STRUCTURE/FUNCTION STUDIES ON THE AUTOTRANSPORTER BrkA AND INVESTIGATION OF THE ROLE OF PERIPLASMIC CHAPERONES IN BrkA SECRETION

by

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ABSTRACT

BrkA is a virulence factor in *Bordetella pertussis* that belongs to the AT1 subfamily of autotransporter proteins in Gram negative bacteria. AT1 members have: (i) an N-terminal signal peptide for secretion across the inner membrane, (ii) a surface expressed passenger domain, and (iii) a C-terminal translocation unit for passenger secretion across the outer membrane.

There are many questions about AT1 secretion across the periplasm and the outer membrane. Studies on the AT1 member IcsA suggests rapid periplasmic transit, and that DegP’s chaperone activity is involved. Comparison of passenger size with estimated and known pore sizes of transporter domains suggest that the passenger is too large for translocation across the outer membrane in a folded state. The mechanism by which an AT1 passenger might maintain an unfolded state in the periplasm while resisting proteolysis is unknown.

BrkA secretion in *Escherichia coli* was used to investigate periplasmic transit and outer membrane translocation of an AT1 passenger. Previous studies revealed the junction domain in BrkA, which functioned in passenger folding. A highly conserved subdomain (region 3) was identified by ClustalW alignment of the junction, and was dispensable for *in vitro* folding. This subdomain might function in secretion by keeping the passenger unfolded for translocation by binding to a periplasmic chaperone. The role of region 3 in BrkA surface expression and *in vivo* folding was tested by trypsin accessibility and immunofluorescence microscopy, and limited proteolysis assays, respectively. In parallel studies, the involvement of periplasmic chaperones in BrkA secretion was investigated by trypsin accessibility assays on Skp, DegP, and SurA knockout strains expressing BrkA.
Residues $A^{681}-E^{693}$ of region 3 were important for secretion of a folding-competent BrkA passenger, but were dispensable for \textit{in vivo} folding. We termed $A^{681}-E^{693}$ the "hydrophobic secretion facilitation" (HSF) domain, after the term coined by Velarde and Nataro for the corresponding region in the AT1 member EspP, which was important for passenger secretion.

SurA, a peptidyl prolyl isomerase (PPIase) that functions in porin secretion, was implicated in BrkA secretion. The PPIase activity of SurA was dispensable, suggesting that SurA's chaperone activity is the necessary activity for BrkA secretion.
# TABLE OF CONTENTS

Abstract ii

Table of Contents iv

List of Tables vi

List of Figures vii

Abbreviations ix

Acknowledgements x

1. INTRODUCTION 1

1.1. Double membrane barrier to secretion in Gram negative bacteria 1
1.2. Sec-independent secretion 2
1.3. Sec-dependent secretion 2
1.4. Type V secretion 3
1.5. The Autotransporter family 4
1.6. Secretion of the AT1 autotransporters 6
1.6.1. Transport across the inner membrane occurs by the Sec system 6
1.6.2. Transit through the periplasm 8
1.6.3. Outer membrane translocation 8
1.6.3.1. Hairpin model 8
1.6.3.2. Common pore model 9
1.6.3.3. Omp85 model 11
1.6.4. Formation of final folded state and processing 11
1.7. Overview of Bordetella pertussis 12
1.8. BrkA 12
1.9. Thesis Overview 14

2. MATERIALS AND METHODS 19

2.1. Bacterial strains, plasmids, and growth conditions 19
2.2. Sequencing and reconstruction of pDO3236-10 deletion (region 3 mutant) 21
2.3. Construction of pYJ5 (autochaperone mutant) 22
2.4. Construction of transporterless mutants 26
2.5. In vivo trypsin accessibility assays 26
2.6. In vivo limited proteolysis assays 27
2.7. SDS-PAGE and immunoblot analysis 27
2.8. Periplasmic extraction 28
2.9. Detection of the BrkA beta-barrel 29
2.10. Immunofluorescence studies 29

3. The BrkA HSF (A\textsuperscript{681}-E\textsuperscript{693}) is required for secretion of a folding-competent passenger 30

3.1. Introduction and Rationale 30
3.2. Residues A\textsuperscript{681}-E\textsuperscript{693} of Region 3 are important for BrkA secretion 32
3.3. Residues A\textsuperscript{681}-E\textsuperscript{693} of Region 3 are not required for \textit{in vivo} folding 37
3.4. Residues A\textsuperscript{681}-E\textsuperscript{693} of Region 3 are required for secretion of a folding-competent passenger 39
3.5. Residues A\textsuperscript{681}-E\textsuperscript{693} of Region 3 and folding competence contribute to levels of BrkA in the periplasm 39
3.6. Discussion 42

4. SurA mediates secretion of BrkA in \textit{E. coli} 54

4.1. Introduction and Rationale 54
4.2. SurA is required for secretion of BrkA in \textit{E. coli} 55
4.3. The BrkA secretion defect in the SurA knockout strain can be complemented 58
4.4. SurA is required for stability or insertion of the BrkA C-terminus into the outer membrane 60
4.5. SurA does not interact with the BrkA HSF to mediate secretion 62
4.6. The periplasmic PPIases PpiD and PpiA are not required for BrkA secretion 65
4.7. The peptidyl-prolyl isomerase activity of SurA is not required for secretion of BrkA 68
4.8. Discussion 71

5. DISCUSSION AND CONCLUSION 80

REFERENCES 89

APPENDIX 1 96
Confirmation of the \textit{ppiD} knockout 96
LIST OF TABLES

Table 2-1. Bacterial strains and plasmids
LIST OF FIGURES

Figure 1-1. General domain architecture of AT1 autotransporters and models for AT1 secretion 7

Figure 1-2. Domain Architecture of AT1 autotransporter BrkA from Bordetella pertussis 13

Figure 1-3. Comparative analysis of the junction region found within several autotransporters reveals two areas of high conservation 16

Figure 1-4. Region 3 is not required for BrkA folding in vitro 17

Figure 2-1. Reconstruction of the region 3 deletion mutant (pYJ3) 23

Figure 2-2. Construction of the autochaperone deletion mutant (pYJ5) 24

Figure 2-3. Stick diagrams of the region 3 and autochaperone mutants 25

Figure 3-1. Examining of BrkA junction domain on homologous region in pertactin 31

