproteins is governed by the lipoprotein localization (Lol) system, composed of five proteins LolABCDE (Figure 4). Following N-terminal acylation, lipoproteins destined for the outer membrane are recognized by the inner membrane complex LolCDE (194). LolCDE has a stoichiometry of 1:2:1, and is a member of the ATP-binding cassette transporter superfamily. LolC and E are integral inner membrane
proteins, and the 2 copies of peripheral protein LolD harbor ATPase function required for release of lipoproteins from the inner membrane to the periplasmic chaperone LolA (195).

The crystal structure of LolA (169) is described as an unclosed β-barrel possessing a hydrophobic pocket ideal for protecting exposed acyl groups. LolA accepts the lipoprotein from LolCDE and shuttles through the periplasm to relay the lipoprotein to LolB (175). LolB itself is a lipoprotein, with a structure similar to LolA (169), despite lack of sequence similarity. LolB claims lipoproteins from LolA due to increased affinity for the acyl groups (100), and proceeds to integrate the lipoprotein into the periplasmic surface of the outer membrane (175). The process yields lipoproteins oriented toward the periplasm, and associated with the outer membrane via hydrophobic interactions between the outer membrane and the acyl groups of N-acyl diacylglycerol-cysteine (Figure 4).

Though periplasmic orientation of lipoproteins is the norm for Gram-negative bacteria, there are some notable exceptions to this rule. Some bacteria, such as the pathogenic spirochete Borrelia burgdorferi, have the ability to express lipoproteins on the bacterial surface (152, 153).

1.7. Secretion systems of Gram-negative bacteria

Gram-negative bacteria must contend with two biological membranes, and employ highly regulated protein machines, to export proteins from the cytoplasm to the exterior of the cell. In addition to the Sec and BAM machinery discussed above, a number of specialized secretion systems are employed to facilitate protein export (Figure 5) (1, 30, 129). Secretion from the bacterial cytoplasm to the cell surface or milieu can be seen as a one or two-step process. Secretion systems Type I, III and some Type IV have the ability to
secrete a protein in a one-step process from the cytoplasm to the exterior of the cell or
directly into a target cell. Type II, V and some Type IV secretion systems rely on protein
translocation across the cytoplasmic membrane via the Sec or TAT machinery; once in the
periplasm translocation across the outer membrane is mediated by Type II, IV or V specific
machinery. A more recent discovery is the Type VI secretion system, which has the ability
to mediate translocation of effector proteins in a one-step process. Type VI effectors can be
targeted to the eukaryotic cytoplasm, or the periplasmic space of other Gram-negative
bacteria (13, 92, 141). Individual secretion systems are briefly highlighted below followed
by an in-depth look at secretion of Type V monomeric autotransporters.
Figure 5: Schematic representation depicting secretion pathways in Gram-negative bacteria

Secretion systems 1, 2, 4 and 5 are able to secrete protein into the milieu in a 2-step process linked with Sec or TAT mediated translocation across the IM. In addition, type 4 secretion is also able to translocate protein or protein/DNA complexes across the entire bacterial envelope directly into target cells in a 1-step process. Secretion systems 3 and 6 are both able to translocate protein across the bacterial envelope and directly into target cells. LPS = lipopolysaccharide; OM, outer membrane; IM, inner membrane. ABC = ATP-binding cassette; MFP, membrane fusion protein; PSR, periplasmic spanning region; MT, multimeric translocon; TCM, target cell membrane. Example of type 4 secretion provided is the Agrobacterium tumefaciens Vir system, with specific VirB proteins indicated by their number designation. Example of type 3 secretion provided is the Yersinia Yop system, with Ysc proteins using letter designations.
1.7.1. Type I secretion

Type I secretion is typically associated with efflux mediated resistance to antibiotics, and the secretion apparatus is generally comprised of 3 components (50). A cytoplasmic ATP-binding cassette (ABC) ATPase forms a complex with a membrane-bound membrane fusion protein (MFP) which spans the periplasmic space. The ABC ATPase/MFP complex will on demand associate with an outer membrane translocon (e.g. TolC) when a secreted moiety is ready for export from the cell. The outer membrane translocon can form a trimer and facilitate export via a gated mechanism triggered by substrate interaction (6, 82).

1.7.2. Type II secretion

Of the 2-step systems, Type II secretion is likely the best understood (63). The Type II secretion system receives periplasmic intermediates from either the Sec or TAT inner membrane translocation machinery. General structure of the Type II apparatus is that of a membrane bound ABC ATPase followed by a periplasmic spanning region composed of pilin-like subunits. Outer membrane translocation is facilitated by a multimeric translocon which functions as the protein conducting channel (16).

1.7.3. Type III secretion

Type III secretion involves injection of protein-based effectors directly from the bacterial cytoplasm into host cells though a needle-like apparatus. Components of the apparatus include a multi-ring basal body spanning the bacterial membranes, a protein-conducting channel (hollow needle structure) extending beyond the cell surface and a pore-forming translocon which interacts with a specific partner on the target cell surface (57). It is interesting to note the Type III secretion apparatus is strikingly similar to the bacterial flagellum assembly, suggesting an evolutionary relationship (5).
1.7.4. Type IV secretion

Similar to that of the Type III secretion system, the Type IV system has the ability to inject effectors directly into target cells. However, the Type IV secretion system is also capable of transporting protein/DNA complexes. In addition, periplasmic intermediates are able to enter the Type IV secretion system, resulting in a 2-step secretion process (4). Noted examples of Type IV secretion are that of the Agrobacterium tumefaciens Vir system (126) and Bordetella pertussis PT (pertussis toxin) secretion (155, 189).

1.7.5. Type V secretion

Members of the Type V secretion system, commonly referred to as autotransporters, are secreted by a 2-step mechanism. Monomeric autotransporters are modular, with an N-terminal signal peptide followed by a passenger domain (harboring functionality) connected via linker region to the C-terminal β-barrel domain. Translocation across the inner membrane, mediated by the autotransporter signal peptide and Sec machinery, precedes partial folding of the C-terminal region into a β-barrel structure, which then contributes to translocation of the N-terminal passenger domain to the cell surface (180).

Trimeric autotransporter adhesins have similar architecture to their monomeric counterparts, but require three identical proteins to combine and form functional passenger and β-barrel domains (96). Each polypeptide contains a portion of the β-domain. During the process of outer membrane assembly and integration, each portion of the β-barrel will combine to form the functional β-domain. The passenger domains form an extended triple α-helical coiled coil stalk which is attached to the β-domain. The adhesin function is contained in the globular domain located at the tip of the stalk (96).
A two-partner secretion (TPS) system is also described, where the autotransporter passenger and translocation unit are expressed as independent proteins though generally encoded on the same operon. The transporter domain (TpsB) facilitates translocation of its cognate passenger domain (TpsA) across the outer membrane (103). Interestingly, the β-domain of the TPS system has striking resemblance to the BamA component of the BAM machinery, where all members of the TpsB family possess anywhere from 1-7 POTRA domain for substrate recognition (103). Numerous members of the autotransporter group are known to contribute significantly to pathogenesis in a wide array of Gram-negative pathogens (26, 66).

1.7.6. Type VI secretion

The Type VI secretion apparatus is structurally and evolutionarily related to bacteriophage (91, 123, 130), facilitating injection of effector proteins directly into other Gram-negative target cells. A protein Hcp (haemolysin co-regulated protein) creates hexameric rings which stack to form a channel through which effector proteins are shuttled (141). The Hcp channel is contained within a VipA/B sheath, which is suggested to retract upon cell-to-cell contact, and with energy provided by ATP hydrolysis drives the Hcp needle through the target cell membrane. Situated atop the Hcp needle is VgrG (valine-glycine repeat protein G) which is responsible for piercing the target cell membrane. Once the target cell membrane is breached, VgrG may move aside permitting passage of effector proteins (13, 141).
1.8. Secretion via the Type V monomeric autotransporter pathway

As described above there are three general types of autotransporters: monomeric, trimeric and two-partner secretion models (Figure 6). The focus of this doctoral thesis centers on secretion of monomeric autotransporters; therefore, a more detailed explanation of their structure, function and biogenesis is provided.

![Figure 6: Comparison of different autotransporter secretion pathways](image)

**Figure 6: Comparison of different autotransporter secretion pathways**

The two-partner secretion (TPS) model utilizes 2 independent proteins, one containing the translocation unit (TU) and other the passenger domain. Each polypeptide is translocated across the inner membrane (IM) by virtue of an N-terminal signal peptide (SP) and Sec translocon. The TU acts as a specific receptor for the passenger domain facilitating outer membrane (OM) translocation. Monomeric autotransporters contain within a single protein both passenger and TU, and N-terminal signal peptide for Sec-mediated translocation across the inner membrane. Trimeric autotransporters utilize 3 independent proteins, each with its own signal peptide and portion of translocation unit. All 3 portions of the translocation unit come together at the outer membrane forming a complete β-barrel, facilitating translocation of the passenger domains to the cell surface where they combine to form an integrated functional passenger. LPS = lipopolysaccharide.
1.8.1. General features of monomeric autotransporters

Members of the monomeric autotransporter family are modular, composed of an N-terminal signal peptide, central passenger domain and C-terminal translocation unit (Figure 7). The signal peptide targets the polypeptide to the inner membrane Sec machinery and subsequent translocation into the periplasm (32). Interestingly, an extended signal peptide region (ESPR) is found in a significant number of autotransporter signal peptides (48), such as EspP from pathogenic *E. coli*, which has an impact on autotransporter biogenesis (167). An extended signal peptide is composed of an N1 and H1 region (the ESPR) followed by N2, H2 and C-regions of a typical Sec-dependent signal peptide. The ESPR demonstrates sequence conservation, where typical Sec-dependent signal peptides do not demonstrate such conservation (93). The passenger is the functional domain of the autotransporter, and is either presented on the bacterial surface or released into the surrounding milieu. An α-helical linker region connects the passenger to the C-terminal β-barrel. Together, the β-barrel and α-helical linker are referred to as the translocation unit (TU). In some cases the linker region is cleaved when the completely formed β-barrel brings together key catalytic residues within the barrel to function as a protease itself (7, 25, 168). In addition, the β-barrel can also function as an anchor for the passenger domain, allowing retention of the passenger on the cell surface (30, 66, 172).

![Figure 7: Domain architecture of monomeric autotransporters](image)

**Figure 7: Domain architecture of monomeric autotransporters**

General structure of a monomeric autotransporter consists of N-terminal signal peptide (SP), central functional passenger domain and C-terminal translocation unit (TU). Connecting the β-barrel to the passenger domain is an α-helical linker region (L) which is commonly the site of cleavage, separating the passenger domain from the β-barrel. Residing at the C-terminus of many autotransporter passenger domains is the autochaperone (AC) region, which once exposed to the cell surface acts as a folding catalyst for the remainder of the passenger domain.
1.8.2. Structure and folding of autotransporter passenger domains

Passenger domains generally fold into a β-helix structure, composed of β-strands sequentially stacked on each other forming a roughly triangular solenoid (15, 34, 39, 62, 75, 120, 198), with known exceptions (177). Numerous loops extend from the sides of the β-helix, which vary in size and location between autotransporters, playing specific roles in the functionality of the protein (34) (Figure 8).

Translocation of the passenger domain across the outer membrane occurs in the absence of an energy source such as ATP. It is proposed that translocation is driven by the collapse of the passenger to its lowest energy state, as it is vectorially folded during the passage through the outer membrane (69). It was discovered that folding of β-helix passenger domains require a critical region located at the C-terminus of the passenger (118) (Figure 7). This region, termed the autochaperone, is conserved in many autotransporters and folds into a unique structure providing a platform onto which the remainder of the passenger folds vectorially in a C-terminal to N-terminal direction (55, 69, 85, 118, 125, 127, 162). The process of folding in a C-terminal to N-terminal fashion, mediated by the autochaperone domain, is proposed to facilitate translocation of the passenger domain through the outer membrane (69).

Though folding of the passenger into its final conformation appears to occur on the bacterial surface, translocation-competent intermediate structures can be formed in the periplasm (54, 197) and protected by resident chaperones. As discussed above, chaperones (such as SurA, Skp and DegP) are involved in outer membrane protein biogenesis; considering autotransporter β-domains fold into a β-barrel conformation it is not surprising similar chaperones are involved in autotransporter biogenesis (139). In addition, interactions between periplasmic chaperones and autotransporter passenger domains have been detected (53, 139, 140).
1.8.3. Translocation unit structure and outer membrane integration

The translocation unit is composed of both β-domain and α-helical linker. Crystallography has determined the β-domain of autotransporters form a β-barrel with the α-helical linker region incorporated into the β-barrel central pore (7, 53, 168, 177) (Figure 9). The translocation unit is integrated into the outer membrane and plays a critical role in passenger domain translocation to the cell surface.

An increasing body of evidence demonstrates that autotransporters require BAM for secretion (53, 55, 58, 136, 147). Data highlighting this critical role of the BAM
machinery in autotransporter biogenesis revealed the interaction of BAM components with both the autotransporter passenger and β-domain. Such an observation raised questions regarding whether the BAM complex was directly involved in passenger domain translocation to the cell surface. This is indeed the case, with data clearly indicating that members of the BAM machinery help to maintain the EspP β-domain in a partially open conformation during the process of integration, facilitating the vectorial translocation of the unfolded/partially folded passenger domain to the cell surface (55).

Figure 9: Structure of an autotransporter translocation unit
Side (A) and top (B) views of the NalP translocation unit reveal β-barrel structure (blue strands) with alpha helical linker (yellow) harbored within. Loop regions are depicted in red. Structures generated using Swiss-Pdb viewer 4.0.4 (43).
1.9. Autotransporters with N-terminal acylation

There exists a subfamily of autotransporters which harbor a lipobox motif within the signal peptide, and therefore become N-terminally acylated during biogenesis. The subtilisin-like autotransporter SphB1 from *Bordetella pertussis*, produces a passenger domain which remains anchored to the cell surface via its acyl groups, and functions to process Filamentous Haemagglutinin B (FhaB) into its mature form FHA (22, 24). In *Neisseria meningitidis* the NalP autotransporter is involved in processing other autotransporters (181) and release of lactoferrin-binding protein B from the cell surface (138). Even though acylation is essential for NalP biogenesis, a subsequent intermolecular autocatalytic event removes a portion of the N-terminus, along with the acyl groups, releasing NalP from the bacterial surface (181). Adhesion of *Helicobacter pylori* to gastric tissue is partially mediated by AlpAB. Both proteins are autotransporters, though only AlpA contains a lipobox and requires acylation for biogenesis (113, 115).

The focus of studies regarding SphB1, NalP and AlpA have been slanted toward elucidating function, leaving the secretion mechanism of such autotransporters poorly understood. Secretion of acylated autotransporters is potentially complex when considering periplasmic interactions occurring during transit to the outer membrane, and surface expression of the passenger. A merger between autotransporter and lipoprotein secretion pathways can be envisioned, raising many questions regarding how cells manage secretion of such proteins. Knowing some acylated autotransporters, such as NalP, do not retain their N-terminal lipid groups raises further questions about why an acylated intermediate is essential for biogenesis.
1.10. Thesis goals

The goal of this doctoral project is to characterize secretion of Bordetella autotransporter F (BapF), a newly identified acylated autotransporter from B. bronchiseptica RB50 (ORF BB2324). The BapF signal peptide is predicted to contain processing sites for both signal peptidase 1 and signal peptidase 2, as shown bioinformatically by SignalP 3.0 (9) and LipoP 1.0 (68). N-terminal acylation of BapF is predicted to occur at the Cys\(^{28}\) residue located at the Lsp processing site; however, the Lep cleavage site is C-terminal to the Lsp site. This situation suggested that acylation may occur during the secretion of BapF, but the acyl groups may be later removed by the activity of signal peptidase 1.

The signal peptidase 1 (Lep in E. coli) and signal peptidase 2 (Lsp in E. coli) enzymes perform an essential function in both Gram-positive and Gram-negative bacteria, with homologues found in a wide array of species including Bordetella. Therefore, studying BapF signal peptide processing in E. coli will yield results which can be extended to help understand BapF secretion in Bordetella. Investigation into BapF biogenesis was moved into an E. coli model system, where genetic tools and reagents are readily available. Secretion of the wild-type BapF autotransporter was examined using various methods including mass spectroscopy, limited proteolysis of surface proteins, fluorescence microscopy and inhibition of Lsp activity using globomycin. Observations of induced phenotypes in E. coli due to expression BapF were also made. Data obtained by these methods addressed the hypotheses that BapF is acylated at its N-terminus during biogenesis, with the final destination of the passenger domain being the cell surface. Results from experiments probing BapF secretion showed that processing between the passenger and translocation unit separates the domains, yet the passenger remains non-covalently associated to the cell surface by virtue of its linker region being retained in the pore of the β-barrel. Once the passenger is presented in its final conformation on the cell surface it is then able to mediate a potential phenotype.
BapF mutants in which processing by signal peptidase 1 or 2 is disrupted were examined in a similar manner as that performed for wild-type BapF. Using these methods, the hypothesis was tested that both signal peptidase 1 and 2 processing have a role in the secretion of BapF to the cell surface; disruption of either of these processing events has a negative impact on the localization or function of the passenger domain, ablating the phenotypes observed in cells expressing the wild-type BapF protein.
Chapter 2: Materials and methods

2.1. Bacterial strains, plasmids and primers

Table 1 lists plasmids and bacterial strains used in this study. *E. coli* strains were grown at 37°C in Luria broth (LB) or on Luria agar supplemented with the appropriate antibiotics. *B. bronchiseptica* RB50 was grown at 37°C either on Bordet-Gengou (BG) agar containing 15% sheep’s blood supplied by RA Media (Calgary, AB.), or in Stainer-Scholte broth (164) containing 10.72 g/liter glutamate and 0.24 g/liter proline and supplemented with 0.15% bovine serum albumin. Table 2 lists primers and oligonucleotides used in this study. Unless otherwise stated most reagents were purchased from Sigma-Aldrich (Oakville, ON.), restriction enzymes from New England Biolabs (Mississauga, ON.), primers from Alpha DNA (Montreal, PQ.) and oligonucleotides from Integrated DNA Technologies (Coralville, IA). Sequencing was performed by GENEWIZ (South Plainfield, NJ) using common in-house primers or those listed in Table 2.
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<td>pBAD24/bapF&lt;sub&gt;im&lt;/sub&gt;</td>
<td>Expression vector containing a fusion of the pertactin signal peptide to the BapF passenger and translocation unit</td>
<td>This work</td>
</tr>
<tr>
<td>pBAD24/bapF&lt;sup&gt;AA34-35S&lt;/sup&gt;</td>
<td>Expression vector containing BapF AA&lt;sup&gt;34-35S&lt;/sup&gt; mutation</td>
<td>This work</td>
</tr>
<tr>
<td>pBAD24/ Bap&lt;sup&gt;A32S,S33P,AA34-35S,A37S&lt;/sup&gt;</td>
<td>Expression vector containing BapF&lt;sup&gt;A32S,S33P,AA34-35S,A37S&lt;/sup&gt; mutation</td>
<td>This work</td>
</tr>
<tr>
<td>plDTSMART/Prn(1-40)-BapF(45-)</td>
<td>plDTSMART cloning vector containing synthesized sequence coding for residue 1-40 of the pertactin signal peptide</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli DH5α</td>
<td>K-12 cloning strain</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E. coli BW27783</td>
<td>lac&lt;sup&gt;I&lt;/sup&gt; rnmB3 ΔlacZ4787 hsdR514 DE(araBAD)567 DE(rhaBAD)568 DE(araFGH) Φ(ΔaraEp P&lt;sub&gt;CP8&lt;/sub&gt;-araE)</td>
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</tr>
<tr>
<td>E. coli BL21(DE3)pLysS</td>
<td>F- ompT hsdSB (rB-mB-) gal dcm (DE3) pLysS (CamR)</td>
<td>Novagen</td>
</tr>
<tr>
<td>B bronchiseptica RB50</td>
<td>Sequenced wild-type strain</td>
<td>A. Weiss, Univ. of Cincinnati</td>
</tr>
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Table 2: Primers and oligonucleotides

<table>
<thead>
<tr>
<th>Primers and Oligonucleotides</th>
<th>Sequence</th>
<th>Restriction site</th>
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<tr>
<td><strong>Primers</strong></td>
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<tr>
<td>BapF-F3</td>
<td>5′–AATAATGGATCCTCAGGGAGCCGGACAGAG–3′</td>
<td>BamHI</td>
</tr>
<tr>
<td>BapF-R3</td>
<td>5′–AGTGTACTCGAGCTAGAAACGCAGGGCTAGGTG–3′</td>
<td>Xho</td>
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<td>BapF-int</td>
<td>5′–AATAGCGATGCTCGTGGTGG–3′</td>
<td>None</td>
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<td>OQc-bapFF1</td>
<td>5′–CTGGCCGCAAGCCCACTGCGACAGGGACAGAG–3′</td>
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<td>5′–CGGTTACCGGAGCTTG–3′</td>
<td>KpnI</td>
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</tr>
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<td>SnaI</td>
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<td>HindIII</td>
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<td>AA34-35Nhe1BapF 5′UTR ORF-K</td>
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<tr>
<td>BapFA32S,S33P, AA34-35S,A37S</td>
<td>5′–</td>
<td>Nhel</td>
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<td>GGGCTAGCAGATCCTCAGGGAGCCGGACAGAGGGGTT</td>
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<tr>
<td></td>
<td>CTGGCGCGTACCGG–3′</td>
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</tbody>
</table>

**Note:** Restriction sites used in cloning procedures are underlined with corresponding restriction enzyme listed in the neighboring column.
2.2. Isolation of genomic DNA from *Bordetella bronchiseptica* RB50

Two plates of BG agar were spread with *B. bronchiseptica* RB50 and incubated overnight at 37°C. 50mg of cells was harvested from the plates and suspended in 1.5mL of 10mM Tris buffer pH8 containing 50mM ethylenediaminetetraacetic acid (EDTA). Cells were centrifuged for 5min at 6000g and supernatant removed. The cell pellet was suspended in 100µL of 10mM Tris pH8 buffer containing 50µM lysozyme and 50mM EDTA. Cells were incubated at 37°C for 15 minutes. Genomic DNA was isolated using a Genomic DNA Purification kit and manufacturer’s protocol (Qiagen, Streetsville, ON.) Two 200µL aliquots of purified genomic DNA were obtained.

2.3. PCR amplification of *bapF* from *B. bronchiseptica* RB50 genomic DNA

Purified genomic DNA from *B. bronchiseptica* RB50 was used in Polymerase Chain Reaction (PCR) with 30pmol/µL each of BapF-F3 and BapF-R3 primers (see Table 2) and Platinum® Pfx DNA Polymerase (Invitrogen, Burlington, ON) with manufacturer’s buffers. Temperature cycles were as follows: 6 minutes initial denaturation at 95°C, 30 cycles of amplification (2 minutes at 95°C, 1 minute 62°C and 2.5 minutes 72°C) and 10 minute final cycle at 72°C. Samples were then loaded into a 1% UltraPure low melting point agarose (Invitrogen, Burlington, ON), and a 2208bp band containing the amplified *bapF* gene was excised. DNA was purified from the agarose using a Gel Purification Kit (Qiagen, Streetsville, ON.). The *bapF* gene amplified using BapF-F3 and BapF-R3 primers produce 5´ *Bam*HI and 3´ *Xho*I restriction enzyme sites.

2.4. Cloning *bapF* from *B. bronchiseptica* RB50 into pBS(KS)

The *bapF* gene, and pBS(KS) vector were digested using *Bam*HI and *Xho*I, ligated together using a standard ligation protocol, transformed into *E. coli* DH5α CaCl₂ competent cells and spread onto LB agar containing 100µg/mL ampicillin and 50µM 5-bromo-4-chloro-3-indoly-
b-D-galactopyranoside (X-gal). Isolated white colonies were grown overnight at 37ºC in LB broth with 100µg/mL ampicillin, and plasmid DNA purified using a standard alkaline lysis protocol. Plasmids were then screened using various restriction enzymes, with three clones passing the screen. All three clones were sequenced using T7, T3 and BapF-int primer (see Table 2), ultimately yielding a single sequence-verified pBS/bapF clone. The pBS/bapF construct was then used for subcloning bapF into various expression vectors.

2.5. Subcloning bapF into pET30b and pBAD24 for inducible expression studies

2.5.1. Subcloning bapF into pET30b

The single positive pBS/bapF clone was digested using BamHI and Xhol, and the 2208bp bapF gene was gel purified as described above and ligated to a similarly digested pET30b vector. The ligation mixture was transformed into E. coli DH5α and spread on LB agar containing 50µg/mL kanamycin. Isolated colonies were grown overnight at 37ºC in LB broth containing 50µg/mL kanamycin. Plasmid DNA was extracted from the overnight cultures using a standard alkaline lysis protocol, and screened using various restriction enzymes, revealing numerous pET30b/bapF positive clones.

2.5.2. Subcloning bapF into pBAD24

The pBS/bapF clone was digested with BamHI and Xhol, and the 2208bp bapF gene was gel purified as described above. An intermediate cloning step using the pMCS5 vector was required to create restriction enzyme sites at the bapF termini matching restriction sites in the pBAD24 multiple cloning site. The pMCS5 vector was digested with BglII and Xhol, gel purified and ligated to the 2.2kb bapF gene previously digested with BamHI and Xhol using a standard ligation protocol. BamHI and BglII sticky ends are compatible, but the restriction site is subsequently destroyed after ligation. The ligated sample was transformed into E. coli DH5α cells then selected on
LB media containing 100µg/mL ampicillin. Potential clones were screened using various restriction enzymes, with positive pMCS5/bapF plasmids subsequently identified. Both pBAD24 expression vector and pMCS5/bapF were digested using Nhel and HindIII. The bapF gene and pBAD24 vector were gel purified as described above then ligated together, transformed into E. coli DH5α cells followed by selection on LB media containing 100µg/mL ampicillin.

2.6. Generating the bapF<sub>C28S</sub> signal peptide mutant

The BapF-oligoF and BapF-oligoR primers (see Table 2) were used to amplify the long oligonucleotide C28SNhe1BapF 5’UTR ORF-K (see Table 2) using Platinum® Pfx DNA Polymerase (Invitrogen, Burlington, ON) with manufacturer’s buffers and conditions. The resulting PCR product was ligated to linear pGEM cloning vector using the pGEM®-T Easy Vector System and protocol (Promega, Madison, WI). Sequence-verified clones were digested using KpnI and Nhel restriction enzymes and the correct 196 base DNA fragment, coding for the bapF signal peptide harboring a C28S mutation, was gel purified as described above. The purified DNA fragment was then ligated to similarly digested pBAD24/bapF plasmid, effectively exchanging wild-type bapF signal peptide for mutant bapF<sub>C28S</sub> signal peptide.

2.7. Generating bapF<sup>AA34-35S</sup> and bapF<sup>A32S,S33P,AA34-35S,A37S</sup> signal peptide mutants

The BapF-oligoF and BapF-oligoR primers were used to amplify the long oligonucleotides AA34-35Nhe1BapF 5’UTR ORF-K and BapFA32S,S33P,AA34–35S,A37S (see Table 2) using Platinum® Pfx DNA Polymerase (Invitrogen, Burlington, ON). The PCR product was ligated to linear pGEM cloning vector using the pGEM®-T Easy Vector System (Promega, Madison, WI). Sequence-verified clones were digested using KpnI and Nhel. The DNA fragments coding for the bapF signal peptide mutants were gel purified as described
above, then ligated to similarly digested pBAD24/bapF plasmid, effectively exchanging wild-type bapF signal peptide for mutant bapF\textsuperscript{AA34-35S} and bapF\textsuperscript{A32S,S33P,AA34-35S,A37S} signal peptides.

2.8. Generating the bapF\textsuperscript{Prn} signal peptide mutant

The pIDTSMART/Prn(1-40)-BapF(45-) plasmid (see Table 1) harbors the synthesized DNA fragment coding for the pertactin signal peptide (residues 1-40). The pIDTSMART/Prn(1-40)-BapF(45-) plasmid was digested using NheI and KpnI restriction enzymes and ligated to similarly digested pBAD24/bapF, exchanging wild-type bapF signal peptide for the pertactin signal peptide.

2.9. Subcloning the BapF passenger into the pET30b expression vector

Two clones were generated containing different coding regions for the BapF passenger domain. The first clone was created for the purpose of raising polyclonal antibody. The bapF passenger from nucleotides 190–1023 (spanning residues 51-330) was excised from the pBS/bapF plasmid using restriction enzymes Sphi and Kpnl, and gel purified using a Gel Purification kit from (Qiagen, Streetsville, ON.). The bapF passenger was then ligated to similarly digested pMCS5 vector using a standard ligation protocol creating pMCS5/bapFp. The pMCS5/bapFp plasmid was digested using EcoRI and HindIII and the bapF passenger was again gel purified then ligated to a similarly digested pET30b expression vector. The final pET30b/bapFp construct codes for an N-terminal 6X histidine tag fused to the BapF passenger (51-330) lacking a signal peptide region.

A second clone containing a larger coding region for the BapF passenger than the previous pET30b/bapFp construct was created, and used for \textit{in vitro} analysis of protease susceptibility. The coding region of the BapF passenger spanning residues 35-407 was amplified by PCR using the BapFpassFw2, and BapFpassRev primers (see Table 1). PCR
cycles utilized the Platinum Pfx polymerase (Invitrogen, Burlington, ON.), with an initial
denaturation step at 95°C for 5 minutes, followed by 30 cycles of amplification 45 s at
95°C, 45 s at 65°C, 1 min 50 s at 72°C). The final elongation step was 10 minutes at 72°C.
PCR amplified products were purified with a gel extraction kit (Qiagen, Streetsville, ON.)
The PCR purified 1.7kb BapF passenger fragment and 5.4kb pET30b vector were
sequentially digested with SacI and HindIII, then ligated together creating the
pET30bBapFpass expression plasmid. Positive clones were PCR screened and sequence-verif-
ified by Genewiz (South Plainfield, New Jersey). The pET30bBapFpass construct codes
for an N-terminal 6X histidine tag fused to the BapF passenger (35-407) lacking a signal
peptide region.

2.10. Purification of the BapF passenger domain

The pET30b/bapFp construct was transformed into E. coli BL21(DE3) cells (see Table 1)
and positive clones selected on LB agar containing 25µg/mL kanamycin. Isolated colonies
were used to inoculate LB broth 25µg/mL kanamycin for overnight growth at 37°C. Aliquots
of overnight culture were then used to inoculate two 50mL LB broth cultures, containing
25µg/mL kanamycin, to an OD_{600} value of approximately 0.1. Cultures were grown at 37°C
until OD_{600} of approximately 0.4 then 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) was
added to induce expression of the BapF passenger domain fused to an N-terminal 6X
Histidine tag. Cultures were incubated for 4 additional hours. Cell lysis and protein
purification were conducted as outlined in the Xpress™ System Xpress System Protein
Purification manual (Invitrogen, Carlsbad, CA). E. coli cells were lysed in 6M guanidine
hydrochloride, and the lysate was loaded atop Ni^{2+}-nitrilotriacetic acid (NTA) agarose
(Qiagen, Streetsville, ON.). Following washes in 8M urea with decreasing pH the BapF
passenger was finally eluted at pH 4 and fractions were pooled. The purified BapF
passenger was refolded by gradual removal of urea during dialysis against 10mM Tris-HCl
pH 8.0 in successive 24 hr washes at 4°C while stirring. Initial wash buffer contained 10 mM Tris-HCl pH 8.0 and 4 M urea, with subsequent washes halving the urea concentration decreasing from 2 M, 1 M, and 0.5 M to final the dialysis solely in 10 mM Tris-HCl pH 8.0.

2.11. Far-UV circular dichroism spectroscopy of refolded BapF passenger

Circular dichroism (CD) analysis was performed on refolded BapF protein using a Jasco J-810 spectropolarimeter (Jasco, Easton, MD) at room temperature using a cell path length of 2 mm. Individual spectra were collected by averaging 3 scans made over a spectral window of 190 nm to 320 nm. BapF passenger protein was analyzed at concentration of 1 µg/mL in 10 mM Tris-HCl at pH 8.0 buffer.

2.12. Raising polyclonal antibodies to the bapF passenger domain

Purified and refolded BapF passenger domain generated from the pET30b/bapFp expression plasmid was used for raising polyclonal antibodies. Protein was sent to Harlan BioProducts for Science (Indianapolis, IN) for inoculation into rabbits. Final bleeds were obtained and BapF polyclonal antiserum was aliquoted and stored at −80°C. BapF antiserum required a pre-absorption step prior to use in Western blots or fluorescence microscopy to reduce non-specific recognition of E. coli proteins and LPS. Pre-absorption was performed using E. coli BW27783 grown in 10 mL LB broth free of antibiotics to a final OD600 of approximately 1.0. Cells were centrifuged at 6000 g for 10 minutes, 5 mL supernatant was then removed and pellet suspended in remaining 5 mL media. Cell suspension was sonicated using 10 x 30 second pulses with 10 second rest on ice between each pulse. Five 1 mL aliquots of broken cells were then centrifuged at 15,000 g for 10 minutes at 4°C. The supernatant was then removed and pellets containing cell fragments used for pre-absorption of BapF antiserum. A 1 mL aliquot of BapF antiserum was used for suspension of a single pellet of cell fragments, and incubated at 4°C for 30 minutes while rocking. Following incubation, the sample was centrifuged for 10 minutes at
15,000g at 4°C. The serum was then removed and used to suspend another pellet of cell fragments. Incubation and centrifugation steps were repeated until all 5 pellets of cells fragments were used for pre-absorption. The final BapF antiserum was then divided into 10µL aliquots for storage at -20°C.

2.13. SDS-PAGE and immunoblot detection of BapF, BrkA and BamA

*E. coli* or *B. bronchiseptica* RB50 cultures were centrifuged, and protein samples derived from cell pellets (whole cell lysate) or supernatant precipitation (see below for protocol) were suspended in sample buffer then boiled for 5 minutes. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (87), and transferred to Immobilon-P membranes (Millipore, Etobicoke, ON) as described (116). Following transfer, SDS-PAGE gels were stained with Coomassie Brilliant Blue to verify equal amounts of protein were loaded into each lane. Immobilon-P membranes were probed using 1:30,000 rabbit anti-BamA (gift from Thomas J. Silhavy), 1:25,000 rabbit anti-BapF or 1:20,000 rabbit anti-BrkA antiserum (116). Following removal of primary antibody, blots were incubated with 1:50,000 horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (ICN Biomedicals, Costa Mesa, CA). PageRule prestained protein ladder (Thermo Scientific, Ont, Can) or Broad Range Protein Marker (New England Biolabs, Ipswitch, MA) were used to estimate molecular mass.

2.14. Expression of BapF in *E. coli*

The plasmid pET30b/bapF was transformed into CaCl₂ competent *E. coli* BL21(DE3)pLysS cells, and plated on LB agar containing 25µg/mL kanamycin. Isolated colonies were used to inoculate LB broth containing 25µg/mL kanamycin for overnight growth at 37°C. Aliquots of the overnight culture were used to inoculate 35mL cultures of similar media to an OD₆₀₀ of approximately 0.1. Cultures were grown at 37°C until OD₆₀₀ value of approximately 0.4, at which time varied concentrations of IPTG from 0.01 to 1mM was added. Cultures were
grown at 37°C for 3 additional hours with OD$_{600}$ readings taken at each successive one hour time point

Plasmids pBAD24/bapF, pBAD24/bapF$^{C28S}$, pBAD24/bapF$^{AA34-35S}$, pBAD24/bapF$^{Prn}$ and pBAD24/bapF$^{A32S,S33P,AA34-35S,A37S}$ were transformed into CaCl$_2$ competent BW27783 cells, and plated on LB agar containing 100µg/mL ampicillin. Isolated colonies were used for inoculation into LB liquid medium containing 100µg/mL ampicillin for overnight growth at 37°C. Aliquots of the overnight culture were used to inoculate larger cultures of similar media to an OD$_{600}$ of approximately 0.1. Cultures were grown at 37°C until OD$_{600}$ value of approximately 0.4, at which time varied concentrations of L-arabinose from 0.0001 to 0.1% was added. Cultures were grown at 37°C for up to 3 additional hours with OD$_{600}$ readings taken at each successive one hour time point. When required, OD$_{600}$ readings were entered into Microsoft Excel spreadsheets for graphing and presentation of growth curves.

2.15. Observation of culture sedimentation differences between BapF clones

BapF clones were expressed in E. coli BW27783 cells as described above for 2 hours. One mL of the least dense culture (as measured by OD$_{600}$) was used to normalize number of cells in all samples for observing sedimentation. Culture aliquots were added to microfuge tubes and centrifuged for 1 minute at 6000g. The supernatants were removed and pellet suspended in 500µL LB broth then rested unmoving at room temperature for 2 additional hours, followed by taking digital photographs of sedimentation.