Figure 3-2. BrkA junction deletion mutants used for study 33

Figure 3-3. The region 3 deletion mutant is deficient in surface expression 35

Figure 3-4. The region 3 deletion mutant is deficient in surface expression 36

Figure 3-5. Residues A$^{681}$$^{E}$$^{693}$ of region 3 are not required for in vivo folding of BrkA 38

Figure 3-6. Residues A$^{681}$$^{E}$$^{693}$ of region 3 contribute to the stability of transporterless constructs in the periplasm 41

Figure 3-7. Model explaining role of residues A$^{681}$$^{E}$$^{693}$ of region 3 in BrkA secretion 44

Figure 3-8. Comparison of the BrkA and EspP junction regions 47

Figure 3-9. Boundaries of the Autochaperone and Hydrophobic Secretion Facilitation domain in BrkA 50

Figure 4-1. SurA is required for BrkA secretion 57
Figure 4-2. The BrkA secretion defect in a SurA knockout strain can be complemented with SurA from a plasmid

Figure 4-3. SurA interacts with the BrkA transporter to facilitate secretion

Figure 4-4. SurA does not require the Hydrophobic Secretion Facilitation domain to mediate BrkA secretion

Figure 4-5. The peptidyl-prolyl isomerases PpiA and PpiD are not required for BrkA secretion

Figure 4-6. The domain architecture and crystal structure of SurA

Figure 4-7. The PPIase activity of SurA is dispensable for BrkA secretion
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Amp (Amp&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>ampicillin (or ampicillin resistant)</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cm (Cm&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>chloramphenicol (or chloramphenicol resistant)</td>
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<tr>
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<td>Heme binding protein</td>
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<tr>
<td>HSF</td>
<td>hydrophobic secretion facilitation domain</td>
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<td>PBS</td>
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<td>PPIase</td>
<td>peptidyl-prolyl isomerase</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium-dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Tet (Tet&lt;sup&gt;r&lt;/sup&gt;)</td>
<td>tetracycline (or tetracycline resistant)</td>
</tr>
<tr>
<td>TU</td>
<td>translocation unit</td>
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1. INTRODUCTION

1.1 Double membrane barrier to secretion in Gram negative bacteria

Certain outer membrane proteins are important to bacteria for pathogenesis and acquisition of nutrients. Secretion of outer membrane proteins in Gram negative bacteria represents a formidable challenge, as the proteins have to cross both the inner and outer membrane, while avoiding degradation by proteases in the periplasm. The secreted proteins, whether destined for association with the outer membrane, or secretion into the extracellular environment, must also fold properly to be functional. Gram negative organisms have many different strategies for secreting proteins across the outer membrane [1]. The study of protein secretion in Gram negative bacteria is important, because the mechanism by which proteins cross the outer membrane is a basic biological question that is not well understood. Also, understanding the secretion mechanism of proteinaceous virulence factors in Gram negative bacteria will contribute to the development of new anti-bacterial agents and treatments. Currently, several distinct pathways for protein secretion across the outer membrane have been described in Gram negative bacteria. Some of these pathways are dependent on the Sec pathway for secretion across the inner membrane, while other pathways are Sec-independent. The Sec pathway is a method of protein secretion across the inner membrane. Proteins that are secreted through the sec pathway have a signal peptide at their N-terminus which targets the protein to the SecYEG translocon at the inner membrane. A cytosolic ATPase called SecA in the complex catalyzes the transfer of the protein through the translocase [2].
1.2 Sec-independent secretion

Secretion Types 1 and 3 proceed through a Sec-independent mechanism. Proteins secreted by a Sec-independent mechanism do not utilize the Sec pathway, because protein transport from the cytoplasm to the cell surface occurs in one step. Type 1 secretion, best studied for α-hemolysin in *Escherichia coli*, occurs in a single step with no periplasmic intermediate. An ATP-binding cassette (ABC) protein, a membrane fusion protein, and a pore-forming outer membrane protein form an apparatus for the secretion of Type 1 proteins [3]. Type 3 secretion is best characterized for the export of the Yop proteins in *Yersinia*. Secretion by the Type 3 pathway involves a number of proteins that constitute a highly regulated channel across the inner and outer membrane [1].

1.3 Sec-dependent secretion

The secretion of Type 2, Type 4, chaperone/usher, two-partner, and autotransporter proteins are Sec-dependent. In sec-dependent transport systems, the protein is first secreted across the inner membrane via the Sec system, and is then routed to a separate system for secretion across the outer membrane. Type 2 secretion occurs in two-steps, and is best characterized in the pullulanase enzyme (PulA) of *Klebsiella oxytoca* [4, 5]. PulA crosses the inner membrane by the sec pathway, then secretion across the outer membrane is mediated by a secretion apparatus composed of 13-14 proteins called a secreton. In Type 4 secretion, the secreted protein utilizes the Sec pathway to cross the inner membrane. For pertussis toxin from *B. pertussis*, the subunits are secreted to the periplasm, before being secreted out of the cell by the ptl system [6-8], while CagA from *Helicobacter* is injected directly into the host cell by the cag system [9-13]. Another Sec
dependent secretion pathway is the chaperone/usher pathway, which is used in the biogenesis of P pili and Type 1 pili in uropathogenic bacteria. In this system, subunits are secreted across the inner membrane, where they bind to a system-specific chaperone in the periplasm [14], which shuttles the subunit to the usher complex in the outer membrane. Subunits are added to the end of the growing pilus at the usher complex, which is an oligomeric structure that forms a pore in the outer membrane [15, 16]. The last group of Sec-dependent secretion systems is termed the Type V family.