2.16. Immunofluorescence microscopy

The Immunofluorescence protocol was adapted from previous methods (117), using slides incubated in 1:10 dilution of poly-L-lysine for 5 minutes, rinsed gently with sterile water and dried overnight at room temperature. All incubation steps were performed in large petri
dishes with a single layer of filter paper soaked in sterile water to maintain humidity. BapF clones were expressed *E. coli* BW27783 cells as described above for 2 hours, at which time 100µL from each culture was placed onto a poly-L-lysine coated slide. Samples were rested at 37ºC for 30 minutes to permit cells to settle and adhere onto the surface of the slides. Following incubation, sample was removed by pipette and bound cells were washed 3 times with phosphate buffered saline (PBS) solution containing 1% bovine serum albumin (BSA). After removal of final wash solution, cells were incubated for 60 minutes at 37ºC in PBS containing 1% BSA and 1:500 dilution of pre-absorbed heat-inactivated anti-BapF antiserum. Slides were washed again as described above, followed by 30 minute incubation at 37ºC in PBS containing 1% BSA and 1:1000 dilution of FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The solution was removed from slides and 10µL Prolong Gold Antifade reagent (Invitrogen, Burlington, ON) then added to reduce signal fading. Bacteria were visualized under fluorescence or differential interference contrast (DIC) microscopy using a Nikon eclipse TE2000 microscope (Nikon Instruments Inc., Mississauga, Ontario, Canada) fitted with appropriate filter sets for detecting fluorescence. Images were digitally captured.

2.17. N-lauryl sarcosyl fractionation and enrichment of BapF

*E. coli* cultures induced to express BapF as described above were centrifuged at 6000g for 15 minutes to pellet cells. Supernatant was decanted and pellets suspended in 5 mL 10mM Hepes buffer at pH8 containing 20µg/mL phenylmethylsulfonyl fluoride (PMSF). Cells were lysed using 3 by 5 second sonication bursts on a Sonicator Ultrasonic Processor® from Mandel Scientific. Lysate was centrifuged at 6000g for 15 minutes to remove unbroken cells and supernatant decanted into fresh centrifuge tubes. Samples were then centrifuged for 30 minutes at 15,500g, supernatant discarded and the pellet suspended in 500µL of 10mM Hepes pH8 followed by addition of 500µL 2% N-lauryl sarcosine. Samples were
allowed to stand at room temperature for 30 minutes then centrifuged at 15,000g for 30 minutes. Supernatant was discarded and the pellet (sarcosine-insoluble fraction) suspended in 100µL 10mM Hepes pH8.

2.18. Mass spectroscopy of full length BapF, passenger and β-barrel domains

The pBAD24/bapF plasmid was transformed into CaCl₂ competent BW27783 cells, grown and expressed in LB broth, then treated with N-lauryl sarcosyl to enrich for membrane proteins as described above. The sarcosine-insoluble fraction was separated by 11% SDS-PAGE then stained with Coomassie Brilliant Blue, followed by a 0.7% acetic acid wash to remove background staining. Unique bands, observed only in samples derived from cultures expressing BapF, were excised for mass fingerprinting using a nano-LC-ESI-MS/MS system and performed by Dr. Julius John in Dr. Francois Jean’s lab at the University of British Columbia as described below.

2.18.1. In-gel labeling and chymotrypsin digestions

Gel slices containing unique bands predicted to be the BapF full-length protein, passenger domain and translocation unit were excised from SDS-PAGE gels. The cysteine residue present in the passenger was reduced with 10µl of 10mM dithiothreitol at 56ºC for 30 minutes and alkylated with 10µl of 55mM iodoacetamide for 45 minutes at 21°C. Gel slices were then washed two times with 150µl of 50% acetonitrile and dried in a SpeedVac.

Chymotrypsin digestions were performed in-gel by addition of 25mM ammonium bicarbonate containing 25ng/µl chymotrypsin and incubated for 2.5 hours at 30°C. An additional 45µl of 50mM ammonium bicarbonate (pH 8.4) was added and incubated at 37°C overnight. Peptides were then extracted with 35µl of 20mM ammonium
bicarbonate at 37°C for 15 minutes, followed by 70µl of 2% formic acid/40% acetonitrile at 37°C for 15 minutes, and dried in a SpeedVac to a final volume of 20µl. The extracted peptides were further desalted using STAGE tips, according to the manufacturer’s instructions, and peptides eluted using 80% acetonitrile and 0.5% formic acid; volume was then reduced to 5µl using a SpeedVac concentrator.

2.18.2. LC-MS/MS and data analysis

Peptides were analyzed by on-line nanoflow liquid chromatography using the EASY-nLC system (Thermo Fisher Scientific). The gradient consisted of 4-35% acetonitrile in 0.5% formic acid at a flow rate of 300nl/min for 35 min, 35-64% acetonitrile in 0.5% acetic acid at a flow rate of 300nl/min for 15 min and 64-90% acetonitrile in 0.5% acetic acid at a flow rate of 300nl/min for 5 minutes. The eluate was electrosprayed into an LTQ Orbitrap Velos ETD (Thermo Fisher Scientific, Bremen, Germany) through a thermo nanoelectrospray ion source (119). The LTQ Orbitrap was set to acquire one MS scan at a resolution of 60000 in the Orbitrap from which the eight top most abundant ions were further CID fragmented in the LTQ.

Raw files were processed to mascot generic format (mgf) using Proteome Discoverer™ (Thermo Fisher Scientific). The SORCERER-SEQUEST™ v 4.0.4 search algorithm run on the SageN Sorcerer (Thermo Electron) was used as a search engine (95). Chymotrypsin was chosen as the cleavage specificity with a maximum number of three allowed missed cleavages. Searches were performed using a peptide tolerance of 10ppm for MS and 0.8Da for MS/MS search. Since BapF contains one cysteine residue, two different database searches were carried out. Carbamidomethylation was considered as a fixed modification in the first search and variable in the second search. Oxidation of methionine, S-palmitoyl cysteine and palmitoylation were considered as variable modifications in both searches. A parallel
database search was done by using the Mascot search engine (Matrix Science) against the NCBI protein database.

2.18.3. Identifying the N-terminus of the BapF passenger and translocation unit

N-terminal labeling, performed by Dr. Julius John, required the synthesis of N-acetoxy-($^1$H$_3$) succinimide using the reagents N-hydroxysuccinimide and acetic anhydride (>99% purity). The protocol for synthesis of N-acetoxy-($^1$H$_3$) succinimide is previously described (61). Briefly, 4 g of N-hydroxysuccinimide was mixed with 19.8 ml of acetic anhydride and incubated overnight at room temperature. White crystal products were washed with hexane and dried in vacuum.

The labeling process began with a 50% acetonitrile wash to the gel slices followed by drying in a SpeedVac concentrator. Approximately 6 mg of N-acetoxy-($^1$H$_3$) succinimide was then added to the dried gel pieces. Fifty microliters of 50 mM HEPES (pH 8.3) was then added to each gel piece, vortexed for 1 minute to dissolve and then centrifuged to spin down material. Reactions were incubated for 3 hours at room temperature. Gel slices were then washed with 170 µl of 100 mM ammonium bicarbonate followed by 20 µl of 50% hydroxylamine solution for 20 minutes then incubated at 21°C. Gel slices were then washed three times with 150 µl of 100 mM ammonium bicarbonate and two times with 50 µl of acetonitrile prior to drying in a SpeedVac. Chymotrypsin digestion, extraction of peptides from gel slices and LC-MS/MS analysis were then performed as described above.

2.19. Separation of membrane proteins using floatation sucrose gradient fractionation

The fractionation protocol used in BapF studies was adapted from Z. Soprova et al (162). Briefly, *E. coli* cultures expressing BapF clones were centrifuged for 10 minutes at 7000g, supernatant removed and pellet suspended in 7 mL 10 mM Tris, 10% sucrose, 20 µM PMSF
and 50µg/mL DNase. The suspension was incubated for 15 minutes at room temperature and then lysed using 2 by 12,000 PSI presses from a standard large French press and immediately placed on ice. The lysate was centrifuged in a Beckman Type 70.1 Ti fixed-angle rotor at 345,000g for 60 minutes at 4°C to pellet membrane fragments. The supernatant was then removed and the pellet suspended in 10mM Tris, 55% sucrose, 20µM PMSF and 50µg/mL DNase. Sample suspended in 55% sucrose buffer was placed at the bottom of an ultracentrifuge tube and layered on top with 51, 48, 45, 39, 36, 33 and 30% sucrose fractions in 10mM Tris, 20µM PMSF and 50µg/mL DNase buffer. Centrifugation was performed in a Beckman SW 41 Ti swing bucket rotor for 60 hours at 4°C and 287,000g. Aliquots were then removed from the top of the centrifugation tube and separated by 11% SDS-PAGE for Western blot analysis as described above.

2.20. Protease susceptibility assays

The protease susceptibility assay used in BapF studies was adapted from previously described protocols (117, 118). BapF clones were expressed in E. coli BW27783 cultures as described above for 3 hours. Aliquots of equal number of cells were removed from cultures and centrifuged at 6000g for 1min. Culture supernatants were removed and pellets containing cells suspended in 150µL PBS buffer at pH7.0 and incubated further at 37°C in final concentration of 100µg/mL trypsin. 15µL aliquots were removed from samples at set times, immediately added to 50µL gel loading buffer and boiled for 5 minutes. In addition, 150µL of in vitro refolded BapF passenger in 20mM Tris-HCl at pH 7.0 buffer was treated with trypsin in a similar manner to test for trypsin susceptibility. In some cases, 80µg/mL lysozyme and 50mM EDTA were added to select cultures to make the outer membrane permeable, allowing trypsin access to the periplasmic compartment.
2.21. Precipitation of protease-released fragments from the cell surface

*E. coli* BW27783 cells containing pBAD24/bapF were inoculated into LB broth to an OD<sub>600</sub> of approximately 0.1. Cultures were grown to OD<sub>600</sub> of approximately 0.4 at which time 0.1% arabinose was added to induce expression of BapF. Following 3 hours of incubation at 37°C, 1mL aliquots were then removed and centrifuged. Culture supernatants were removed and pellets containing cells suspended in 1mL PBS buffer at pH 7.0 and incubated for 0, 1, 5, 15, 30, 60 minute and overnight at 37°C in the presence of 100µg/mL trypsin. Following incubation, pellet and supernatant fractions were separated for each sample. Pellets were suspended in SDS gel loading buffer then boiled for 5 minutes. The supernatant fractions were incubated on ice with an equal volume of 20% TCA for 1 hour, followed by centrifugation at 15,000g for 1 hour to pellet precipitated protein. The pellets were then washed in ice-cold acetone and centrifuged at 15,500g. Remaining acetone was discarded and final samples suspended in 50µL SDS gel loading buffer, followed by boiling for 5 minutes.

2.22. Globomycin treatment of *E. coli* cultures expressing BapF or BrkA

*E. coli* BW27783 cultures expressing BapF or BrkA were inoculated into LB broth to an OD<sub>600</sub> of approximately 0.1. Cultures were grown to OD<sub>600</sub> of approximately 0.4-0.6 at which time 0.1% L-arabinose was added to induce expression, with 5µg/mL globomycin (gift from Shunichi Miyakoshi, Sankyo Co., Japan) added simultaneously to selected samples. Cultures were then incubated for 3 hours and aliquots removed for 5 minute centrifugation at 6000g. Supernatant was discarded and pellets suspended in SDS gel loading buffer, boiled for 5 minutes and protein separated in 11% SDS-PAGE for Western blot analysis as described above.
Chapter 3: Characterizing secretion of the novel *Bordetella* autotransporter BapF

3.1. Introduction

*Bordetella* species are the causative agent of respiratory infection in a wide range of mammals (42), with disease mediated by a large number of virulence factors (101). *Bordetella pertussis* is a strictly human pathogen. *Bordetella parapertussis* is also able to produce a respiratory infection humans (B. *parapertussis* HU) as well as sheep (B. *parapertussis* OV) with no indication of cross-over infection between species. *Bordetella bronchiseptica* is reported to infect humans, though rarely and these infections are generally in immunocompromised individuals. The primary hosts for *B. bronchiseptica* are from the *Canis* family, such as dogs, coyotes and wolves (101). Other species of *Bordetella* have been identified, and reported to cause respiratory infections in humans, but these species are not characterized as thoroughly as those mentioned above.

The virulence determinants mediating *Bordetella* colonization include adhesins such as fimbriae (FIM), filamentous hemagglutinin (FHA) and the autotransporter pertactin (Prn). Toxins released during infection include adenylate cyclase/hemolysin (CyaA), dermonecrotic toxin (DNT), tracheal cytotoxin (TCT) and pertussis toxin (PT). Infection is initiated by attachment and colonization of the host’s nasopharynx. The infection progresses to the upper respiratory tract where toxin-mediated damage to epithelial tissue produces the distinctive cough which *Bordetella* infection is known for. During *B. pertussis* infection, factors such as the autotransporter BrkA (*Bordetella* resistance to killing), CyaA and PT aid in immune evasion and persistence (101). During *B. bronchiseptica* infections some differences are observed in the virulence factors expressed compared to *B. pertussis*. Factors such as Tracheal Colonization factor (TcfA) and PT are not observed at either the gene expression or protein expression level in *B. bronchiseptica* infections (101). Though some variance in expressed virulence factors is observed in *B. pertussis* and *B. bronchiseptica* infections, the symptoms observed are very similar. However, *B. pertussis*
is predicted to remain in the Bvg⁺ phase, for it is a strictly human pathogen and exists in an environment which does not modulate the BvgAS system into the Bvg⁻ state (for a full description of the BvgAS system refer to section 3.3). Conversely, *B. bronchiseptica* is capable of living outside of its host, and can persist in the environment and observed to be in a Bvg⁻ phase.

Genome sequencing of *Bordetella pertussis* Tohama1, *Bordetella bronchiseptica* RB50 and *Bordetella parapertussis* 12822 (122) helped to facilitate identification of numerous autotransporters in these species (47). Identification of the C-terminal autotransporter domain and N-terminal signal peptide are the main criteria in determining whether a protein has the potential to be secreted by the autotransporter pathway.

The open reading frame encoding the *Bordetella* autotransporter F (BapF) autotransporter is predicted to be expressed in both the *B. bronchiseptica* and *B. parapertussis* sequenced strains; however, the open reading frame is not predicted to be expressed in the *B. pertussis* Tohama1 strain due to a frame-shift mutation. BapF is different from most autotransporters due to a lipobox motif contained within an N-terminal canonical signal peptide. It was also observed by phylogenetic analysis that the BapF translocation unit is an outlier when compared to other *B. bronchiseptica* translocation units (47) (Figure 10), suggesting it is more distantly related to other *B. bronchiseptica* translocation units. When the function of the related passenger domains was factored into the analysis, a general trend was observed. The analysis suggests autotransporters with similar function tend to group with phylogenetically similar translocation units (S. Pleasance and R. Fernandez, unpublished).

Investigating secretion of BapF will be informative for a number of reasons. It is predicted to be N-terminally acylated, a modification rarely observed in the autotransporter
superfamily. Also, the BapF \( \beta \)-domain appears to be evolutionarily different when compared to other \( B. \ bronchiseptica \) autotransporters, possibly indicating a unique role in secretion of BapF. Events occurring in the periplasm during secretion of BapF may involve interactions between the autotransporter and lipoprotein biogenesis pathways, interactions which to date have not be analyzed. A general insight into the secretion of autotransporters may also be achieved by studying the biogenesis of BapF, an unusual autotransporter which may test the extremes of secretion by having to contend with both N-terminal acyl groups and an autotransporter \( \beta \)-domain.

Figure 10: Phylogenetic analysis of known and predicted transporter domains from \( B. \ bronchiseptica \) autotransporters. A CLUSTALW alignment of predicted \( \beta \)-domains from \( B. \ bronchiseptica \) was used in generation of a phylogenetic tree presented using TreeView. Data from the alignment was subjected to neighbor-joining and distance matrix analysis using PHYLIP. The BB2324 (BapF) \( \beta \)-domain is observed as an outlier in this analysis. Figure reproduced with permission.
3.2. Bioinformatic analysis

3.2.1. Primary sequence analysis

Within the *B. bronchiseptica* RB50 and *B. parapertussis* 12822 genomes an open reading frame was identified coding for a potential 718 amino acid autotransporter. The same open reading frame in *B. pertussis* Tohama1 contains a frame-shift mutation resulting in a predicted truncation. The open reading frame from *B. bronchiseptica* RB50 (BB2324, accession NP_888867) was chosen for study and named in our lab *bapF* (for Bordetella autotransporter F).

The Basic Local Alignment Search Tool (BLAST) (3), which finds regions of similarity between biological sequences, was used to find proteins which are potentially similar to the BapF passenger domain. The proteins identified by BLAST to be most similar to BapF from *B. bronchiseptica* RB50 were the open reading frames from *B. parapertussis* and of course the BapF open reading frame from *B. bronchiseptica* RB50. Following the *Bordetella* matches to BapF is a protein from *Methylomonas methanica* MC09 with unknown function and from the HAF family of extracellular repeat-containing proteins was also found to be 44% similar to BapF. Next, an autotransporter with unknown function from *Janthinobacterium marseillense* was found to have 35% similarity to the BapF passenger domain. The BLAST search also identified a region of the BapF passenger domain spanning residues 166-370 belonging to a group of proteins in the Cluster of Orthologous Groups (COG) database (170), which are predicted integral membrane proteins with unknown function containing uncharacterized repeats (COG5563). In fact, the list of proteins which are similar to the BapF passenger domain contains numerous uncharacterized autotransporters, and proteins which are members of the HAF family of extracellular repeat-containing proteins. The HAF repeat is generally about 40 amino acids in length and contains the HAF tripeptide occurring regularly usually with 4 intervening amino acids. Other
members of this family also contain a C-terminal autotransporter domain, suggesting the HAF repeat is exported to the cell surface.

The BapF autotransporter domain was identified using the Pfam 26.0 tool for predicting protein families (8). Pfam 26.0 predicts the BapF autotransporter domain (PF03797) at coordinates 450-709 (alignment region spanning residues 452-700), and places BapF into the clan CL0193 which is described as an outer membrane β-barrel protein superfamily. Identification of the autotransporter domain was also performed using PROSITE 20.77 (28), another tool for predicting protein families. PROSITE predicted that BapF contains a C-terminal autotransporter domain (PDOC51208) spanning residues 449-718, and an N-terminal acylation motif (PDOC00013), also termed a lipobox, which terminates in a cysteine residue at position 28.

In addition to the lipobox motif identified by PROSITE, the BapF open reading frame codes for a canonical N-terminal signal peptide as identified using SignalP 3.0 (107, 108) (Figure 11a). Cleavage and removal of the signal peptide by signal peptidase 1 (SP1) is most strongly predicted to occur between the Ala\textsuperscript{34} and Ala\textsuperscript{35} residues. The signal peptide prediction was supported using LipoP 1.0 (67) which also identified a lipobox motif (Figure 11b). In the case of BapF, the lipobox sequence is LAA/C where the cysteine residue is at position 28, and cleavage between Ala\textsuperscript{27} and Cys\textsuperscript{28} is performed by a signal peptidase 2 (SP2) enzyme (Lsp in \textit{E. coli}). The primary amino acid sequence with signal peptide, lipobox motif and autotransporter domain highlighted can be found in Figure 12a, with a stick model of BapF presented in Figure 12b.
Figure 11: SignalP and LipoP results for BapF

A. According to SignalP 3.0 analysis, the N-terminal amino acid sequence of BapF contains a canonical signal peptide with 3 typical regions: N-region is the hydrophilic cytoplasmic region; H-region is the hydrophobic transmembrane segment; and C-region is the site of cleavage and removal of the signal peptide. Probabilities are scored on a scale of 0 to 1, with the overall BapF signal peptide probability measuring 0.977. Cleavage of the signal peptide is predicted to occur between the alanine residues at positions 34 and 35.