1.4 Type V secretion

Type V secretion includes the two-partner secretion (TPS) families and the autotransporter (AT) family [17]. Type V secretion is distinguished from the other secretion pathways by the low number of specialized proteins hypothesized to be involved in secretion; one for the AT family and two for the TPS family. The two-partner secretion system is aptly named because it consists of a secreted protein (TpsA), and its cognate transporter (TpsB) [18]. Both TpsA and TpsB are secreted into the periplasm through the sec system. The TpsB protein then integrates into the outer membrane, and allows for secretion of TpsA to the surface. Tps systems are characterized by filamentous hemagglutinin (Fha) from *B. pertussis*, and its cognate transporter FhaC.
1.5 The Autotransporter family

This family of proteins was named "autotransporters" because they are hypothesized to mediate their own secretion across the outer membrane. All autotransporters of known function have a role in virulence [19]. The autotransporter family is further subdivided into the AT1, AT2, and AT3 families, based on sequence homology [20]. The first member of the autotransporter family that was characterized, IgA protease from Neisseria gonorrhoeae, is a member of the AT1 family. The AT1 family of autotransporters are modular proteins, consisting of an N-terminal signal peptide, the passenger domain, and the C-terminal translocation unit (Figure 1-1A). The signal peptide utilizes the sec pathway to mediate secretion of the autotransporter across the inner membrane into the periplasm. The translocation unit is the minimal sequence required for secretion of the passenger, and is composed of a beta-core (transporter) and an upstream alpha-helical linker [21, 22]. The beta-core of the AT1 protein NalP has been shown to be a 12-stranded beta-barrel [23], and has been predicted to be a 12-14 stranded beta-barrel for all other AT1 proteins [24]. The beta-core is hypothesized to insert into the outer membrane to form a channel [25], and the linker is thought to direct secretion of the passenger across the outer membrane [26]. The secreted passenger is cleaved from the transporter domain in most cases, and may be released into the extracellular milieu, or remain anchored to the cell surface, depending on the autotransporter [27]. The passenger represents the mature form of the protein on the surface. The structures of two passenger domains of AT1 proteins have been solved. The structure of the pertactin passenger is a 16-stranded parallel beta-helix [28], while the structure of the Hbp passenger is a 24-stranded parallel beta-helix with a large N-terminal
globular domain and also a smaller domain present mid-way along the length of the beta-helix [29]. The AT1 family of autotransporters represent the largest family of Gram negative secreted proteins [30]. Phylogenetic analyses have shown that members of the AT1 family are present in a wide range of Gram negative bacteria, such as the α-, β-, γ-, and the ε-proteobacteria. AT1 family members are also found in Chlamydia [18].

The prototypical member of the AT2 family is YadA from Yersinia enterolitica. The passenger domain of several AT2 family autotransporters have been shown to form trimers, with a head and neck region mounted on a stalk region [31, 32]. The stalk is connected to a linker, which joins the AT2 passenger to a homotrimeric transporter domain [31, 33], with each monomer predicted to contribute 4 β-strands to form a 12-stranded transporter.

The proposed AT3 subfamily of autotransporters includes intimin from enterohaemorrhagic and enteropathogenic E. coli, and invasin from Yersinia enterolitica [20]. Intimin is a modular protein, with a signal peptide, an N-terminal flexible periplasmic domain, a predicted beta-barrel that is conserved, and the surface expressed passenger of three immunoglobulin-like domains capped by a C-type lectin domain [34]. Invasin has a similar architecture [35], but lacks the N-terminal flexible domain present in intimin. The predicted beta-barrel of intimin formed channels of 50 picoSiemens, suggesting that it might form a pore [36]. The export of heterologous passengers attached to a C-terminal truncated intimin construct composed of the 1st Ig-like domain, the putative transporter, and flexible N-terminal domain has been demonstrated, mirroring the ability of AT1 autotransporters to secrete heterologous passengers [37]. Furthermore, domain swap experiments have shown that the intimin transporter can
secrete the invasin passenger, and vice versa [38]. The AT3 family seems to have a reversed architecture in comparison to the AT1 and AT2 families, as the transporter domain is N-terminal to the passenger.

1.6 Secretion of the AT1 autotransporters

1.6.1 Transport across the inner membrane occurs by the Sec system

All autotransporters have a signal peptide at the N-terminus that directs their secretion across the inner membrane by the sec system (Figure 1-1A). The SRP chaperone has been implicated in secretion of Hbp, but SecB was able to compensate secretion of HBP to some extent [39]. In the case of the autotransporter IcsA from *Shigella flexneri*, the SecB chaperone has been demonstrated to mediate secretion through the Sec system [40]. Many autotransporters have extended signal peptides. It was recently found that the N-terminal extension of the signal peptide of the serine protease autotransporter EspP functioned to transiently anchor the protein to the inner membrane after export through the Sec system. The truncation of the N-terminal extension or the substitution of the native EspP signal peptide with the signal peptide from the periplasmic maltose-binding protein led to reduced efficiency of EspP translocation across the outer membrane, but not across the inner membrane. The authors suggested that the N-terminal extension of the EspP signal peptide may function to prevent misfolding of the autotranporter in the periplasm [41].
A. Domain architecture of AT1 autotransporters. Two processing events occur during autotransporter secretion: 1) cleavage of the signal peptide (SP) after secretion across the inner membrane by the sec system, and 2) processing at the surface to separate the alpha and beta domains. The translocation unit (TU) represents the minimal sequence required for passenger secretion. It is composed of two highly conserved regions, the beta-core (transporter) and the linker, which is predicted to be an alpha helix. The passenger on the surface represents the mature form of the AT1 autotransporter.

B. Models of AT1 secretion. The autotransporter is translated in the cytoplasm. The signal peptide mediates secretion of the autotransporter across the inner membrane through the sec system. There are many unanswered questions about AT1 transit through the periplasm. Studies have suggested that this stage is highly transient, and that periplasmic chaperones such as DegP may be involved. The beta-core is predicted to insert into the outer membrane, forming a pore. There are currently three models for translocation of the passenger through the outer membrane. In the i) hairpin model, the alpha-helical linker domain forms a hairpin structure, and mediates translocation of the passenger through the pore formed by the beta-core in a C-terminal to N-terminal direction. In the ii) common pore model, the passenger crosses through a central pore formed by an oligomer of beta-core domains. For clarity, only one passenger is depicted in the model. A role for iii) Omp85, an essential outer membrane protein has also been suggested in AT1 secretion. It has been suggested that Omp85 might be forming a channel to allow translocation of an AT1 passenger. Folding of an AT1 passenger may occur concurrently with or after secretion to the surface. In the case of most AT1 members, a cleavage event occurs after secretion, separating the passenger from the rest of the protein. This cleavage event is either autoproteolytic, mediated by a specific protease, mediated by a general outer membrane protease, or the mechanism is unknown. After cleavage, the passenger either remains non-covalently associated with the cell surface, or is released into the extracellular milieu. Figure adapted from [61].
1.6.2 Transit through the periplasm

Little is known about autotransporter secretion through the periplasmic space (Figure 1-1B). Pulse-chase studies on IcsA have shown that the periplasmic intermediate is highly transient [42]. There is evidence that periplasmic enzymes and chaperones are able to interact with the passenger domain. The periplasmic proteins FkpA and DsbA were shown to influence the final folded state on the cell surface of heterologous antibody passengers coupled to the IgA protease translocation unit [43]. The chaperone activity of the periplasmic protease DegP was found to be important for polar localization and secretion of IcsA [44]. This was the first evidence supporting a role of a periplasmic chaperone in secretion of a native ATI passenger.

1.6.3 Outer membrane translocation

Three models of autotransporter secretion across the outer membrane have been proposed: the hairpin, the oligomer, and the Omp85 model. (Summarized in Figure 1-1B).

1.6.3.1 Hairpin model

Klauser et al. studied the autotransporter secretion mechanism by using cholera-toxin B subunit (CtxB) coupled to the transporter domain of IgA protease. The cholera-toxin B subunit contained disulfide bonds, which were required for folding and function of the protein. Formation of disulfide bonds impeded translocation of the heterologous passenger to the cell surface. However, prevention of disulfide bond formation through point mutations or growth in reducing media rescued secretion of the CtxB subunit. The authors proposed a model in which the transporter domain contained a pore through
which the passenger was secreted (Figure 1-1Bi). Their CtxB studies suggested that a folded passenger could not translocate through the pore. However, it is known that the CtxB subunit is able to pentamerize, and thus the multimer formed was probably too large for secretion. They also found that in cases where the CtxB subunit was trapped in the periplasm, that a surface loop was accessible to trypsin cleavage [26]. Klauser et al. suggested that the passenger might be secreted through the pore in a hairpin conformation, by threading in a C to N-terminal direction. Pore-forming activity has been demonstrated for the translocation units of BrkA [45], IgA protease [46], PalA [47], and NalP [23], suggesting that they might form a channel. Structure-function studies on the AT1 members IgA protease [48], AIDA-1 [49], BrkA [21], and IcsA [50] have revealed that a linker region predicted to be an alpha-helix directly upstream of the beta-barrel is required for passenger secretion. The linker might be associating with the pore, initiating the formation of a hairpin structure. The hairpin might then facilitate the threading of the passenger domain through the pore formed by the beta-barrel. [19]. In support of this model, the crystal structure of the autotransporter NalP translocation unit revealed that the alpha-helical linker resides within the hydrophilic channel of the beta-core [23].

1.6.3.2 Common pore model

Cryo-electron microscopy pictures showed that the transporter domain of IgA protease formed hexamers, which formed a central stain-filled region with a 2 nm diameter [46]. Cross-linking studies on the IgA protease transporter also suggested hexamer formation. This data presented the possibility that translocation of the autotransporter passengers might occur through a central common pore formed by several
β-domain subunits. Coexpression of two different heterologous passengers coupled to the IgA protease transporter domain showed that the bulky passenger impeded translocation of the smaller passenger, suggesting that both passengers were competing for transport through the central channel [46] (Figure 1-1Bii). However, one caveat to this model is that the central channel would be hydrophobic, and thus likely would be lipid-filled. It is not known how a passenger domain might displace the lipids in order to translocate through the common pore. Multimer formation by the transporter domains of other AT1 members has not yet been reported.

Although the formation of a common pore raises the possibility of a larger channel size, it is not large enough for transport of an autotransporter passenger domain in a folded conformation. The structure of the pertactin passenger is a beta-helix with a width of 2 nm [28]. The Hbp passenger has a beta-helix of 2 nm width, and also a large N-terminal globular domain that combined with the beta-helix gives the N-terminus of Hbp a 7 nm width [29]. Midway along the beta-helix, there is also another domain that juts out, giving that portion of Hbp a 6nm width [29]. In comparison, the channel size of the NalP transporter domain is 1 nm, with part of the channel occluded by the linker, and the estimated channel sizes of the IgA protease hexamer [46], and PalA [47] are both 2nm. This would suggest that a native autotransporter passenger domain might have to assume an extended, translocation-competent conformation for secretion. It is not known how a protein in this conformation might resist degradation by periplasmic proteases.
1.6.3.3 Omp85 model

Omp85 is an essential, highly conserved outer membrane protein in Gram negative bacteria. In *Neisseria meningitides*, it has been found to be important for secretion of a wide range of outer membrane proteins, such as the trimeric porins PorA and PorB, the secretin PilQ, the siderophore receptors FrpB and RmpM, and the phospholipase OmpLA. Interestingly, Omp85 was also found to be important for secretion of the autotransporter IgA protease [51]. It has been suggested that Omp85 might form a pore, allowing secretion of the autotransporter passenger [23] (Figure 1-1Biii).

1.6.4 Formation of final folded state and processing

It is thought that folding of the passenger domain either occurs concurrently with secretion, or after secretion to the cell surface. A region termed the “junction” in the autotransporter BrkA was found to be required for folding of the passenger domain [52]. *Trans* complementation of folding experiments with BrkA suggested that passenger folding can occur at the surface. After surface expression, autotransporter proteins are usually processed, separating the passenger from the transporter domain. This processing event may be performed by another protease such as the cleavage of IcsA by SopA [53, 54], or may be autocatalytic, as in the case of IgA protease [25]. The processing mechanism of certain autotransporters is still unknown. Depending on the autotransporter, the passenger domain is either released after processing, or remains associated with the cell surface in a non-covalent manner.
1.7 Overview of *Bordetella pertussis*

*B. pertussis* is a Gram negative bacterium that has coccobacilliar morphology. It is the causative agent of whooping cough. *B. pertussis* expresses a variety of virulence factors that function in adherence to host cells, serum resistance, and other factors that interfere with host immune functions. The majority of virulence factors in *B. pertussis* are regulated by the two component BvgA/BvgS system [55].