B. In the LipoP 1.0 analysis, the best score for BapF being 7.1305 for the SP1 cleavage site. Margin describes the difference between the best and second best scores. SP2 cleavage is scored at 2.74722. Amino acid sequences surrounding predicted cleavage sites are presented, and confirm the best scored SP1 cleavage site falls between two alanine residues found at positions 34 and 35. The SP2 cleavage site is found at the cysteine residue in the BapF LAAC lipox. Graphical representation of cleavage probabilities shows SP1 sites as red bars with varying scores, and the single SP2 site as a green bar. SPI = SP1; SPII, SP2.
A. Amino acid sequence of BapF

MKKQVVEYVRCGPSLRVPLSATLACLAHCGLASAAPALPPATVGMGMHQLAVPMGTTFP
MAMSSATVLAGTGSVPGLPHAAAMWPLSNPFYVYVIAGDRSSFHALSQDGSGVGVVVS
GGLSQAVRWDDNGSWQPGVGRTGATQSYASGVSDSGSVGWLTDGAGERPRFRWCTQA
TGVQTLSGFESYSAASISADGKVSVGVVDYTVTDALITPNAKRTATDGIITLLPHVAGG
TSSRAATNSDGSVVGVTAYGSSFGGARAFRTQAGGSANLGTIAGEGAEQSFASAVNAD
GSVGVYVDRTGILILITEQSAFRWCTQAGGMLTVEQWLRHGYTDQGQTWRALAVNADG
IVMGMNGDRTFVARTPADKVPDPVSPGGEPAPQPQGQPGPGGGLITLREFASSLG
GTALPASLTSTDMLHGAAHGSPLRLSPAGKVGMWTTGDGRTHEHGGGDGAIAAVGEAG
LSYRFNEQVQLNLAVGGGNMRQYLEDGKSTAQRQTYVMPELLWNLPSPLWLTASALYYAD
GDNLVRRGYPNAGVDDRSGKASLRTAAARLRLDWRDANWERAALTPYLDYSYRTVGV
SYTEHGGGFPARWDARTDHANVARLGVDATVQGNNQVQLGTVAEASHRFESHNSGASGQV
IGLMPFELDGASTORNWLRFGAGVQAPVGPITATMLNTTTHSSMPSYWLAATYRLAF

B. Stick model of BapF

![Stick model of BapF](image)

Figure 12: Amino acid sequence and stick model of BapF

A. Amino acid sequence of BapF. The signal peptide is underlined, terminating at the alanine in position 34 as predicted by both SignalP 3.0 and Lipop 1.0 predictions. The Lipobox is colored red with the acylated cysteine residue bolded. The predicted autotransporter domain is also bolded, and start site selected based on PROSITE 20.77 analysis.

B. Stick model of BapF includes the signal peptide region in grey, with SP1 and SP2 cleavage sites labeled with arrows. The BapF passenger domain and autotransporter domain are in red and green respectively, with PROSITE 20.77 autotransporter domain designation included. The alpha-helical linker region (in yellow) bridges the passenger and autotransporter domains and is found within the β-barrel.
3.2.2. Secondary structure prediction

Secondary structure prediction was carried out via PSIPRED (64). This analysis predicts that BapF contains significant β-structure, with alpha helical structure found near the N-terminus and central regions matching predictions for the signal peptide and linker region respectively (Figure 13) (see Chapter 1 for description of the linker region).

3.2.3. Tertiary structure prediction

The vast majority of autotransporter passenger domains either have or are predicted to have a β-helix conformation (48, 59). BetaWrap is a tool which can be used to predict if a protein is capable of forming a β-helix structure (14). The first 430 residues of BapF (the predicted passenger domain) were input for BetaWrap analysis, and data suggest the BapF passenger domain does not form a β-helix, or has very little β-helix conformation. The confidence value (P-value) reported by BetaWrap for the BapF passenger is 0.023, where 0.01 is the minimum confidence value required to suggest a β-helix may be formed. Though the BetaWrap result for the BapF passenger might appear close to the minimum threshold to identify a potential β-helix structure, known β-helix proteins have incredibly low P-values, such as pertactin from *Bordetella pertussis* with a BetaWrap P-value of 0.000017.

To help determine if the BapF passenger domain attains a structure different than a β-helix, a structural prediction was performed using Phyre (74), a threading tool designed to predict potential tertiary structures based on sequence alignment to known structures. The result from the Phyre prediction for the BapF passenger domain indicates a strong similarity to known β-propeller proteins composed of WD40 repeat-like motifs (Figure 14). The β-propeller architecture is generally composed of 4-8 blade-shaped β-sheets arranged around a central axis. The WD40 repeat is a
structural motif of approximately 40 amino acids, often terminating in a tryptophan-aspartic acid (WD). Several repeats combine to form a WD domain. A common function of WD-repeat proteins is to aid in coordinating protein complexes, where repeat regions can serve as scaffolds to mediate protein interactions (160). To date there are no reported autotransporters with a known or predicted β-propeller structure of the passenger domain.

3.2.4. Repeats and regions of hydrophobicity identified in the BapF passenger

Though Phyre predicted a β-propeller structure for the BapF passenger domain, only 2 WD-repeats are present in the BapF primary sequence. It was hypothesized that the BapF passenger contains other repeats which may functionally replace or complement the 2 existing WD-repeats. To this end, the BapF passenger domain was searched for repeats using the tool HHreplD (12) which is designed to identify sequence signatures within structural repeats. The HHreplD results predict 7 structural repeats harboring valine-rich regions and in many cases a VVV repeat (Figure 15A).

Analysis of hydrophobicity along the BapF passenger sequence was used to gain insight into what elements may be facilitating β-propeller formation. Such an analysis may uncover areas of potential aggregation, which may in turn coincide with amino acid repeats within the BapF passenger. The tool used for such an analysis was Tango (36, 94, 137), which has the ability to assess the relationship between protein structure and β-aggregation. Results clearly reveal areas of hydrophobicity within the BapF passenger domain, which also coincide with stretches of amino acids rich in valine (V) residues, and often containing the VVV repeats also identified in the HHreplD analysis (Figure 15B).
Figure 13: PSIPRED secondary structure prediction for BapF

The entire amino acid sequence was used in the PSIPRED analysis which revealed significant β-sheet structure, as indicated by arrows in the graphical representation. Alpha helix structures are represented as cylinders, and random coiled regions as straight lines. Also included are the letter representations for secondary structure elements, with C for random coil, E for β-sheet and H for alpha helical structure. Confidence levels are represented as blue bars above the structural data, with the height of the bar representing level of confidence.
Figure 14: Phyre prediction of tertiary structure for the BapF passenger domain

Tertiary structure prediction was performed by finding sequence alignments to known structures. The Phyre prediction tool uses the BapF PSI-BLAST profile and that of known structures to predict remotely homologous regions. In regards to E-values and estimated precision values, the authors of Phyre state “E-values should be considered an internal scoring scheme. We have benchmarked the system on an extensive set of known remote homologies and calculated how often a given e-value is assigned to true and false homology. This is the basis for our ‘Estimated precision’ calculation. A typical result will present an e-value and next to it, an estimated precision value. The estimated precision is the percentage of times a match with the given e-value was found to be a true homology.” (74) The β-propeller structures to which BapF is aligned are composed of repeating WD-regions.
A.

Seven structural repeats were identified in the BapF passenger domain and labeled A1-7. Sequence segments corresponding to each structural repeat are presented, with the red box encompassing the valine-rich regions and VVV repeats.

B.

Values on the X-axis refer to positions 1–439 in the primary sequence of the BapF passenger. Y-axis values refer to degree of β-aggregation. Peaks represent areas of potential β-aggregation, and are matched below to the amino acids found at those positions. One of the VVV repeats observed did not match with a predicted region of β-aggregation, as represented by the question mark (†).

Figure 15: Tango and HHrepID analysis of the BapF passenger domain
The BapF passenger sequence spanning residues 35-407 was used in the Tango (A) and HHrepID (B) analysis.
3.3. Expression of BapF in \textit{B. bronchiseptica} RB50 is regulated by BvgAS

The BvgAS two-component signal transduction system regulates a wide array of virulence factors in \textit{Bordetella}, which includes autotransporters (176). Once the BvgS sensor kinase receives a stimulus it forms a dimer and signals via a phosphor-transfer cascade to the cytoplasmic regulator BvgA, initiating expression of certain genes and repression of others (176). When the BvgAS system is active, the cell is in the Bvg$^+$ phase; alternatively, the cell is in the Bvg$^-$ phase when the BvgAS system is not stimulated. A Bvg intermediate phase (Bvg$^i$) has also been identified, in which the gene expression profile includes the early expressed genes from the Bvg$^+$ phase, and proteins unique to the Bvg$^i$ phase such as BipA (166). Each phase can activate and repress different genes, and during transition between phases, gene expression can be more accurately viewed as a gradient where the expression profile is gradually altered (78).

It is possible to artificially modulate the Bvg system by growing cells in the presence of MgSO$_4$ (149); under these conditions the Bvg system is in the Bvg$^-$ phase and a vast array of virulence genes are repressed. When \textit{B. bronchiseptica} RB50 is grown in either the presence or absence of 50mM MgSO$_4$, and whole-cell lysate is then probed by Western blot using pre-absorbed BapF antiserum, the resulting data indicate the BapF passenger is visible only under Bvg$^-$ conditions (Figure 16). Independent microarray studies also reported increased expression of BapF in \textit{B. bronchiseptica} RB54 (a Bvg$^-$ phase-locked mutant) when compared to the wild-type \textit{B. bronchiseptica} RB50 (105).

A higher molecular weight protein was also identified in the Western blots of whole-cell lysates derived from \textit{B. bronchiseptica} RB50 in both Bvg$^-$ and Bvg$^+$ conditions. This protein may be the BapF full-length protein or some other \textit{B. bronchiseptica} RB50 protein recognized by the BapF polyclonal antibody by cross-reactivity. Generally, in the native strain background, it is not unusual to only see the passenger expressed as most of the
full-length protein is efficiently secreted, processed and surface expressed. If the higher molecular weight protein is indeed full-length BapF, then data may suggest that processing of BapF is regulated by the BvgAS system. In addition, production of the BapF passenger could possibly be growth-phase dependent, where expression is reduced at lag and late-log/stationary phases. This result may not be surprising for growth-phase regulation of virulence factors in *B. bronchiseptica* has already been observed (106).
Figure 16: Expression of BapF in *B. bronchiseptica* RB50 under Bvg- conditions
Both panels are Western blots of *B. bronchiseptica* RB50 whole-cell lysates, using pre-absorbed polyclonal antiserum to the BapF passenger domain. Passenger domain (indicated by red arrow) at approximately 45kDa is observed only in cultures maintained in 50mM MgSO$_4$. Overnight *B. bronchiseptica* RB50 cultures were grown in SS broth and either presence or absence of 50mM MgSO$_4$, and used for inoculation of larger cultures in similar media to an initial OD$_{600}$ of approximately 0.1. Cultures were grown further at 37°C and aliquots removed at designated times post-inoculation, then centrifuged and supernatant removed. Pellets containing cells were suspended in gel loading buffer and probed for BapF. Molecular mass markers in kDa are displayed to the left of each blot.
3.4. *E. coli* model system to study BapF biogenesis

There exist many genetic tools for expressing recombinant proteins in *E. coli*, which are not easily available or generated in *Bordetella*; therefore, moving the project into an *E. coli* model system greatly increased the ease with which experiments were conducted. In addition, the work done to characterize BapF secretion is in-part performed to broaden the understanding of acylated autotransporter biogenesis in Gram-negative bacteria, a goal more easily achieved in an *E. coli* background. The *E. coli* BW27783 expression strain was employed to study BapF biogenesis. This strain is ideal due to elimination of genes involved in arabinose metabolism and import, and addition of a constitutively expressed low-affinity arabinose importer. Therefore, the concentration of exogenous arabinose induces expression of genes governed by the arabinose promoter (araBAD) in a directly proportional manner (104). Cloning of genes into the pBAD24 expression vector places them under control of the arabinose promoter; thus, pBAD24 plasmids harboring bapF can be transformed into the *E coli* BW27783 expression strain and cultures induced via addition of arabinose into the growth medium (156).

3.5. Phenotype of *E. coli* cells expressing BapF

Expression of BapF in *E. coli* BW27783 cells produced phenotypes of auto-aggregation and rapid culture sedimentation, where pBAD24 vector control cultures did not show any signs of similar phenotypes (Figure 17). Whether BapF is self-associating or binding to structures on the *E. coli* surface was not determined. Neither auto-aggregation nor rapid sedimentation phenotypes are observed in *B. bronchiseptica* RB50 grown in MgSO₄ (Bvg- conditions). The absence of a phenotype in *B. bronchiseptica* RB50 may be due to factors such as a low copy number of BapF in *B. bronchiseptica* RB50 under native expression conditions, the possible release of the BapF passenger from the *B. bronchiseptica* RB50 surface, or differences in cell surface composition between *B. bronchiseptica* RB50 and *E. coli* BW27783.
Figure 17: Sedimentation and aggregation of *E. coli* cells expressing BapF

*E. coli* BW27783 cells harboring pBAD24 vector (top panels) or pBAD24/bapF expression plasmid (bottom panels) were either not induced (left panels) or induced (right panels) using 0.1% arabinose for 3 hours. Each panel shows both DIC microscopic image of cellular aggregation immediately following the 3 hour period of induction, and photograph of culture sedimentation, where following the 3 hour induction period cells were rested for an additional 2 hours at room temperature.
3.6. Molecular analysis of BapF expressed in *E. coli*

3.6.1. Enrichment of outer membrane fractions

Attempts to observe BapF in whole-cell lysates from *E. coli* BW27783 cells were initially conducted prior to raising a polyclonal antibody to the BapF passenger domain. These attempts used Coomassie Brilliant Blue to stain SDS-PAGE gels containing whole-cell lysate samples, which were ultimately unable to distinguish between native *E. coli* proteins and BapF. The inability to identify BapF in whole-cell lysate samples led to the use of N-lauryl sarcosine for enrichment of outer membrane proteins, the fraction in which BapF is predicted to be found. This approach made possible the observation of three unique bands in SDS-PAGE gels stained with Coomassie Brilliant Blue. The observed bands appeared to represent BapF full-length protein, passenger domain and translocation unit (Figure 18).
E. coli cultures harboring the pBAD24/bapF expression plasmid were grown in the absence or presence of 0.1% L-arabinose. Membrane proteins were then enriched using 2% sarcosine (see Methods) enabling observation of three unique bands in SDS-PAGE gels stained with Coomassie Brilliant Blue. Observed sizes of bands were similar to predicted sizes of BapF full-length, passenger domain and translocation unit at approximately 70.9, 40.8 and 30.1kDa respectively (calculated from signal peptidase 1 cleavage site and the cleavage site between the passenger and translocation unit at position 439). These bands were excised and used for mass spectrometry analysis. Molecular mass markers in kDa are displayed to the left of the gel.
3.6.2. Identifying unique bands isolated from *E. coli* cells expressing BapF

The three unique bands observed in SDS-PAGE gels stained with Coomassie Brilliant Blue, and predicted to be BapF full-length protein, passenger and translocation unit were excised. Gel slices were then incubated with chymotrypsin for in-gel digestion; peptides were extracted from the gel-slices, then desalted and concentrated under vacuum for analysis by LC-MS/MS (see methods). Briefly, for mass spectroscopy analysis, peptides were subjected to liquid chromatography and eluate electrosprayed through a thermo nanoelectrospray ion source into an LTQ Orbitrap Velos ETD (Thermo Fisher Scientific, Bremen, Germany) (119).

Mass spectroscopy analysis produced a complete sequence for the entire BapF protein, and also confirmed the prediction that the unique bands were indeed the BapF full-length protein, passenger and translocation unit. The method was able to clearly map the signal peptidase 1 cleavage site between residues Ala$^{34}$ and Ala$^{35}$, as predicted by SignalP 3.0 and LipoP 1.0; however, the processing site between the passenger and β-barrel domains was not determined. To obtain these data, gel slices were subjected to in-gel labeling with N-acetoxy-$^{(1}H_3$) succinimide prior to chymotrypsin digestion (see methods). N-acetoxy-$^{(1}H_3$) succinimide labels the free N-terminus of any protein present in the sample. LC-MS/MS results obtained using the N-terminally labeled samples confirmed the signal peptidase 1 processing site, and identified the processing site between the passenger and β-barrel lying between residues Gly$^{439}$ and Ala$^{440}$.

3.6.3. Globomycin specifically affects BapF biogenesis

Mass spectroscopy results told us that N-terminal processing occurs at the predicted signal peptidase 1 cleavage site. However, the signal peptidase 2 processing site is N-terminal to the signal peptidase 1 processing site (refer to Figure 12), and may still
be acylated during BapF biogenesis. Acylation of lipobox-containing proteins in Gram-negative bacteria is performed by three enzymes which are anchored to the periplasmic side of the inner membrane (see Chapter 1). Briefly, phosphatidylglycerol/prolipoprotein diacylglycerol transferase (Lgt), adds a diacylglycerol via thioether linkage to the Cys, followed by prolipoprotein signal peptidase (LspA or signal peptidase 2) which cleaves and removes the signal peptide leaving a free N-terminal Cys residue. Phospholipid/apolipoprotein transacylase (Lnt) will then amino acylate the N-terminal cysteine residue, yielding the final product N-acyl diacylglycerol-cysteine.

Globomycin is an antibiotic which specifically inhibits the activity of LspA (56), yielding a partially acylated intermediate which can be targeted for degradation. This antibiotic therefore provides a method for tracking whether or not N-terminal acylation has occurred. Importantly, complete inhibition of lipoprotein production is fatal for cells. Therefore, titration of globomycin in growing *E. coli* cultures was necessary to find a concentration which permitted growth, yet resulted in a detectable reduction of lipoprotein production.

Titration experiments were conducted prior to production of the BapF antiserum, and therefore required N-lauryl sarcosine enrichment of outer membrane fractions in order to observe BapF in SDS-PAGE gels stained with Coomassie Brilliant Blue. Results from titration experiments determined that 5µg/mL globomycin was permissive for cell growth, as observed by tracking OD$_{600}$ values, yet adequately inhibited BapF biogenesis (Figure 19). Following production of the anti-BapF antiserum, N-lauryl sarcosine enrichments were no longer required, and whole-cell lysates were analyzed by Western blot. When 5µg/mL globomycin was added to *E. coli* cultures as described above, BapF biogenesis was dramatically reduced as previously observed; however,
expression of BrkA (a *Bordetella* autotransporter lacking a lipobox motif) was minimally effected (Figure 20).