1.8 BrkA

BrkA (Bordetella resistance to killing) is a virulence factor of *B. pertussis* that is involved in serum resistance and adherence to host cells [56]. BrkA was discovered in a transposon-mutagenesis screen for Bvg-regulated proteins [57], and the BrkA mutant was shown to have 10-fold lower virulence in infant mice [58]. It is a member of the AT1 family of autotransporters. There are 21 other known or predicted autotransporter proteins in *Bordetella* [27]. BrkA is a 103 kDa protein that undergoes two processing events (Figure 1-2). The signal peptide is removed during Sec translocation [21], and the second processing event is hypothesized to occur at the surface, resulting in a 73 kDa passenger [45]. The 73 kDa passenger remains non-covalently associated with the cell surface [59].

Using a passenger consisting of residues M¹-G²²⁹ of BrkA, the N-terminal limit of the minimal translocation unit required for secretion has been shown to reside within residues E⁶⁹²-L⁷⁰² [21]. The translocation unit is made up of two highly conserved domains; the linker, which is predicted to be an alpha-helix, and the beta-core, which is predicted to be a 12-stranded beta-barrel (Figure 1-2). The translocation unit has been shown to form channels of 3.2 nanoSiemens in black lipid bilayer membranes [45]. It is
BrkA is a 103 kDa protein that undergoes two cleavage events, removal of the signal peptide, and processing to separate the alpha and beta-domains to produce a 73 kDa passenger. This second cleavage event is hypothesized to occur at the cell surface, after secretion. The N-terminal boundary of the minimal translocation unit was mapped to E693-L702 [21]. The translocation unit is composed of two highly conserved domains, the linker (grey box) and the beta-core (transporter) (orange box) [21]. The linker is predicted to be an alpha-helix, while the beta-core is predicted to be a beta-barrel. The translocation unit has been shown to form channels of 3.2 nanosiemens in black lipid bilayer studies [45]. Secretion of the BrkA passenger might occur through this channel. The highly conserved junction domain was found to be required for folding of the BrkA passenger [52]. Figure adapted from [61].
not known whether the BrkA passenger translocates through this channel. Oligomers of the translocation unit have not been detected in BrkA, as they have in the case of IgA protease.

A putative intramolecular chaperone termed the “junction” was previously identified in PrtS, an AT1 autotransporter from *Serratia marcescens* [60]. PrtS is a serine protease, and in the absence of the junction, Ohnishi *et al.* found that when expressed in *E. coli*, no enzymatic activity or mature PrtS could be found [60]. They hypothesized that the PrtS passenger was not able to fold without the junction, and thus was being degraded by proteases at the cell surface. Recently, it was shown that a region at the C-terminus of the passenger was required for folding of BrkA [52]. Although lacking sequence identity, this BrkA region functionally corresponded to the junction region in PrtS. This domain was found to be highly conserved within subfamilies of AT1 autotransporters [52], and was termed the junction domain, in accordance with the term coined by Ohnishi *et al.* [60].

1.9 Thesis Overview

There are many unanswered questions in AT1 secretion. For example, (i) it is not clear how a passenger domain might assume a translocation competent state for secretion across the outer membrane, and (ii), the mechanism by which autotransporters resist proteolysis, [21] misfolding, and aggregation in the periplasm is also not known.

Recently, a highly conserved domain termed the “junction” at the C-terminus of the BrkA passenger was found to be required for BrkA folding [52]. The folded state of the passenger is likely to be intimately linked to its secretion. Distinct sub-domains within the junction might mediate separate activities of folding and secretion of the
passenger. To further localize the sequence required for BrkA folding, ClustalW sequence alignment of the junction domain was performed with a range of AT1 autotransporters [52] (Figure 1-3). The sequence alignment showed two highly conserved subdomains termed region 1 and region 3, sandwiching a region of lower conservation termed region 2. N-terminally His-tagged BrkA passengers with a deletion in region 3 were constructed to determine the effect on folding (Figure 1-4). The region 3 deletion fusion construct was capable of folding, as indicated by circular dichroism spectroscopy analysis during in vitro refolding assays (Figure 1-4). This fusion construct was also able to bind host cells, indicating that the construct had adopted its proper fold [61]. This suggested that regions 1 and 2 were required for in vitro BrkA folding, while region 3 was dispensable. Accordingly, region 1 and 2 were collectively termed the autochaperone domain (Figure 1-4). Region 3 was not required for in vitro folding, but its high conservation suggests it has an important function. Based on these results, our hypothesis is that region 3 has a role in secretion of BrkA, possibly by facilitating transit through the periplasm or the outer membrane. The preceding work that led to my project was performed by Dave Oliver, a PhD student in our lab (Figures 1-1, 1-2, 1-3, 1-4) [61].

The role of region 3 in secretion was tested using trypsin accessibility and immunofluorescence microscopy to assay surface expression of BrkA, and limited proteolysis assays to investigate the folded state of BrkA on the surface. We found that region 3 is required for secretion of a folding-competent passenger, and is not required for in vivo folding of BrkA.
Figure 1-3. Comparative analysis of the junction region found within several autotransporters reveals two areas of high conservation.

The position of the amino acids within the boxed region is noted for each protein. (*), >80% identity; (.), >40% identity; (:), >60% similarity. Grey shading denotes regions predicted to form β-sheet structure by the secondary structural prediction program PsiPred [52]. Unshaded regions are predicted to have coil structure. PsiPred scores were assigned at a confidence level of >2. ¹From *H. influenzae*, ²From *N. gonorrhoeae*. The blue and pink bars denote regions of high conservation, while the white region denotes a region of lower relative conservation. Figure adapted from [61].
Region 1 and 2: Autochaperone