This result demonstrated the specificity of globomycin as an inhibitor of BapF biogenesis, and not that of proteins lacking a lipobox motif. The slight reduction observed in BrkA biogenesis can be attributed to the pleotropic effects of globomycin, specifically in regards to the production of lipoproteins BamB-E. Disruption of the BAM complex will have a global impact on outer membrane protein biogenesis, but when used at an appropriate concentration globomycin will have a more significant impact on lipoprotein biogenesis. In addition, exposure of cells expressing BapF to globomycin inhibits the ability to produce the aggregation and sedimentation phenotypes, and instead results in phenotypes similar to negative control cultures (Figure 21).
Figure 19: Titration of globomycin

A. SDS-PAGE gel, stained with Coomassie Brilliant Blue, containing samples derived from BapF-expressing *E. coli* BW27783 cells enriched for membrane proteins using N-lauryl sarcosine. *E. coli* cultures were grown in either the absence (−) or presence (+) of arabinose and simultaneously exposed to globomycin concentrations at either 5 or 10µg/mL. Red arrows indicate 3 unique bands each representing the BapF full-length protein (≈ 70kDa), passenger domain (≈ 40kDa) or translocation unit (≈ 30kDa). Molecular mass markers in kDa are displayed to the left of the gel.

B. OD₆₀₀ values were tracked throughout the course of the experiment for cultures “Not induced” (− arabinose), “Induced” (+ arabinose) and induced in the presence of globomycin at 5 or 10µg/mL.
Figure 20: Globomycin inhibition of BapF biogenesis

Western blot of whole-cell lysates using anti-BapF polyclonal antibody (left and middle panels) and anti-BrkA polyclonal antibody (right panel). Cultures were grown in the absence (−) or presence (+) of arabinose and/or globomycin at 5µg/mL. In the presence of arabinose, and absence of globomycin, BapF full-length protein and passenger domain are observed at predicted sizes of approximately 70 and 40kDa respectively. However, with addition of globomycin expression of both BapF full-length and passenger domain are significantly inhibited. BrkA full-length and passenger domain is observed in the presence of both arabinose and globomycin, suggesting inhibition of BapF biogenesis by globomycin is specific and not due to pleotropic effects. Molecular mass markers in kDa are displayed to the left of the blot.
Figure 21: Globomycin inhibits aggregation and sedimentation phenotypes in BapF-expressing E. coli cultures
Data from Figure 17 juxtaposed with images of E. coli cells induced to express BapF and grown in the presence of 5µg/mL globomycin (right panels). Lower right panel: DIC image of cells harboring the pBAD24/bapF expression plasmid, and grown in the presence of both arabinose and globomycin, reveal the inability of cells to form aggregates; neighboring photograph shows similar inability of cultures to rapidly sediment, in contrast to cells expressing BapF in the absence of globomycin (lower middle panel).
3.6.4. BapF is localized to the outer membrane

Autotransporters are outer membrane proteins; therefore, regardless of any N-terminal processing which may occur, BapF at minimum should be targeted to the outer membrane by virtue of its autotransporter β-domain. Previous work using N-lauryl sarcosine produced crude preparations enriched for outer membrane proteins. To clearly determine whether BapF reached the outer membrane compartment, a sucrose gradient fractionation approach was employed, along with Western blot analysis using pre-absorbed BapF antiserum. Results show that both BapF full-length protein and passenger domain co-localize with the known outer membrane protein BamA, suggesting the final destination of BapF is the outer membrane compartment as predicted (Figure 22), and as supported by the sarcosine enrichment results (Figure 18).
Figure 22: Sucrose-gradient fractionation of membrane fragments from cells expressing BapF

*E. coli* cultures induced to express BapF for 3hr period were subjected to sonication and subsequent stepwise sucrose gradient ultracentrifugation (see Methods). Resulting fractions were then analyzed by Western blot using pre-absorbed BapF polyclonal antibody (upper panel) or BamA polyclonal antibody (lower panel). BapF full-length protein and passenger domain fractionated to the high-density regions of the gradient which generally contain outer membrane proteins. BapF also co-fractionated with the known outer membrane protein BamA. Molecular mass markers in kDa are displayed to the left of the blot.
3.6.5. Surface expression of the BapF passenger domain

Sucrose gradient fractionation results indicate BapF reaches the outer membrane compartment, but not whether the passenger domain is presented on the cell surface, although the aggregation phenotype (Figure 17) indirectly suggests that this is the case. Fluorescence microscopy and trypsin susceptibility experiments were employed to definitively determine whether BapF is surface expressed. As shown below, both methods indicate the BapF passenger domain localizes to the cell surface.

3.6.5.1. Fluorescence microscopy

Fluorescence microscopy can produce convincing data suggesting surface expression of a protein. *E. coli* BW27783 cells expressing BapF were labeled using pre-absorbed heat-inactivated anti-BapF antiserum, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibody which is specific to the Fc region of rabbit IgG (see Methods). Results show a greater fluorescence signal from cells expressing BapF than from cells harboring a pBAD24 vector control (Figure 23).

3.6.5.2. Limited trypsin susceptibility assay

If the BapF passenger domain is exposed on the cell surface, as demonstrated by fluorescence microscopy, it will be susceptible to trypsin digestion. Keeping trypsin at a concentration low enough to not disrupt the outer membrane, yet at high enough concentration to digest surface-exposed protein, will help determine if the BapF passenger is on the cell surface. *E. coli* BW27783 cells expressing BapF were exposed to 100µg/mL trypsin in a similar manner to that previously used to study surface expression of the BrkA autotransporter (117).
Western blot analysis revealed that in the absence of trypsin the full-length BapF protein diminishes over long time intervals of approximately ≥60 minutes, with the passenger domain remaining very stable over the same time period (Figure 24, top panel). However, when exposed to 100μg/mL trypsin for ≤60 minutes, the BapF full-length protein (∼70kDa) diminishes slightly more rapidly, with the passenger domain (∼40kDa) remaining stable. Yet, when exposed to 100μg/mL trypsin overnight, the BapF passenger domain shows evidence of digestion, resulting in a slightly smaller trypsin-resistant fragment (Figure 24, bottom panel). In some experiments, this smaller, trypsin-stable fragment appeared as early as 15 minutes. These data suggest that at least a portion of the BapF passenger is present on the surface of the cell and susceptible to trypsin digestion, with a fraction of the full-length BapF protein potentially digested by trypsin as well.
Figure 23: Fluorescence microscopy confirms surface expression of the BapF passenger

*E. coli* BW27783 cells harboring either pBAD24 vector control or pBAD24/bapF expression plasmid were grown in the presence of arabinose for 3 hours and exposed to BapF polyclonal antibody and FITC-conjugated anti-rabbit IgG secondary antibody for subsequent exposure to fluorescence (see Methods). Vector controls (left panels) and BapF expressing cultures (right panels) were imaged using Differential interference contrast microscopy (DIC, upper panels) or fluorescence microscopy (lower panels). Results suggest the BapF passenger is exposed on the cell surface.
Figure 24: Trypsin susceptibility assay suggests surface expression of the BapF passenger domain

*E. coli* cells expressing BapF were aliquot, suspended in PBS and exposed to 100µg/mL trypsin for various times (see Methods). Western blot analysis of samples in the absence (upper panel) or presence (lower panel) of trypsin reveal that when the BapF passenger domain exposed to trypsin over time, it is digested into a slightly smaller molecular weight fragment which is trypsin resistant (red arrow). Molecular mass markers in kDa are displayed to the right of each blot.
3.6.6. Trypsin releases the BapF passenger from the cell surface

The nature of the trypsin experiments did not permit differentiation between whether the BapF passenger remains on the cell surface, or is released into the environment due to the activity of trypsin. It was therefore important to determine whether the trypsin-resistant passenger fragment, appearing at a slightly smaller molecular weight than the entire BapF passenger domain, remained attached to the surface or was released into the milieu. *E. coli* BW27783 cells expressing BapF were exposed to trypsin as described above, followed by centrifugation to separate cells from the supernatant fraction. Whole-cell lysate was compared by Western blot to precipitated protein from supernatant fractions. Data indicate the vast majority of the trypsin-resistant BapF passenger was released from the cell surface (Figure 25).
Figure 25: Trypsin-released BapF passenger found in supernatants
Trypsin susceptibility experiments conducted exactly as outlined for Figure 24 were modified such that following various periods of exposure to trypsin whole-cells were separated from supernatants (see Methods). Protein released from the cell surface by trypsin digestion was TCA precipitated from supernatants. Western blot analysis was used to compare whole-cells lysates (left panel) to precipitated protein from supernatants (right panel). Results suggest that nearly the entire BapF passenger domain was released from the cell surface by trypsin into the supernatant (red arrow). Molecular mass markers in kDa are displayed to the left of the blot.
3.6.7. *In vitro* refolded BapF passenger exhibits trypsin resistance

As described above, the BapF passenger domain exhibits significant trypsin resistance, yet the data does not determine whether resistance is due to the inherent properties of the folded BapF protein. The tertiary structure of the BapF passenger domain is predicted to be similar to known β-propeller proteins, and these structures have been reported to be trypsin resistant (173). *In vitro* digestion of the refolded BapF passenger was performed to determine whether the trypsin resistance observed for the BapF passenger is conferred by the structure itself. The pET30bBapFpass expression construct (encoding for residues 35-407 of the BapF passenger) was transformed into *E. coli* BL21(DE3) and expression induced via IPTG. The BapF passenger is expressed with an N-terminal 6XHis tag which was used for protein purification under denaturing conditions over Ni-NTA agarose. The purified BapF passenger was then refolded by gradual removal of denaturant by dialysis against Tris-HCl (see methods). The refolded BapF passenger was analyzed for secondary structure using circular dichroism spectroscopy with results indicating significant β-sheet structure was attained, suggesting a correct tertiary structure was at least in-part restored.

The refolded BapF passenger was assayed for trypsin susceptibility in the exact manner as described above for whole-cell experiments. Results obtained suggest the BapF passenger domain attains a structure which is resistant to trypsin digestion, producing a trypsin-resistant fragment similar to that observed in the whole-cell assays. In addition, it appears a population of the BapF passenger sample is incompletely folded. Trypsin is able to digest the partially folded protein, and produce a distinct profile of smaller molecular weight fragments (Figure 26). This profile of lower molecular weight fragments may indicate the BapF passenger is in the process
of folding independent sub-domains, possibly correlating to blades of a β-propeller which are flanked by predicted trypsin digestion sites.
Figure 26: In vitro trypsin digestion of refolded BapF passenger

A. The BapF passenger domain was purified under denaturing conditions and refolded via dialysis against Tris-HCl (see Methods). Trypsin digestion was performed exactly as done with whole-cells (Figure 24), and Western blot analysis was performed using the anti-BapF antiserum. Upon trypsin exposure a population of larger molecular weight protein is fragmented into a reproducible profile (red box), with longer exposure to trypsin producing a fragment slightly smaller than the passenger domain (red arrow). The slightly smaller passenger fragment produced at long time intervals matches that observed in trypsin susceptibility assays using whole-cells. Molecular mass markers in kDa are displayed to the left of the blot.

B. Circular Dichroism spectroscopy results for the refolded BapF passenger domain ranging from amino acids 35-468. Graph produced (blue line) indicates the passenger domain has folded into a secondary structure rich in β-sheet conformation. The refolded passenger domain demonstrated significant β-structure, suggesting adequate refolding into the natural tertiary structure.
3.7. Conclusions

Bioinformatics predicted that BapF is unique in a number of ways. The N-terminus of BapF may be acylated by virtue of a lipobox motif, located N-terminal to a predicted signal peptidase 1 cleavage site. These predictions suggest that BapF may only be N-terminally acylated at the Cys\textsuperscript{28} residue, and have the acyl groups remain attached. Alternatively, BapF could be processed solely by signal peptidase 1. Finally, BapF may initially be N-terminally acylated at the Cys\textsuperscript{28} residue, but subsequent cleavage by signal peptidase 1 would remove the acyl groups. Bioinformatics also predicted a potential β-propeller tertiary structure for the BapF passenger, a structure not reported in any other characterized autotransporter. Regions of the BapF passenger exhibiting potential β-aggregation may facilitate folding into its final structure. Proteins to which the BapF passenger domain is similar were found to be uncharacterized autotransporters from various species of bacteria, and also members of the HAF family of extracellular repeat proteins.

Expression of BapF is observed in both \textit{B. bronchiseptica} RB50 and \textit{E. coli}. In \textit{B. bronchiseptica}, BapF appears to be under Bvg regulation with the passenger domain only observed in the Bvg\textsuperscript{+} phase. In \textit{E. coli}, the BapF full-length protein, passenger and translocation unit are clearly observed. Mass spectroscopy was used to map the signal peptidase 1 processing site to lie between Ala\textsuperscript{34}/Ala\textsuperscript{35}, and the cleavage site between passenger and translocation unit to lie between residues Gly\textsuperscript{439}/Ala\textsuperscript{440}. N-terminal acylation of the BapF passenger was shown to have a role in biogenesis, yet the acyl groups are subsequently removed by activity of signal peptidase 1. The purpose of having an acylated intermediate in the BapF secretion pathway is unclear at this time, yet it may be important for regulating folding or secretion of the passenger to the cell surface. Of note, the BapF passenger domain exhibits trypsin resistance, which may be attributed to a potential β-propeller structure.
Chapter 4: The role of signal peptidase 1 and signal peptidase 2 processing in BapF secretion

4.1. Introduction

The globomycin data indicated that LspA (E. coli signal peptidase 2) is required for biogenesis of BapF. In addition, mass spectroscopy revealed that both full-length BapF and the passenger domain are ultimately processed by signal peptidase 1. This scenario describes BapF as having an acylated intermediate in its secretion pathway. This intermediate is then processed by signal peptidase 1 to remove the N-terminal acyl groups, permitting release from the inner membrane and transit to the outer membrane for translocation of the passenger to the cell surface. In this chapter, the impact of disrupting signal peptidase 1 or 2 activity on BapF biogenesis will be described. The hypothesis is that both signal peptidase 1 and 2 activity is required for secretion of the BapF passenger domain to the cell surface.

4.2. Disrupting signal peptidase 2 processing results in accumulation of unprocessed BapF

4.2.1. Generating mutants which block acylation

To address the role of signal peptidase 2 processing in BapF biogenesis, mutants were generated which lack the ability to be acylated. Two routes were chosen to disrupt acylation of BapF: site-specific mutation of the cysteine residue present in the lipobox to a serine (BapF\textsuperscript{C28S}); and replacement of the entire BapF signal peptide with the signal peptide from pertactin (BapF\textsuperscript{Prn}) (Figure 27). Pertactin is a Bordetella autotransporter with a signal peptide lacking a lipobox motif, but similar in size to that of the BapF signal peptide. Blocking acylation would dictate that BapF secretion should follow a route similar to that of autotransporters with a canonical N-terminal signal peptide (see Chapter 1, and Figure 27C).
Figure 27: BapF\textsuperscript{C28S} and BapF\textsuperscript{Prn} signal peptides and predicted secretion pathway

A. ClustalW alignment comparing signal peptide regions for BapF\textsuperscript{WT}, BapF\textsuperscript{C28S} and BapF\textsuperscript{Prn}. The blue arrow identifies the predicted signal peptidase 1 cleavage site for the BapF\textsuperscript{WT} and BapF\textsuperscript{C28S} clones, and the red arrow identifies the signal peptidase cleavage site for the BapF\textsuperscript{Prn} clone. Small and large red boxes identify the C28S mutation and pertactin signal peptide respectively.

B. Graphical representation of signal peptidase 2 mutant signal peptides. Red star in the BapF\textsuperscript{C28S} signal peptide depicts the site-specific mutation. The pertactin signal peptide in purple is depicted fused to the BapF passenger domain.

C. Predicted pathway for secretion of signal peptidase 2 mutants. See Chapter 1 for description on roles for various components in the secretion pathway. Briefly, \textit{E. coli} signal peptidase 1 cleaves and removes the signal peptide from the secreted protein following inner membrane translocation via the SEC translocation machinery. The BAM machinery directs insertion of the BapF autotransporter domain, ultimately leading to cleavage between the passenger and translocation unit and presentation of the passenger on the cell surface.
4.2.2. Phenotypes induced in *E. coli* by expression of BapF<sup>C28S</sup> and BapF<sup>Prn</sup>

BapF<sup>C28S</sup> and BapF<sup>Prn</sup> were expressed using the pBAD24 vector and *E. coli* BW27783 expression strain as outlined in chapter 3. Initial observations revealed that BapF<sup>C28S</sup> and BapF<sup>Prn</sup> still retain the ability to induce cellular aggregation and culture sedimentation phenotypes similar to that observed for cells expressing the BapF<sup>WT</sup> clone. Minor variation in size of aggregates was observed, with BapF<sup>Prn</sup> consistently producing larger aggregates than either BapF<sup>WT</sup> or BapF<sup>C28S</sup> clones. The BapF<sup>C28S</sup> mutant also consistently produced smaller aggregates. Cells expressing BapF<sup>C28S</sup> also appeared more stressed than cells expressing either BapF<sup>WT</sup> or BapF<sup>Prn</sup> clones, which was apparent when OD<sub>600</sub> values were measured throughout the induction and averaged over five experiments (Figure 28).
Figure 28: Sedimentation and aggregation phenotypes for BapF<sup>C28S</sup> or BapF<sup>Prn</sup> mutants
Similar to that outlined in Figure 17, *E. coli* BW27783 cells harboring pBAD24 vector, BapF<sup>WT</sup>, BapF<sup>C28S</sup> or BapF<sup>Prn</sup> expression plasmid were induced using 0.1% arabinose for 3 hours.
A. DIC microscopic image of cellular aggregation immediately following the 3 hour period of induction.
B. Photographs of culture sedimentation, where following the 3 hour induction period cells were rested for an additional 2 hours at room temperature.
C. OD<sub>600</sub> values tracked over the 3 hour period of induction, and averaged over 5 experiments. Error bars represent the standard deviation.
4.2.3. Detection of BapF<sup>C28S</sup> and BapF<sup>Prn</sup> in <i>E. coli</i> whole-cell lysates

Sedimentation and aggregation data suggested BapF<sup>C28S</sup> and BapF<sup>Prn</sup> were expressed in <i>E. coli</i>. Expression was confirmed using the pre-absorbed anti-BapF antiserum in Western blot analyses (Figure 29). Processed passenger domain is produced from both BapF<sup>C28S</sup> and BapF<sup>Prn</sup> mutants and in similar amount to that observed from the BapF<sup>WT</sup> clone; however, the amount of full-length protein produced from the BapF<sup>C28S</sup> and BapF<sup>Prn</sup> mutants is substantially greater than that observed for BapF<sup>WT</sup>. The observed doublet of full-length BapF protein very likely represents the presence (upper band) or absence (lower band) of a signal peptide. Considering the passenger domain is the functional moiety of autotransporters, having roughly similar amounts of expressed passenger may account for the aggregation and sedimentation phenotypes observed in cultures expressing the BapF<sup>WT</sup>, BapF<sup>C28S</sup> and BapF<sup>Prn</sup> clones.
Figure 29: Western blot detection of BapF<sup>C28S</sup> or BapF<sup>Prn</sup> mutants in *E. coli* BW27783 cells
Western blot analysis was performed using pre-absorbed anti-BapF antiserum. Full-length protein produced by BapF<sup>WT</sup>, BapF<sup>C28S</sup> or BapF<sup>Prn</sup> is identified and appears as a doublet, which very likely represents the full-length protein with either the signal peptide present (top band), or removed via signal peptidase 1 processing (bottom band). Passenger domain is also identified for all clones. BapF<sup>C28S</sup> or BapF<sup>Prn</sup> mutants produce what appears to be an increase in amount of observable degradation products at molecular weight less than that of the passenger domain. Molecular mass markers in kDa are displayed to the left of the blot.
4.2.4. Sucrose gradient fractionation to determine localization of BapF\textsuperscript{C28S}

The production of a BapF\textsuperscript{C28S} or BapF\textsuperscript{Prm} processed passenger domain suggests the autotransporter has localized to the outer membrane, and the autotransporter β-domain has formed a functional β-barrel (see Chapter 1). For the BapF\textsuperscript{C28S} clone, outer membrane localization was demonstrated using a sucrose density gradient fractionation assay as described in chapter 3. When samples obtained are probed using Western blots and the pre-absorbed anti-BapF antiserum, it was observed that both the BapF\textsuperscript{C28S} full-length protein and passenger domain co-fractionated with the known outer membrane protein BamA (Figure 30).
Figure 30: Sucrose gradient fractionation of whole-cell lysate from cells expressing $\text{BapF}^{C28S}$.
Increasing concentration of sucrose is represented by the ramp. Transition from inner membrane to outer membrane localized proteins is represented by the arrow. $\text{BapF}^{C28S}$ full-length and passenger domain are identified in samples also containing $\text{BamA}$, as identified by Western blot using polyclonal antibody to the BapF passenger domain (upper panel) or BamA (lower panel). Molecular mass markers in kDa are displayed to the left of the blot.
4.2.5. The BapF_{C28S} and BapF_{Prn} passenger domains are surface expressed

Observations thus far suggest that both the BapF_{C28S} and BapF_{Prn} passenger are presented on the cell surface. To clearly demonstrate surface expression of the BapF_{C28S} and BapF_{Prn} passenger domains, fluorescence microscopy and trypsin susceptibility experiments were performed as described in chapter 3.

Fluorescence data obtained from *E. coli* cells expressing BapF_{C28S} and BapF_{Prn} clones reveal that the intensity of fluorescence produced by these mutants is similar to that observed for cells expressing BapF_{WT} (Figure 31). The strong fluorescence signal, well above that produced from pBAD24 vector controls, supports the hypothesis that BapF_{C28S} and BapF_{Prn} clones are producing a surface expressed passenger domain. Aggregates observed in growing cultures (Figure 28) are not as prominent in the DIC or fluorescence images; this may be due to the mounting procedure used in the preparation of slides for microscopy experiments, where numerous washes of the slide surfaces may have had an impact on the retention or integrity of aggregates.

Exposure of *E. coli* cells expressing BapF_{C28S} or BapF_{Prn} to trypsin resulted in partial digestion of the passenger domain, producing a slightly smaller trypsin-resistant product similar to that observed in BapF_{WT} experiments. However, in contrast to the BapF_{WT} results, BapF_{C28S} and BapF_{Prn} full-length protein appears to be digested at early time intervals, producing a distinct profile of digestion products in the range of 15-25kDa (Figure 32), similar to that observed in the *in vitro* trypsin digestion of refolded BapF passenger (Figure 26).
Figure 31: Fluorescence microscopy reveals surface expression of BapF^{C28S} and BapF^{Pm} passenger domains

Fluorescence microscopy was performed using heat-killed pre-absorbed anti-BapF antiserum, and FITC-conjugated secondary antibody specific to the rabbit IgG Fc region (see Methods). DIC microscopy was used for light images of mounted cells (upper panels), with fluorescence microscopy (lower panels) performed on the same fields shown in DIC images.
Figure 32: Trypsin susceptibility assay performed on cells expressing BapF<sup>C28S</sup> or BapF<sup>Prn</sup>
Western blot analysis of samples derived from cells expressing BapF<sup>C28S</sup> and BapF<sup>Prn</sup> mutants and exposed to 100µg/mL trypsin over various periods of time (see methods). Molecular mass markers in kDa are displayed to the left of each blot.

A. Results from BapF<sup>C28S</sup>-expressing cells. Graphical representation of the BapF<sup>C28S</sup> signal peptide (SP) is presented on the left. BapF<sup>C28S</sup> full-length protein and passenger domain are identified (black arrows), along with the trypsin-resistant passenger fragment (red arrow). Reproducible profile of trypsin-derived fragments is found at lower molecular weight (red box).

B. Results from BapF<sup>Prn</sup>-expressing cells. Graphical representation of the BapF<sup>Prn</sup> signal peptide (SP) is also presented on the left, with the same identifiers for the Western blot as in A.
4.2.6. When outer membrane integrity is disturbed, *E. coli* cells expressing BapF<sup>WT</sup> can produce a similar profile of lower molecular weight fragments as those produced from BapF<sup>C28S</sup> and BapF<sup>Prn</sup> mutants

In the absence of trypsin, cells expressing BapF<sup>C28S</sup> and BapF<sup>Prn</sup> appear to accumulate full-length protein. It was therefore hypothesized that accumulation of full-length protein may promote outer membrane leakiness allowing trypsin to enter the periplasm and have access to partially folded full-length protein. To test this hypothesis, cultures expressing BapF<sup>WT</sup> protein were subjected to outer membrane disruption using EDTA and lysozyme in an isotonic environment (see methods), after which trypsin susceptibility was assayed in the same manner as described above. The BapF<sup>WT</sup> clone expressed under these conditions was able to give rise to a profile of lower molecular weight fragments closely matching those observed in BapF<sup>C28S</sup> and BapF<sup>Prn</sup> experiments (Figure 33). This result is in contrast to what is seen in the absence of EDTA/lysozyme pre-treatment (Figure 24) and in-part supports the hypothesis that trypsin is accessing the periplasm in cultures expressing the BapF<sup>C28S</sup> and BapF<sup>Prn</sup> mutants.
Figure 33: Trypsin susceptibility assay performed on cells expressing BapF<sup>WT</sup> but with the OM made permeable

Western blot analysis of samples derived from cells treated with 80µg/mL lysozyme and 50mM EDTA, followed by exposure to 100µg/mL trypsin for various times (see methods). Full-length protein and passenger domain are identified (black arrows) along with the trypsin-resistant passenger fragment (red arrow). Reproducible profile of trypsin-derived fragments is found at lower molecular weight (red box), mirroring those produced from trypsin susceptibility assay performed on cells expressing either BapF<sup>C28S</sup> or BapF<sup>Prn</sup> mutants (see Figure 32). Molecular mass markers in kDa are displayed to the left of the blot.
4.3. Disrupting signal peptidase 1 processing in BapF biogenesis results in lower levels of BapF expression

4.3.1. Generating a BapF signal peptidase 1 mutant

To help determine the role of acylation in BapF secretion, a mutant was generated in which signal peptidase 1 processing was predicted to be blocked. This mutant contained a site-specific mutation, resulting in removal of the Ala\textsuperscript{34} and Ala\textsuperscript{35} residues and replacing them with a single serine residue (BapF\textsuperscript{AA34-35S}). The likelihood of signal peptidase 1 processing was significantly reduced in this mutant, as predicted by SignalP 3.0 and LipoP 1.0 (Figure 34). The BapF\textsuperscript{AA34-35S} mutant should therefore retain any acyl groups attached during biogenesis. By forcing the retention of N-terminal acyl groups, the predicted secretion pathway for the BapF\textsuperscript{AA34-35S} mutant would differ significantly from the BapF\textsuperscript{WT} secretion pathway (Figure 35). The BapF\textsuperscript{AA34-35S} mutant would require the BAM machinery for assembly and integration of its \(\beta\)-barrel domain, and also require assistance from the Lol pathway for protection and transit of the N-terminal acyl groups through the periplasm to the outer membrane. Yet, the fate of the passenger domain is difficult to predict, for it could be oriented toward the periplasm similar to other Gram-negative lipoproteins, or presented on the cell surface similar to other autotransporter passenger domains.
Figure 34: SignalP and LipoP predictions for the BapF\textsuperscript{AA34-35S} signal peptide

A. SignalP 3.0 results for the BapF\textsuperscript{AA34-35S} signal peptide. Regions are depicted for the cytoplasmic region (n-region, green line), hydrophobic transmembrane region (h-region, dark blue line) and cleavage region (c-region, light blue line) with probable cleavage sites (vertical red lines). Final determination from SignalP 3.0 is that the BapF\textsuperscript{AA34-35S} mutant is a non-secretory protein.

B. LipoP 1.0 results for the BapF\textsuperscript{AA34-35S} signal peptide. The signal peptidase 2 processing site is still identified (green bar), with minor signal peptidase 1 cleavage sites also identified (red bars). SPI=SP1; SPII, SP2.
Figure 35: The BapF<sup>AA34-35S</sup> signal peptide and predicted secretion pathway

A. ClustalW alignment comparing signal peptide regions for BapF<sup>WT</sup> and BapF<sup>AA34-35S</sup>.

B. Graphical representation of the BapF<sup>AA34-35S</sup> signal peptide, with the red star depicting the site-specific mutation.

C. Predicted pathway for secretion of BapF<sup>AA34-35S</sup>. See Chapter 1 for description of roles for various components in the secretion pathway. Briefly, following inner membrane translocation via the Sec translocation machinery, <i>E. coli</i> Lgt, LspA and Lnt acylate the cysteine residue at position 28 in the BapF lipobox. The Lol sorting machinery is responsible for outer membrane localization of lipoproteins. The BAM machinery directs insertion of the BapF<sup>AA34-35S</sup> autotransporter domain, ultimately leading to cleavage between the passenger and translocation unit and presentation of the passenger on the cell surface. Note that most Gram-negative lipoproteins are anchored to the periplasmic side of the outer membrane.
4.3.2. Expression of BapF$^{AA34-35S}$ in *E. coli*

Expression of BapF$^{AA34-35S}$ was tested using the pBAD24 expression vector and *E. coli* BW27783 cells as described above. Aggregation and sedimentation phenotypes were observed and compared to the BapF$^{WT}$, BapF$^{C28S}$ and BapF$^{Prn}$ results (Figure 36). Aggregation and sedimentation rates were somewhat similar to those observed in BapF$^{WT}$ experiments, suggesting the BapF$^{AA34-35S}$ mutant was being expressed and responsible for these phenotypes. Whole-cell lysates were then probed, and Western blot results using pre-absorbed anti-BapF antiserum confirmed the BapF$^{AA34-35S}$ mutant produces a processed passenger domain similar to that produced by the BapF$^{WT}$ clone. However, in contrast to results from BapF$^{WT}$, BapF$^{C28S}$ or BapF$^{Prn}$ expression studies, the BapF$^{AA34-35S}$ full-length protein was undetectable in Western blots (Figure 37).
Figure 36: Sedimentation and aggregation phenotype produced in BapF\textsuperscript{AA34-35S} expressing cells

The first four panels are the same as presented in Figure 28, and now reveal the BapF\textsuperscript{AA34-35S} clone in the far right panel. As described above, \textit{E. coli} BW27783 cells harboring pBAD24 vector, BapF\textsuperscript{WT}, BapF\textsuperscript{C28S}, BapF\textsuperscript{Prn} or BapF\textsuperscript{AA34-35S} expression plasmid were induced using 0.1% arabinose for 3 hours.

A. DIC microscopic image of cellular aggregation immediately following the 3 hour period of induction.

B. Photograph of culture sedimentation, where following the 3 hour induction period cells were rested for an additional 2 hours at room temperature.

C. OD\textsubscript{600} values tracked over the 3 hour period of induction, and averaged over 5 experiments. Error bars represent the standard deviation.
Figure 37: Expression of BapF^{AA34-35S} in *E. coli* BW27783 cells

The first four lanes of the gel were presented in Figure 29, and now reveal the BapF^{AA34-35S} data in the far right lane. Western blot analysis was performed using pre-absorbed anti-BapF antiserum. Full-length protein produced by BapF^{WT}, BapF^{C28S}, or BapF^{Prn} is identified and appears as a doublet, which very likely represents the full-length protein with either the signal peptide present (top band), or removed via signal peptidase 1 processing (bottom band). Full-length protein is not observed in any Western blots of BapF^{AA34-35S} samples. Processed passenger domain is identified for all clones. Molecular mass markers in kDa are displayed to the left of the blot.
4.3.3. Probing surface expression of the BapF$^{AA34-35S}$ passenger domain

Production of a passenger domain and increased aggregation and sedimentation rates suggest surface expression of the BapF$^{AA34-35S}$ passenger. Fluorescence microscopy and trypsin susceptibility were used as described above to determine whether the BapF$^{AA34-35S}$ passenger domain is expressed on the cell surface.

Fluorescence microscopy data reveals that cells expressing the BapF$^{AA34-35S}$ clone fluoresce at a similar intensity to that produced in cells expressing the BapF$^{WT}$, BapF$^{C28S}$ or BapF$^{Prn}$ clones, suggesting the passenger domain is presented on the cell surface (Figure 38).

Trypsin susceptibility assays show the BapF$^{AA34-35S}$ passenger is digested similarly to the BapF$^{WT}$ passenger, producing over time a trypsin-resistant fragment of slightly lower molecular weight than the intact passenger domain (Figure 39). The profile of lower molecular weight bands observed in trypsin susceptibility experiments involving BapF$^{C28S}$ and BapF$^{Prn}$ was not observed in the BapF$^{AA34-35S}$ assays. The absence of this constellation of trypsin-generated fragments supports the hypothesis that these fragments are derived from protease digestion of partially folded full-length protein.
Figure 38: Fluorescence microscopy reveals surface expression of the BapF\textsuperscript{AA34-35S} passenger

The first four panels were presented in Figure 31, and now reveal data from the BapF\textsuperscript{AA34-35S} clone. As described above, fluorescence microscopy was performed using heat-inactivated pre-absorbed anti-BapF antiserum, and FITC-conjugated secondary antibody specific to the rabbit IgG Fc region (see methods). DIC microscopy was used for light images of mounted cells (upper panels), with fluorescence microscopy (lower panels) performed on the same fields shown in the DIC images. \textit{E. coli} cells expressing BapF\textsuperscript{AA34-35S} (right panels) fluoresce with similar intensity to cells expressing other BapF clones.
Figure 39: Trypsin susceptibility assay performed on *E. coli* cells expressing BapF$^{AA34-35S}$
Western blot analysis of samples derived from cells expressing BapF$^{AA34-35S}$ and exposed to 100µg/mL trypsin over various periods of time. Experiments were conducted exactly as outlined previously (see methods). The BapF$^{AA34-35S}$ passenger domain is identified (black arrow), along with the trypsin-resistant fragment (red arrow). No full-length protein is observed in any exposure of samples derived from BapF$^{AA34-35S}$-expressing cells. A graphical representation of the BapF$^{AA34-35S}$ signal peptide is presented underneath the Western blot data. Molecular mass markers in kDa are displayed to the left of the blot.
4.3.4. Mass spectroscopy analysis of the BapF^{AA34-35S} passenger domain reveals signal peptidase 1 processing was not blocked

To confidently interpret data from experiments involving the BapF^{AA34-35S} mutant, confirmation was required that N-terminal lipid groups are retained on the final form of the passenger domain. LC-MS/MS was employed as described for the BapF^{WT} protein, to determine whether the BapF^{AA34-35S} passenger domain remained acylated. Briefly, N-lauryl sarcosine was used to enrich the outer membrane fraction from cells expressing the BapF^{AA34-35S} mutant. This fraction was separated using SDS-PAGE, and protein visualized via Coomassie Brilliant Blue. Unique bands representing the BapF^{AA34-35S} passenger and translocation unit were observed and excised, and the gel slices were subjected to in-gel chymotrypsin digestion. Peptides produced from the digestion were extracted from the gel slices and analyzed using LC-MS/MS. Results clearly showed the signal peptidase 1 cleavage site found at residues Ala^{34} and Ala^{35} was effectively destroyed, but surprisingly cleavage occurred at an alternate signal peptidase 1 site located between residues Ala^{32} and Ser^{33} (GLA/SSPAL). All predicted signal peptidase 1 processing sites in BapF^{AA34-35S} have very low probabilities, such that SignalP 3.0 predicts BapF^{AA34-35S} to not even be secreted in a signal peptidase 1-mediated mechanism (Figure 34). However, the GLA/SSPAL cleavage site is predicted in the LipoP 1.0 analysis with a score of -1.92116.

With BapF^{AA34-35S} processed at the alternate GLA/SSPAL signal peptidase 1 site, secretion of the BapF^{AA34-35S} passenger becomes comparable to that of the BapF^{WT} passenger. Production of a passenger domain similar to the BapF^{WT} passenger very likely explains the aggregation and sedimentation similarities observed between cultures expressing these clones. However, it is important to note the BapF^{AA34-35S} mutant produces no observable full-length protein, signifying some alteration to the BapF^{WT} secretion pathway.
4.3.5. Generating a new BapF signal peptidase 1 mutant

Due to the unexpected results obtained from the mass spectroscopy analysis of BapF\textsuperscript{AA34-35S}, it was necessary to generate a new BapF mutant incapable of being processed by signal peptidase 1. The passenger domain generated from the new clone would be expected to retain all acyl groups. The BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} mutant was therefore designed, and SignalP 3.0 and LipoP 1.0 analysis shows an even greater reduction in the probability of signal peptidase 1 cleavage (Figure 40).
Figure 40: SignalP and LipoP results for BapF$^{A32S,S33P,AA34-35S,A37S}$

A. SignalP 3.0 results for the BapF$^{A32S,S33P,AA34-35S,A37S}$ signal peptide. No signal peptide regions are identified, resulting in final determination that the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant is a non-secretory protein.