Region 3: function unknown

Figure 1-4. Region 3 is not required for BrkA folding in vitro
BrkA fusion constructs with a deletion in region 3, and deletion of regions 2, 3 and part of region 1 were used to determine the minimal sequence required for in vitro folding. Circular dichroism spectroscopy was performed during in vitro refolding assays on these constructs. The circular dichroism profiles showed that region 1 and 2 are required for in vitro folding, while region 3 is dispensible for in vitro folding. Thus, region 1 and 2 are termed the autochaperone domain, because they represent the minimal sequence required for BrkA folding. Figure adapted from [61].
In parallel with the region 3 study, we investigated the role of periplasmic chaperones in BrkA secretion. We hypothesized that a periplasmic chaperone might have a role in mediating BrkA secretion. Knockouts in periplasmic chaperones were transduced into an *E. coli* strain or made with the lambda red system, and trypsin accessibility assays were used to determine whether BrkA was being secreted. We discovered that SurA, a peptidyl-prolyl isomerase, is necessary for BrkA surface expression, by mediating secretion of the BrkA transporter to the outer membrane. The requirement of SurA for BrkA secretion was independent of the peptidyl-prolyl isomerase activity of SurA.
2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

Strains and plasmids used in this study are listed in Table 2-1. The *E. coli* OmpT⁻ strain UT5600 was used as the host strain for all studies [62]. JY101 (*degP::Kan*) was created by P1 phage transduction of the *degP::Kan* allele from GPE101 [44] into UT5600. JY236 (Δaskp zae-502::Tn10) was created by P1 phage transduction of the Δaskp zae-502::Tn10 allele from AR236 [63] into UT5600. JY250 (*surA::Kan*) was created by P1 phage transduction of the *surA::Kan* allele from JMR250 [63] into UT5600. JY565 (*ppiA::Kan*) was created by P1 phage transduction of the *ppiA::Kan* allele from TR565 (T. Raivio, University of Alberta, unpublished) into UT5600. GPE101, AR236 and JMR250, and TR565 were generous gifts from Dr. Georgianna Purdy (University of Texas, Austin), Dr. Thomas Silhavy (Princeton University, Princeton), and Dr. Tracy Raivio (University of Alberta, Edmonton), respectively. The PpiD knockout strain was created by use of the one-step inactivation strategy, which relies on the red recombinase and homology in the primers to delete the gene [64]. Utilizing this method, a kanamycin resistance cassette was used to replace the *ppiD* gene in UT5600, generating the PpiD knockout strain JY665. The kanamycin resistant cassette was amplified from pKD4 using the primers 5'-GGC TGC AAA CAG TCT CGT GCT CAA GAT TAT TTT CGG TAT CAT TAG TGT AGG CTG GAG CTG CTT C -3' and 5'- CCA ATT TTG ATT TTC GCC TCT TTA CGC AGG TTA CTC ATC AGA GCC ATA TGA ATA TCC TCC TTA G -3'. Amplification of an internal fragment of 1059 bp of *ppiD* using the primers 5'- ATC CTC AAC CAG ATG GGG ATG ACC G -3' (binds to 461559-461583 of *E. coli* chromosome) and 5'- TGT TGT GCT GAA CCA ATG CCT TAA CTT G -3' (binds to 462591-462618 of the *E. coli* chromosome).
## Table 2-1. Bacterial strains and plasmids

<table>
<thead>
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<th>Strain/ plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/ source</th>
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<tr>
<td><strong>E. coli</strong></td>
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</tr>
<tr>
<td>UT5600</td>
<td>UT2300 derivative, ΔompT-fepC266</td>
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</tr>
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<td>DH5α F'</td>
<td>K-12 cloning strain</td>
<td>Invitrogen</td>
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<tr>
<td>GPE101</td>
<td>UT4400 degP::Kan'</td>
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<td>MC4100 Δskp zae-502::Tn10</td>
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<td>MC4100 surA::Kan’</td>
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<td>TR565</td>
<td>MC4100 ppiA::Kan’</td>
<td>T., Raivio, unpublished</td>
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<table>
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<th><strong>Plasmids</strong></th>
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<td>Stratagene</td>
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<td>Amp’, BrkA Δ (E602-A692), junction mutant</td>
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<tr>
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<td>Amp’, BrkA Δ (R603-A692), autochaperone mutant</td>
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</tr>
<tr>
<td>pDO6935 Δ TU</td>
<td>Amp’, BrkA Δ (W750-F1010), wild type transporterless mutant</td>
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<tr>
<td>pKD4</td>
<td>Kan’ cassette, template for linear PCR product for PpiD inactivation</td>
<td>64</td>
</tr>
</tbody>
</table>

Kan’, Tet’, Cm’ and Amp’ refer to resistance to kanamycin, tetracycline, chloramphenicol, and ampicillin, respectively.

ss’ = signal peptide sequence including the two adjacent 3' codons of surA (codons 1-22)

cf’ = 43 C-terminal codons of surA
were used to confirm that $ppiD$ was knocked out in JY665 (Appendix 1). The SurA expression constructs pQE60, pPER93, pSurA-2A and pSurA-NCt constructs were gifts from Susanne Behrens (Institute for Microbiology and Genetics, Goettingen). *E. coli* strains were grown at 37 °C on Luria broth or agar with appropriate antibiotics.

Ampicillin was added at a concentration of 100 µg/ml for DH5α, and 200 µg/ml for UT5600 and all UT5600 derivative strains. The use of twice the normal amount of ampicillin for selection of these strains is necessary because they have mutations in outer membrane proteins involved in iron siderophore transport, leading to leakage of beta-lactamase from the cell. This leakage might protect untransformed cells from normal levels of ampicillin. Thus, double the amount of ampicillin was used to ensure selection of transformants. Ampicillin, kanamycin, chloramphenicol, and tetracycline were used at final concentrations of 200, 50, 34, and 10 µg/ml, respectively for non-complementation experiments. For SurA complementation experiments, ampicillin, kanamycin, chloramphenicol were used at final concentrations of 200, 20, 20 µg/ml, respectively for SurA knockout mutants, and 200, 0, 34 µg/ml for the wild type strain. For non-complementation experiments, all strains were selected in liquid culture with chloramphenicol and ampicillin at final concentrations of 34 and 200 µg/ml, respectively. For induction of SurA construct expression, IPTG was added to a final volume of 0.1 mM when the cultures reached an optical density at 600 nm ($OD_{600}$) of 0.2 to 0.4.