B. LipoP 1.0 results for the BapF$^{A32S,S33P,AA34-35S,A37S}$ signal peptide. With fewer than four predicted cleavage sites no plot is produced. No signal peptidase 1 processing sites are identified, yet the signal peptidase 2 processing site remains intact.
4.3.6. **Expressing BapF$^{A32S,S33P,AA34-35S,A37S}$ in *E. coli***

Expression of the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant using the pBAD24 expression plasmid and *E. coli* BW27783 cells was performed as described above. Very little, or no aggregation and sedimentation were observed compared to the phenotypes produced by the BapF$^{WT}$ clone. Rather, the phenotypes produced by the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant more closely resemble those produced in cells harboring a pBAD24 vector control (Figure 42).

Expression of the new BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant in *E. coli* was confirmed using Western blot analysis (Figure 43A). Both BapF$^{AA34-35S}$ and BapF$^{A32S,S33P,AA34-35S,A37S}$ produce a distinct passenger domain, though possibly of a slightly larger size than passengers derived from the BapF$^{WT}$, BapF$^{C28S}$ or BapF$^{Pm}$ clones. As mentioned above, BapF$^{AA34-35S}$ full-length protein is not observed in Western blots; however, the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant produces a small amount of observable full-length protein when Western blots are overexposed (Figure 43B), signifying a potential difference in secretion pathways between the two clones.

4.3.7. **Mass spectroscopy analysis of the BapF$^{A32S,S33P,AA34-35S,A37S}$ passenger domain**

Mass spectroscopy analysis was performed to confirm whether the BapF$^{A32S,S33P,AA34-35S,A37S}$ passenger domain retains its N-terminal acyl groups. Preliminary data has identified residues starting from Gly$^{30}$, leaving only the His$^{29}$ and acylated Cys$^{28}$ residues to be identified (Figure 41). Labeling experiments using N-acetoxy-(1'H$_3$) succinimide followed by LC-MS/MS, as described above, have failed to identify the N-terminus of the purified passenger domain. One possible explanation for this may be that the N-terminus of the Cys$^{28}$ residue is acylated and blocking the labeling reaction. Difficulty in identifying these remaining amino acids may perhaps be due to the lipid
groups not being completely extracted from the polyacrylamide gel, or not eluted from the LC column due to their hydrophobicity. A detailed search of unassigned mass spectroscopy peaks may yet identify the remaining Cys\textsuperscript{28} and His\textsuperscript{29} residues.
Figure 41: Chymotrypsin cleavage sites within the range of Cys$^{28}$–Lys$^{60}$ of the BapF$^{A32S,S33P,AA34-35S,A37S}$ passenger

A graphical display of the chymotrypsin low cleavage sites found near the N-terminus of the BapF$^{A32S,S33P,AA34-35S,A37S}$ passenger domain. Cleavage by chymotrypsin occur C-terminal to the indicated amino acids. Residues highlighted in yellow have been identified by mass spectroscopy in the passenger, with the underlined His$^{29}$ and Cys$^{28}$ residues remaining to be identified.
4.3.8. Assessing surface expression of the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger domain

A processed BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger domain is detected when \textit{E. coli} cells express this mutant (Figure 43), but no obvious aggregation or sedimentation phenotype is observed (Figure 42). To probe the location of the passenger domain, immunofluorescence and trypsin susceptibility experiments were performed as described above. Fluorescence results suggest an intermediate level of fluorescence for the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} mutant, falling roughly between that observed for the pBAD24 vector control and BapF\textsuperscript{WT} clone (Figure 44). Though, in these preliminary experiments it is observed that the intensity of fluorescence can vary between individual bacteria.

Preliminary data (not shown) indicates that the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger domain is resistant to trypsin digestion for up to 60 minutes, in that the smaller band shown in Figures 24, 32 and 39 is not observed. After overnight exposure to trypsin the passenger domain seems to completely disappear. Interestingly, on some occasions the passenger domain was completely digested upon exposure to trypsin. These results are inconclusive at this time and require additional experimental repeats.
Figure 42: Aggregation and sedimentation results for *E. coli* cells expressing BapF<sup>A32S,S33P,AA34-35S,A37S</sup>

Similar to that outlined in Figure 28, *E. coli* BW27783 cells harboring either pBAD24 vector, BapF<sup>WT</sup> or BapF<sup>A32S,S33P,AA34-35S,A37S</sup> expression plasmid were induced using 0.1% arabinose for 3 hours.

A. DIC microscopic image of cellular aggregation immediately following the 3 hour period of induction.

B. Photograph of culture sedimentation, where following the 3 hour induction period cells were rested for an additional 2 hours at room temperature.

C. OD<sub>600</sub> values tracked over the 3 hour period of induction.
Figure 43: Expression of BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} detected in \textit{E. coli} via Western blot
Similar to Figure 29: Western blot analysis was performed using pre-absorbed anti-BapF antiserum. Molecular mass markers in kDa are displayed to the left of the blot.

A. Slight overexposure of the Western blot still reveals full-length protein produced by BapF\textsuperscript{WT}, BapF\textsuperscript{C28S}, or BapF\textsuperscript{Prn} as a doublet, which very likely represents the full-length protein with either the signal peptide present (top band), or removed via signal peptidase\textsuperscript{1} processing (bottom band). Processed passenger domain is observed for all clones.

B. Increased overexposure of the Western blot reveals BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} full-length protein (red arrow). This result is in sharp contrast to cells expressing BapF\textsuperscript{AA34-35S} where full-length protein is never observed.
Figure 44: Fluorescence microscopy suggests less surface expression of the $\text{BapF}^{A32S, S33P, AA34-35S, A37S}$ mutant compared to the $\text{BapF}^{\text{WT}}$ clone

As described above, fluorescence microscopy was performed using heat-inactivated pre-absorbed anti-BapF antiserum, and FITC-conjugated secondary antibody specific to the rabbit IgG Fc region (see methods). DIC microscopy was used for light images of mounted cells (upper panels), with fluorescence microscopy (lower panels) performed on the same fields shown in the DIC images. E. coli cells expressing $\text{BapF}^{A32S, S33P, AA34-35S, A37S}$ (right panels) fluoresce with intermediate intensity between cells expressing the $\text{BapF}^{\text{WT}}$ clone and cells harboring the pBAD24 vector control.
4.4. Conclusions

Blocking signal peptidase 2 processing in the BapF biogenesis pathway appeared to have little effect on the ability to produce a surface expressed BapF passenger domain. Both BapF\textsuperscript{C28S} and BapF\textsuperscript{Prn} mutants produce to greater or lesser extent a similar aggregation and sedimentation phenotype to that observed in cells expressing BapF\textsuperscript{WT}. Yet, globomycin clearly has an effect on the biogenesis of BapF, signifying that acylation does play a role in secretion of the BapF\textsuperscript{WT} passenger domain. Though generation of a processed passenger domain is observed for the BapF\textsuperscript{WT}, BapF\textsuperscript{C28S} and BapF\textsuperscript{Prn} clones, accumulation of full-length protein at the outer membrane, potentially poised for secretion, is observed only in cultures expressing the BapF\textsuperscript{C28S} and BapF\textsuperscript{Prn}. This accumulation is hypothesized to stress the outer membrane, potentially causing leakiness and permitting trypsin access to the periplasm where it can digest the partially folded full-length protein into distinct fragments. These fragments resemble those observed in the trypsin susceptibility assay performed on \textit{in vitro} refolded BapF passenger domain (Figure 26). Taken together, it is hypothesized that the constellation of trypsin-generated fragments may indeed be individual blades of the predicted BapF passenger structure.

The BapF\textsuperscript{AA34-35S} mutant was discovered to be processed at an alternate signal peptidase 1 cleavage site, producing a passenger domain quite similar in size to that derived from the BapF\textsuperscript{WT} clone. This similarity in passenger domains may be the reason why phenotypes induced by these clones in \textit{E. coli} are somewhat similar. The BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} mutant was then generated and predicted to retain the N-terminal acyl groups. The new mutant did not induce similar aggregation and sedimentation phenotypes as that observed in cultures expressing the other BapF clones. Yet, the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} mutant still produced a processed passenger domain. This passenger is likely to be acylated as its N-terminus could not be labeled, likely because it is blocked, and there is no evidence for
processing by signal peptidase 1. Assays to assess surface expression of the BapF$^{A32S,S33P,AA34-35S,A37S}$ passenger domain showed cells with moderate surface fluorescence, and a passenger domain that was resistant to trypsin up to 60 minutes. These results are preliminary and await further confirmation. Nevertheless, it is clear that removing the signal peptidase 1 processing site affects biogenesis of BapF. The absence of an induced phenotype, yet presence of a processed passenger suggest the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant may indeed retain the N-terminal lipid groups, but be oriented toward the periplasm or possibly presented on the cell surface in a non-functional state.
Chapter 5: Discussion

5.1. Overview of findings from studies describing BapF biogenesis

Autotransporters are a very large class of secreted proteins in Gram-negative bacteria, many of which play a significant role in pathogenesis. Studies focused on their secretion have produced insights into processes such as periplasmic transit, outer membrane integration and surface presentation. In many cases autotransporters possess a canonical N-terminal signal peptide; however, a subgroup of autotransporters harbor an N-terminal extended signal peptide region (discussed below) or a lipobox motif which is targeted for N-terminal acylation. BapF possess a lipobox motif within its signal peptide, suggesting it is an acylated autotransporter. The function of acylated autotransporters has been studied (20, 21, 23, 114, 182), leaving questions regarding secretion for the most part unaddressed. BapF was therefore chosen to help model events occurring during the secretion of acylated autotransporters.

Bioinformatic predictions suggest the BapF passenger domain does not form a β-helix, but instead attains a structure similar to known β-propeller proteins. Tango (36, 94, 137) and HHrepID (12) analysis identified areas of potential β-aggregation and 7 structural repeats respectively, supporting the hypothesis that the BapF passenger folds into a β-propeller. In many cases these regions identified by Tango and HHrepID contain a VVV repeat. In addition, protein-protein interactions are a common attribute of β-propellers, along with an inherent resistance to trypsin digestion, which may explain the phenotypes observed in E. coli cells expressing BapF. A crystal structure of the BapF passenger domain will unequivocally determine the final tertiary structure. If the BapF passenger is found to truly be a β-propeller, it will be the first of its kind to be reported.
Studies of BapF biogenesis benefitted greatly from the analysis of signal peptide mutants. One set of mutants focused on removing the acylation step, while the other set was designed to block signal peptidase 1 cleavage.

5.2. The effects of removing acylation from the BapF secretion pathway

Two methods were utilized to generate mutants which removed the acylation step from the BapF secretion pathway: replacement of the entire BapF signal peptide with the pertactin signal peptide; or site-directed mutagenesis of Cys\textsuperscript{28} to Ser\textsuperscript{28}. The choice of pertactin for the signal peptide swap was justified due to it belonging to a known \textit{Bordetella} autotransporter, and also the pertactin signal peptide is of similar size as the BapF signal peptide. It is commonly accepted that signal peptides do not demonstrate a great deal of sequence conservation, yet still perform their designed function. In this regard, both BapF\textsuperscript{Prn} and BapF\textsuperscript{C28S} mutants are unable to undergo N-terminal acylation, yet their signal peptides retain all characteristics necessary for Sec-dependant translocation.

Surprisingly, when expressed in \textit{E. coli} BW27783 cells, both BapF\textsuperscript{Prn} and BapF\textsuperscript{C28S} mutants confer somewhat similar aggregation and sedimentation phenotypes to that produced by the BapF\textsuperscript{WT} clone (Figure 28). The mutants were also able to produce a very similar amount of processed passenger to that observed in cells expressing the BapF\textsuperscript{WT} clone (Figure 29). Surface expression of the BapF\textsuperscript{Prn} and BapF\textsuperscript{C28S} passenger domains was confirmed by both fluorescence microscopy (Figure 31) and trypsin susceptibility (Figure 32).

These were striking observations, for previous work done with the BapF\textsuperscript{WT} protein demonstrated sensitivity to globomycin, suggesting a dependency on acylation for biogenesis (Figure 20). However, when looking closely at the mechanism of inhibition, it becomes obvious why globomycin experiments demonstrate a significant loss of BapF
expression. Globomycin is an allosteric inhibitor of Lsp (signal peptidase 2), the second enzyme in the pathway responsible for N-terminal acylation. When Lsp is inhibited, it results in a potentially toxic accumulation of partially acylated intermediates tethered to the periplasmic side of the inner membrane. These intermediates can be degraded by enzymes such as DegP, as part of a stress-induced response brought on by protein accumulation in the inner membrane (84, 148). The BapF_{Prn} and BapF_{C28S} mutants do not produce partially acylated intermediates, for the pathway of acylation is completely bypassed, effectively avoiding a similar stress response as that proposed to occur in cells treated with globomycin (Figure 45). That being said, cultures expressing the BapF_{C28S} clone demonstrated what appeared to be a slight stress response, with OD{sub 600} values plateauing soon after induction and slightly reduced aggregation and sedimentation phenotypes (Figure 28). It is possible that signal peptidase 2 is still recognizing the lipobox in the BapF_{C28S} signal peptide, though it is unable to cleave due to the C28S mutation. This activity may be sufficient to induce a stress response by stalling protein secretion at the periplasmic face of the inner membrane, and thus produce a stressed phenotype in these cells.

The strong similarities between the BapF_{WT} clone, and BapF_{Prn} and BapF_{C28S} mutants, were put into a new perspective when mass spectrometry data became available for the BapF_{WT} protein. These results revealed that both the mature BapF_{WT} passenger domain and full-length protein are processed at the predicted signal peptidase1 site between residues Ala{sup 34} and Ala{sup 35}. This painted a picture where the BapF_{WT} clone produced an acylated intermediate in the biogenesis pathway, where acylation of the Cys{sup 28} residue occurs initially, followed by cleavage between residues Ala{sup 34} and Ala{sup 35} by the activity of signal peptidase 1.
Cumulatively, results obtained from BapF<sup>Prn</sup> and BapF<sup>C28S</sup> expression studies suggest that acylation, though not required to produce a surface-expressed passenger domain, may play a different role in BapF biogenesis. Insight into this role may come from the observation of how in comparison to cultures expressing BapFWT, cultures expressing either BapF<sup>Prn</sup> or BapF<sup>C28S</sup> accumulate full-length protein, as observed in Western blots (Figure 29). Removing the N-terminal acylation process may result in release of the protein from the inner membrane prematurely, causing accumulation of the full length protein in the periplasm. Data from trypsin susceptibility experiments support the hypothesis that full-length protein accumulates in the periplasm (Figures 26 and 32), though it cannot be completely ruled out that some full-length protein may be presented on the cell surface and susceptible to trypsin digestion. This periplasmic accumulation may create a rate-limiting step at the point of BAM-mediated assembly and integration of the β-barrel domain, resulting in the observed accumulation of full-length protein awaiting recognition by the BAM complex. Generally, such an accumulation of periplasmic protein can trigger a stress response, resulting in targeted removal of the protein by periplasmic proteases such as DegP (84, 148). However, the accumulated full-length protein produced by the BapF<sup>Prn</sup> and BapF<sup>C28S</sup> clones appears to be protected from degradation (Figure 29). This observation may suggest the full-length BapF<sup>Prn</sup> and BapF<sup>C28S</sup> intermediates are protected in a secretion-competent state with the assistance of periplasmic chaperones. Alternatively, the accumulated protein may instead, or also, form aggregates which are somewhat resistant to degradation.

An unexpected result obtained from these studies was the production of a distinct constellation of digestion products when cells expressing BapF<sup>Prn</sup> and BapF<sup>C28S</sup> are exposed to a trypsin (Figure 32). It was hypothesized that accumulation of BapF<sup>Prn</sup> and BapF<sup>C28S</sup> full-length protein may induces periplasmic stress, resulting in outer membrane leakiness. The increased permeability of the outer membrane may permit trypsin to enter
the periplasm and digest the partially folded full-length BapF protein. This hypothesis was in-part confirmed by production of the same constellation of trypsin digested bands from cells expressing BapF\textsubscript{WT} but with their outer membrane made permeable (Figure 33). In addition, this distinct profile of digestion fragments may be evidence that the BapF passenger domain forms a partially folded $\beta$-propeller structure in the periplasm. The individual blades may form in the periplasm, limiting accessibility of trypsin to sites flanking the individual blades, and as a result the full-length protein can only be digested into discrete fragments. Therefore, unexpectedly, experiments designed to determine whether the BapF\textsuperscript{Pm} and BapF\textsuperscript{C28S} passenger is presented on the cell surface, may also support the hypothesis that the passenger domain folds into a $\beta$-propeller structure. Future studies can focus on confirming whether a periplasmic stress response is actually induced, using tools such as reporter constructs with the ability to respond to periplasmic stress by virtue of a $\sigma^E$ regulated promoter governing expression of the reporter gene (97). In addition, assaying for increased outer membrane permeability using dyes which label peptidoglycan, will allow microscopic observations of cells which have their outer membranes disrupted.
Polypeptide is post-translationally translocated to the periplasm via the Sec machinery. Signal peptide (SP) cleavage occurs at the periplasmic face releasing the autotransporter from the inner membrane. Secretion-competent autotransporter, bound to periplasmic chaperones such as SurA, transit to the outer membrane for β-barrel assembly and integration mediated by the BAM. A rate-limiting step is created at the point of β-barrel integration, producing accumulation of secretion-competent intermediates. Passenger translocation occurs in conjunction with β-barrel assembly, producing surface expressed passenger which then acquires a β-propeller structure. Cleavage occurs between the passenger and β-barrel domains, with the passenger remaining associated with the cell surface by non-covalent interaction with the β-barrel. IM = inner membrane; OM, outer membrane; LPS, lipopolysaccharide; N, N-terminus; C, C-terminus; P, passenger; TU, translocation unit; SP1, signal peptidase 1; Bam accessory proteins designated by single letters B,D,E,C.
5.3. The effects of blocking signal peptidase 1 cleavage in the BapF secretion pathway

Mass spectroscopy revealed the full-length BapF<sup>WT</sup> protein and passenger domain are processed by signal peptidase 1, suggesting the acylated form of BapF is an intermediate in the secretion pathway. The importance of signal peptidase 1 processing was addressed by the creation of two mutants. The first mutant blocked cleavage at the predicted processing site located between residues Ala<sup>34</sup> and Ala<sup>35</sup> (BapF<sup>AA34-35S</sup>). It was thought that such a mutant would be forced to retain its N-terminal acyl groups, and proceed down a secretion pathway involving the Lol system, differing from that of BapF<sup>WT</sup> which subverts the Lol pathway by virtue of cleavage and removal of the N-terminal acyl groups.