### 2.2 Sequencing and reconstruction of the pDO3236-10 deletion (region 3 mutant)

Previously, Dave Oliver had constructed pDO3236-10 [65], which contains a BrkA mutant with a deletion in region 3. The plasmid pDO3236-10 was constructed by
generating a PCR fragment of BrkA from pDO6935 containing residues A^{52}-K^{680}. The PCR fragment had an Ascl linker at the N-terminus and an XbaI linker at the C-terminus. The plasmid GD10 [21] was cut using Ascl and XbaI, generating a fragment containing residues M^{1}-G^{51} and D^{694}-F^{1010} of BrkA. This GD10 fragment was ligated with the PCR fragment, creating pDO3236-10. This deletion was sequenced. To accomplish this, pDO3236-10 was cut with NcoI and HindIII and then blunt ligated to itself, bringing the deletion sequence close to the universal primer site. This construct was called pYJ2. The T7 universal primer 5'-TAATACGACTCACTATAGGG-3' was used for sequencing. Sequencing was performed by the Nucleic Acid Service Protein Service unit on UBC campus. Approximately 660 bp could be read from the sequencing data. The residues A^{681}-E^{693} were found to be deleted from BrkA in pDO3236-10. The sequence obtained from the T7 primer site did not cover the entire reading frame of BrkA. Thus, we were not sure that no other mutations existed within the sequence. To bypass this problem, a 411 bp fragment containing the sequenced region 3 deletion was cut using NcoI and StuI from pDO3236-10 and then subcloned into pDO6935, reconstructing the region 3 mutant (Figure 2-1, 2-3). This yielded pYJ3, which contained BrkA with the sequenced deletion in the C-terminal region of the passenger domain. DH5α was used for this genetic manipulation.

2.3 Construction of pYJ5 (autochaperone mutant)

The plasmid pDO6935 was cut with StuI and HindIII. pGD9 was cut with XbaI, the ends were filled with Klenow, and then cut with HindIII. The 1kb fragment containing BrkA residues N^{677}-F^{1010} from the pGD9 digestion was then ligated with the 4kb fragment containing BrkA residues M^{1}-A^{602} generated from the pDO6935 digestion.
A subcloning strategy was used to reconstruct the region 3 deletion mutant. The 411 bp fragment containing the region 3 deletion was excised from pDO3236-10 using Ncol and Stul, and ligated to the 6.5 kbp fragment generated by digesting pDO6935 with Ncol and Stul. The reconstructed region 3 deletion mutant was named "pYJ3".

Figure 2-1. Reconstruction of the region 3 deletion mutant (pYJ3)
Figure 2-2. Construction of the autochaperone deletion mutant (pYJ5).
The pGD9 plasmid was digested with XbaI, the ends were filled with Klenow, and then digested with HindIII. The pDO6935 plasmid was digested with Stul and Hind III. The 5.5 kb fragment from the pDO6935 digestion was ligated with the 1kb fragment from the pGD9 digestion, generating pYJ5.
Figure 2-3. Stick diagrams of the region 3 and autochaperone mutants.
A. A section of pYJ3 containing the BrkA region 3 mutant is shown, with relevant restriction enzyme sites. The hatched bar represents the fragment from pDO3236-10 which contains the sequenced deletion within the region 3 subdomain. The white bars represent fragments containing BrkA from the plasmid pD06935. The arrow represents the BrkA region 3 mutant gene product.

B. A section of pYJ5 containing the BrkA mutant from pYJ5 is shown. The hatched bar represents the fragment from pGD9, which contains residues N677-F1010 of BrkA. The white bar represents the fragment from pDO6935 that contains residues M602-A676. The arrow represents the BrkA autochaperone mutant gene product. These diagrams are not drawn to scale.
digestion, generating pYJ5 (Figure 2-2, 2-3). The construct pYJ5 coded for the BrkA autochaperone mutant. DH5α was used for this genetic manipulation.

2.4 Construction of transporterless mutants

The transporterless constructs were created by digestion of the appropriate full-length construct with \textit{NcoI} and \textit{HindIII}, and then the larger fragment containing the passenger domain was blunt-ligated to yield the following constructs: pGH3-13 ΔTU, pYJ3 ΔTU, and pYJ5ΔTU, corresponding to the junction mutant transporterless construct, region 3 mutant transporterless construct, and autochaperone transporterless constructs, respectively. DH5α was used for this genetic manipulation.

2.5 In vivo trypsin accessibility assays

Trypsin accessibility assays were performed as previously described [21], with a few modifications. \textit{E. coli} cultures were grown in LB with appropriate antibiotics to an optical density at 600 nm (OD$_{600}$) of approximately 2. A one milliliter volume of the cultures were spun down, and resuspended in 200 μl of phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 9.67 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$). The sample was then split into two 100 μl aliquots. To one aliquot, 1μl of 10mg/ml trypsin was added to a final concentration of 100 μg/ml. The other aliquot was left untreated. The aliquots were then incubated at 37 °C for 10 minutes. The samples were pelleted by centrifugation, washed twice with PBS containing 10% fetal bovine serum, and once with PBS. Samples were resuspended in 2x sample buffer (0.25 M Tris [pH 6.8], 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% bromophenol blue), and boiled for 10
minutes before resolution by SDS-PAGE [56]. Staining of SDS-PAGE gels with Coomassie brilliant blue was performed to show that equal amounts of protein were loaded.

2.6 In vivo limited proteolysis assays

Limited proteolysis assays were performed as described previously [52], with a few modifications. Briefly, the cultures were grown to \( \text{OD}_{600} < 2 \), and 1 ml was harvested. The cells were spun down and suspended in 150 \( \mu \)l of PBS. For the no trypsin treatment control, a 15 \( \mu \)l aliquot was removed from each sample, added to 50 \( \mu \)l of disruption buffer, and boiled for two minutes. To the remaining culture, trypsin was added to a concentration of 0.01 mg/ml. At the 1, 5, and 15 min. time points after trypsin addition, 15 \( \mu \)l samples were taken, added to 50 \( \mu \)l of disruption buffer, and boiled for 2 min. Equivalent amounts of protein were then run on SDS-PAGE, transferred to PVDF membranes, and probed with anti-BrkA antibody.