The BapF<sup>AA34-35S</sup> mutant failed to perform as designed, for mass spectroscopy indicated that signal peptidase 1 cleavage was still occurring. However, the cleavage site identified was not between residues Ala<sup>34</sup> and Ala<sup>35</sup>, but rather at an alternate cleavage site located between residues Ala<sup>32</sup> and Ser<sup>33</sup> (CHGLA<sup>32</sup>/SSPALP), which is predicted with very low probability by both SignalP 3.0 and LipoP 1.0 (see Figure 34). This was a surprising observation, for cleavage by signal peptidase 1 at such a poor recognition site was not anticipated to occur when a fully functional lipobox is also present. Why the cell would choose to still remove the N-terminal acyl groups under these conditions is uncertain. It may be hypothesized that signal peptidase 1 recognizes the region containing the cleavage site, and refuses to release the protein until processing is complete. Therefore, it appears that signal peptidase 1 processing may still occur at an alternate site, regardless of how inefficient it may be. Supporting this observation are findings that when amino acid changes are made to the cleavage region of a signal peptide, processing can occur at an alternate site (37, 88, 111).

Even though signal peptidase 1 cleaves both the BapF<sup>AA34-35S</sup> and BapF<sup>WT</sup> signal peptides, differences between these clones were still observed in various assays, with the most
obvious being the complete absence of detectable BapF^{AA34-35S} full-length protein in Western blot analyses (Figure 37). Yet, the mutant retains the ability to produce a surface-expressed passenger domain, and appears to produce phenotypes in *E. coli* similar to those observed in cells expressing BapF^{WT} (Figures 36 and 38). It is hypothesized that reduced efficiency of signal peptidase 1 processing, due to the poor cleavage site within the C-region of the BapF^{AA34-35S} signal peptide, slows the release of protein from the periplasmic side of the inner membrane. Such a process would induce a common stress response, where degradation of the accumulated protein would be mediated by enzymes such as DegP. This situation would result in removal of any full-length protein which is not being actively processed by signal peptidase 1. However, protein which is effectively cleaved by signal peptidase 1 would traverse the periplasm and follow secretion via the autotransporter pathway. Preliminary data has been obtained which in-part supports this hypothesis. DegP protease activity has been reported to be negligible at temperatures ≤ 28°C (163). When *E. coli* cells expressing the BapF^{AA34-35S} mutant were maintained at 27°C, a trace amount of full-length BapF^{AA34-35S} protein was observed for the first time (data not shown).

Though analysis of the BapF^{AA34-35S} mutant may provide some insight into the function of signal peptidase 1 in the biogenesis of BapF, it was necessary to generate a new mutant incapable of being processed by signal peptidase 1. To this end the BapF^{A32S,S33P,AA34-35S,A37S} mutant was designed and expressed in *E. coli*. Though a processed passenger domain is observed in Western blots (Figure 43), the BapF^{A32S,S33P,AA34-35S,A37S} mutant did not produce any obvious phenotypes (Figure 42). It is unclear whether the absence of an obvious phenotype is due to the passenger domain being oriented toward the periplasm, or whether it is presented on the cell surface but in a non-functional state, perhaps tethered by the N-terminus instead of the C-terminus. Continued work using fluorescence microscopy and trypsin susceptibility assays will help clarify if the reason for the lack of an obvious
phenotype in *E. coli* is due to the passenger domain not being localized to the cell surface. Importantly, confirmation that the new mutant retains its N-terminal acyl groups is required. Thus far, mass spectroscopy data has sequenced nearly the entire BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger, but the Cys\textsuperscript{28} (with or without acyl groups) and His\textsuperscript{29} residues are yet to be identified, likely due to technical difficulties. Alternatively, metabolic labeling of lipoproteins using fatty acid chemical reporters (132) may provide a method to determine whether the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger is acylated. Experiments using this approach have been initiated.

If indeed the passenger domain of BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} is oriented toward the periplasm, and retains its acyl groups, then this suggests that surface expression of an acylated passenger domain may require elements other than just the autotransporter β-barrel to traverse the outer membrane. For example, the acylated autotransporters SphB1 from *Bordetella pertussis* and NalP from *Neisseria spp.* are both surface expressed, and according to models of their function, the acyl groups are still present when the passenger domain is at the cell surface (23, 182). SphB1 harbors within its N-terminal region areas which are rich in glycine or proline residues. Removal of one of these regions still permits surface expression of the protein, with no effect on the function of the passenger domain; however, removal of both regions results in no observable protein in any of the fractions assayed, possibly due to intracellular degradation (23). Though SphB1 harbors these N-terminal regions which appear to play a role in secretion of the passenger to the surface, or maintaining its stability, NalP does not harbor any similar such regions. Therefore, it is unclear why the BapF\textsuperscript{A32S,S33P,AA34-35S,A37S} passenger domain may be oriented toward the periplasm, when clearly there are examples of other acylated autotransporters which can localize to the cell surface.
In addition, processing of an autotransporter passenger domain generally occurs following β-barrel formation and exposure of the passenger to the cell surface (see Chapter 1). This processing may be intramolecular, mediated by a catalytic mechanism within the hydrophilic pore of the β-barrel such as that observed in the SPATE (serine protease autotransporters of the *Enterobacteriaceae*) family of autotransporters (25, 27), and in *Bordetella pertussis* BrkA (25). Alternatively, processing may be intermolecular, mediated by other surface proteases such as that observed in the processing of the *Neisseria* autotransporters Iga protease and App by NalP (182). It is therefore puzzling how the BapF<sup>A32S,S33P,AA34-35S,A37S</sup> mutant may produce a passenger domain oriented toward the periplasm, when observations of other autotransporters suggest that translocation to the cell surface precedes cleavage and release of the passenger domain.

Moreover, the BapF<sup>A32S,S33P,AA34-35S,A37S</sup> full-length protein is observed in Western blots, though in very low abundance when compared to the BapF<sup>WT</sup>, BapF<sup>C28S</sup> and BapF<sup>Prn</sup> clones (Figure 43). As described above, the BapF<sup>AA34-35S</sup> clone is hypothesized to be released more slowly from the periplasmic side of the inner membrane than the BapF<sup>WT</sup> protein, resulting in a stress response which degrades any excess BapF<sup>AA34-35S</sup> full-length protein. The BapF<sup>A32S,S33P,AA34-35S,A37S</sup> clone appears to invoke a similar stress response, though it may be induced in a different manner than that of BapF<sup>AA34-35S</sup>. Considering the BapF<sup>A32S,S33P,AA34-35S,A37S</sup> mutant is predicted to retain its N-terminal acyl groups, it is possible a rate-limiting step is produced at the point of release from the inner membrane by LolCDE. Build-up of the fully acylated protein on the periplasmic face of the inner membrane may induce a similar DegP-mediated stress response as that hypothesized to occur in cells expressing the BapF<sup>AA34-35S</sup> mutant. Supporting this observation is an independent report demonstrating that excess lipoprotein can trigger a DegP-mediated stress response (161).
Data suggest the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant may retain the N-terminal acyl groups as predicted, and may therefore provide necessary information to help clarify why an acylated intermediate may be required in the BapF secretion pathway. A potential model for secretion of the BapF$^{A32S,S33P,AA34-35S,A37S}$ mutant is presented below (Figure 46). The model proposes that secretion proceeds along the lipoprotein modification route to the point of interaction with LolCDE. Any full-length protein stalled at this point would be degraded by DegP, while LolA shuttles the remainder of the acylated full-length protein to LolB for subsequent insertion of the acyl groups into the outer membrane. Assembly and integration of the autotransporter β-barrel is mediated by the BAM complex; however, whether the BapF$^{A32S,S33P,AA34-35S,A37S}$ passenger domain remains oriented toward the periplasm, or expressed on the cell surface in a non-functional state has not been definitely determined at this time.
Figure 46: Potential model for secretion of Bapo\textsuperscript{A32S,S33P,AA34-35S,A37S}

Polypeptide is post-translationally translocated to the periplasm via the Sec machinery. N-terminal acylation ensues by recognition and modification of the Cys residue present in the lipobox. Acylation proceeds on the periplasmic side of the inner membrane by Lgt, Lsp and Lnt (see Chapter 1 for full description). Release from the inner membrane via LolCDE limits the amount of free protein in the periplasm. Subsequent build-up of full-length protein on the periplasmic side of the inner membrane induces a DegP-mediated stress response, resulting in degradation. Full-length protein is shuttled across the periplasm by virtue of its N-terminal acyl groups and LolA. β-barrel assembly and integration via the BAM, and N-terminal acyl group integration into the periplasmic side of the outer membrane via LolB occur simultaneously or successively. Final result of the pathway is a fully integrated outer membrane β-barrel, and processed passenger domain which either faces the periplasm or is surface-expressed but potentially in a non-functional state. IM = inner membrane; OM, outer membrane; LPS, lipopolysaccharide; SP, signal peptide; P, passenger; TU, translocation unit; C, C-terminal; Bam accessory proteins designated by single letters B,D,E,C.
5.4. Biogenesis of the *B. bronchiseptica* RB50 autotransporter BapF

Data suggest the BapF\(^{WT}\) autotransporter is acylated during the initial stages of secretion. Surprisingly, the N-terminal acyl groups are removed by the activity of signal peptidase1, and the passenger domain is subsequently translocated to the cell surface in a presumably similar mechanism to that observed for other monomeric autotransporters.

It is reasonable to think that energy put into N-terminal acylation has a purpose, and is likely required for secretion or function of the BapF passenger. Analysis of data obtained from experiments involving the BapF\(^{C28S}\) and BapF\(^{Prn}\) mutants may lead to the conclusion that acylation is not required to produce a functional surface-expressed passenger. However, the accumulation of full-length protein in cells expressing these mutants suggests the pathway of secretion was somehow altered. Similarly, cells expressing the BapF\(^{A32S,S33P,AA34-35S,A37S}\) mutant produce a processed passenger domain, yet the amount of observable full-length protein is greatly reduced, also suggesting the normal secretion pathway was altered. Importantly, the absence of an obvious phenotype in cells expressing BapF\(^{A32S,S33P,AA34-35S,A37S}\) suggests that signal peptidase 1 processing is either required for translocation across the outer membrane, or required for obtaining a functional structure of the passenger domain.

Production of an acylated intermediate in the BapF\(^{WT}\) secretion pathway entails multiple enzymatic reactions at the periplasmic surface of the inner membrane. One hypothesis is that retention of BapF\(^{WT}\) at the inner membrane, during the process of N-terminal acylation, may provide a window of time in which to regulate the formation of secretion-competent intermediates in the periplasm, reducing any periplasmic stress due to increased build-up of incorrectly folded off-pathway protein. Data obtained from trypsin susceptibility assays support this hypothesis, where discrete bands are created upon exposure of cells expressing the BapF\(^{C28S}\) and BapF\(^{Prn}\) mutants to trypsin. Production of these bands...
coincides with the disappearance of the full-length protein, suggesting this is the source from which the constellation of bands originates. Though the BapF<sub>C28S</sub> and BapF<sub>Prm</sub> mutants still appear to create periplasmic intermediates with a partially folded structure, possibly formed of individual blades of the predicted β-propeller, the removal of the acylation process from the secretion pathway is suspected to stress the cells and potentially damage the outer membrane.

If acylation of BapF provides a method of retention at the periplasmic side of the inner membrane, then it may resemble some observations made regarding the extended signal peptide region (ESPR) of other autotransporters. As described in Chapter 1, an extended signal peptide is composed of 5 regions, where N1 and H1 represent the ESPR and N2, H2 and C regions comprise a canonical Sec-dependant signal peptide. Data obtained from studies of the trimeric autotransporter EmaA (extracellular matrix protein adhesin A) of *Aggregatibacter actinomycetemcomitans* may support the hypothesis of inner membrane retention mediated the ESPR. EmaA demonstrated dependence of the ESPR for stability, assembly and production of functional passenger on the cell surface (60). However, a mechanism by which residues of the extended signal peptide perform their function in EmaA biogenesis is not clearly described, though a role in regulating periplasmic stress responses is hypothesized. In contrast to the EmaA observations is that of FHA, a two-partner secreted protein from *Bordetella pertussis*, where secretion was only marginally effected, or not effected at all, by deletion of the ESPR (19, 89).

ESPR studies performed using *E. coli* SPATE monomeric autotransporters have produced conflicting data. When EspP biogenesis was observed in the absence of its ESPR, secretion defects were detected and attributed to miss-folding in the periplasm. Conclusions suggest the ESPR of EspP mediates a delay at the inner membrane by a unique interaction with the Sec machinery (167). Results from EspP studies supported
early studies describing secretion of the Pet autotransporter (31), where inner membrane translocation was severely impaired in the absence of the Pet ESPR. Later studies in Pet biogenesis refuted previous conclusions, demonstrating secretion and folding were not obviated in Pet mutants lacking their ESPR (93). Results obtained in Hbp studies, demonstrate a lack of dependence on the ESPR region for SRP-mediated translocation across the inner membrane, however, outer membrane translocation of the Hbp passenger domain appeared to be impaired (65).

The exact role of the ESPR remains unclear. There may in fact be other elements contributing to the function of extended signal peptides, such as passenger domain structure or size. Creating a delay at the periplasmic face of the inner membrane may in some cases play a role in autotransporter biogenesis, and creation of an N-terminally acylated intermediate may be another method by which the cell accomplishes this goal.

A potential secretion model based on current data obtained from studies involving BapF is presented below (Figure 47). The model proposes that secretion of BapF proceeds along the lipoprotein biogenesis pathway to the point of having a fully acylated cysteine residue at the N-terminus. During this process the passenger and translocation unit begin to fold, with assistance from periplasmic chaperones, into a secretion-competent form: the passenger forming individual blades of the β-propeller, and the translocation unit forming a partially folded β-barrel with linker region aligned with the inside of the barrel. The N-terminal acyl groups are removed by signal peptidase 1, and the protein is released from the inner membrane to traverse the periplasm to the outer membrane. Assembly and integration of the BapF β-barrel is mediated by the BAM machinery, with translocation of the passenger domain to the cell surface proceeding in a C-terminal to N-terminal fashion. Processing then occurs between the β-barrel and passenger domain. Once on the surface, the individual blades of the BapF passenger assemble into the final β-propeller structure.
However, at this time it is unclear whether the passenger is associated to the bacterial surface by its amino or carboxy terminus. It may indeed be possible that the BapF passenger is non-covalently associated with the cell surface via its linker region remaining within the β-barrel pore, similar to that suggested for the *Bordetella pertussis* autotransporter BrkA (118).

Numerous data obtained from this study suggest that the BapF passenger forms a β-propeller structure. BLAST results using the BapF passenger as the query revealed a similarity to a number of proteins containing HAF extracellular repeats. This repeat is named for a tripeptide motif HAF, with member proteins found in species with no outer membrane (archaea and Gram-positive bacteria) while others have C-terminal autotransporter domains, suggesting the repeat region is translocated across the outer membrane. Interpretations of bioinformatic predictions using Tango and HHrepID suggest that 7 repeats, composed of valine-rich regions, may form the individual blades of the β-propeller.

Interestingly, the VVV repeat motif found within the BapF passenger domain is also found in some of the autotransporter passengers picked-up in the BLAST search, when BapF was used as the query. Trypsin susceptibility experiments conducted on *in vitro* refolded BapF passenger, and also on cells expressing BapF<sup>ΔC28S</sup> and BapF<sup>Prn</sup> mutants, and even on cells expressing BapF<sup>WT</sup>, but with the outer membrane made permeable, show distinct digestion products which may be the individual blades of the β-propeller. Obtaining a crystal structure of the BapF passenger domain will ultimately provide the definitive data describing the final structure.

A number of proteins which assume a β-propeller structure are reported to be involved in protein-protein interactions (38). It is this property which may produce the observed
phenotypes in *E. coli* cells expressing BapF. However, the aggregation and sedimentation data produced in these experiments, though potentially useful in tracking surface expression of the BapF passenger, may only apply to *E. coli*. This is supported by the observations made in *B. bronchiseptica* RB50, where BapF expression is observed in the Bvg phase, yet neither aggregation nor sedimentation phenotypes are observed. Creation of a BapF deletion strain in *B. bronchiseptica* RB50 will help identify the function of BapF in its native background. Once a function is elucidated, then questions regarding the necessity of acylation for the function of BapF in *Bordetella* can be addressed.

The analysis of BapF secretion has highlighted a number of questions regarding secretion of acylated autotransporters. Members of this subfamily appear to vary in regards to whether or not the N-terminal acyl groups are retained in the final structure. However, for the case of NalP and SphB1, N-terminal acylation is required for the passenger to ultimately perform its function at the cell surface. Current models for these autotransporters suggest the acyl groups are required to anchor the passenger domain, at least temporarily, to the cell surface. However, in the case of BapF, acylation is not required for the passenger to translocate to the *E. coli* cell surface and induce the aggregation and sedimentation phenotypes. Though, as described above, acylation may be required for BapF to perform its function in *Bordetella*. Therefore, does N-terminal acylation perform different functions depending on the individual requirements of the particular autotransporter? Some acylated autotransporters may require N-terminal acyl groups to anchor their passenger domain to the cell surface. In the case of BapF, the acylation process may play a role in the formation of secretion-competent intermediates in the periplasm, facilitating efficient secretion of the passenger domain to the cell surface, where it can achieve its final structure. If the secretion-competent BapF intermediate is composed of independent blades of a β-propeller, then how does that impact translocation across the outer membrane?
Finally, the dual processing sites identified in the BapF signal peptide are also observed in a large number of other *E. coli* proteins (P. Sims and R. Fernandez, unpublished). The probabilities of cleavage at the signal peptidase 1 and signal peptidase 2 sites vary amongst these proteins, though processing at these sites is a definite possibility. Therefore, understanding the mechanism which regulates processing of the BapF signal peptide may also help to illuminate how the Gram-negative cell regulates secretion of many other outer membrane proteins.
Figure 47: Potential model for Bap\(^{WT}\) secretion
Polypeptide is post-translationally translocated to the periplasm via the Sec machinery. N-terminal acylation ensues by recognition and modification of the Cys residue present in the lipobox. Acylation proceeds on the periplasmic side of the inner membrane by Lgt, Lsp and Lnt (see Chapter 1 for full description). Release from the inner membrane occurs via (signal peptidase 1 (SP1) activity, producing a secretion-competent form in the periplasm which is bound and protected by chaperones such as SurA. The secretion competent form of the passenger consists of individual blades of the \(\beta\)-propeller. Assembly and integration of the autotransporter \(\beta\)-barrel mediated by the BAM complex initiates the process of passenger translocation across the outer membrane, where the passenger achieves its final conformation. Cleavage occurs between the passenger and \(\beta\)-barrel domains, with the passenger remaining associated with the cell surface by non-covalent interaction with the \(\beta\)-barrel. IM = inner membrane; OM, outer membrane; LPS, lipopolysaccharide; SP, signal peptide; P, passenger; TU, translocation unit; C, C-terminal; SP1, signal peptidase 1; Bam accessory proteins designated by single letters B,D,E,C.
References


73. Kedrov, A., I. Kusters, V. V. Krasnikov, and A. J. Driessen. 2011. A single copy of SecYEG is sufficient for preprotein translocation. The EMBO journal.