2.7 SDS-PAGE and immunoblot analysis

Samples were resolved on 11% polyacrylamide gels. The protein was then transferred to Immobilon-P membranes (Millipore, Etobicoke, ON) as described previously [59]. Blots were probed for BrkA using heat inactivated rabbit anti-BrkA antiserum diluted to 1:50 000. The anti-BrkA antiserum recognizes residues Met\(^1\) to Glu\(^{693}\) of BrkA [59]. SurA was detected using rabbit anti-SurA antiserum, a gift from S. Behrens, diluted to 1:10 000 [66]. Rabbit anti-His antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) diluted to 1:1500 was used to probe for histidine-tagged proteins. The secondary antibody used for all western blots was horseradish peroxidase-conjugated
goat anti-rabbit antibody (ICN Biomedicals, Costa Mesa, CA) at a dilution of 1: 10 000. Renaissance chemiluminescence reagent (NEN Life Science Products, Boston, MA) was used to develop the blots. Kaleidoscope pre-stained molecular weight markers were purchased from Bio-Rad (Hercules, CA). The molecular weight markers shown on the western blots are approximations.

2.8 Periplasmic extraction

Periplasmic extraction was performed as previously described [67], with some modifications. A 5ml LB culture was grown to the appropriate density. A volume of cells were spun down that was equivalent to 3.25 OD. The pellet was then washed with 500ul of PBS++ (1.47 mM KH$_2$PO$_4$, 2.68 mM KCl, 137 mM NaCl, 1.26 mM Na$_2$HPO$_4$, 2.46 μM Mg$_2$ x 6H$_2$O, 4.51 μM CaCl$_2$), before resuspension in 500ul of periplasmic extraction buffer (0.05 M Tris [pH 7], 20% sucrose, 1 tablet complete Mini EDTA-free (Roche Diagnostic, Mannheim, Germany)). The permeabilization of the outer membrane was done by addition of 20 μl of 2.5 mg/ml lysozyme in 0.25 M EDTA, and incubation for 10 minutes at room temperature. The soluble fraction, representing the periplasmic extract, was separated from the insoluble fraction containing the intact cells and membrane by centrifugation at 10 000 rpm for 10 minutes at 4 °C. The insoluble fraction was resuspended in 1 ml of 5x periplasmic extraction buffer and 250 μl of 5x disruption buffer (0.08 M Tris [pH 6.8], 2% SDS, 24% glycerol, 1% bromophenol blue). A volume of 125 μl of 5x disruption buffer was added to the periplasmic extract. Both insoluble and soluble fractions were then boiled for 5 min. before loading on a gel, or storage at – 20 °C. For the scale-up experiments, 50 ml of culture was used for the experiment.
2.9 Detection of the BrkA beta barrel

The SDS-PAGE gels that were used for running the trypsin accessibility assay samples were transferred to PVDF membrane using either transfer buffer pH 8.4 (24.8 mM Tris base, 0.192 mM glycine, 20% methanol) or CAPS buffer pH 9.0 (10 mM 3-(Cyclohexylamino)-1-propanesulfonic acid, 10% methanol). After transfer overnight at 30 volts, the gel was stained with Coomassie brilliant blue.

2.10 Immunofluoresence studies

Strains were transformed, plated on the appropriate antibiotics, and incubated overnight. Liquid cultures were grown to an $\text{OD}_{600} > 2$ at 37 °C. A volume of 100 μl of culture was then pipetted onto a slide previously treated with O-polylysine (Sigma diagnostics, St. Louis, MO). The bacteria were left to bind for 10 minutes at room temperature. The slide was then washed three times with 100 μl of 1% BSA in PBS. A 1/100 dilution of anti-BrkA in 1% BSA in PBS was used as the primary antibody. A 100 μl aliquot of the antibody dilution was added to the slide, and incubated for 15 minutes at room temperature. The slide was then washed three times with 100 μl of 1% BSA in PBS. The slide was then stained with 100 μl of a 1/100 dilution of goat anti-rabbit FITC for 15 minutes at room temperature. The slide was then washed with 1% BSA in PBS as previously mentioned. A 10 μl aliquot of MOWIOL was added to the sample, and allowed to dry for 5 min. A coverslip was then placed on the slide, and painted with nail polish to seal the coverslip onto the slide. The slides were viewed on a Zeiss Axioskop 2 MOT microscope, using the 100x oil immersion lens. Pictures were recorded with Eclipse version 6.0 (Empix Imaging Inc.).
3. The BrkA HSF (A$^{681}$-E$^{693}$) is required for secretion of a folding competent passenger

3.1 Introduction and Rationale

ClustalW sequence alignment of the junction domain of various AT1 members revealed the presence of the highly conserved region 3 (Figure 1-3). Region 3 was found in in vitro refolding assays to be dispensable for BrkA folding (Figure 1-4). Examination of the sequence homologous to region 3 in pertactin, which has highly sequence homology to BrkA, showed that it might form its own subdomain separate from the rest of the junction (Figure 3-1). The high sequence conservation of region 3 suggests that it has an important role in BrkA secretion. I hypothesize that region 3 keeps the passenger in an unfolded translocation-competent state during periplasmic transit.

Studies on heterologous passengers have demonstrated that some folded passengers can be secreted. A folded heterologous camel antibody passenger of 2 nm width was shown to be secreted through the IgA protease transporter [43], presumably through the central channel which measures 2 nm in diameter [46]. However, studies on mitochondrial import systems have shown that a heterologously expressed titin passenger, which has an immunoglobulin fold, could undergo local unfolding during import [68]. Thus, it is possible that the heterologous camel antibody may not have been secreted through the IgA protease transporter in a fully folded conformation. The passenger domain of pertactin is a beta-helix with a width of 2 nm [28]. The Hbp passenger is a long beta-helix with a width of 2 nm, and has a globular domain at the N-terminus that combined with the beta-helix gives the region a 7 nm width [29]. The channel sizes of PalA and IgA protease have both been estimated by liposome swelling assays to be approximately 2 nm in width [46, 47], while the crystal structure of the
Figure 3-1. Examination of junction domain in pertactin.

To gain insight into the structure of region 3, the homologous region to the BrkA junction was examined on the pertactin crystal structure [28]. Region 3 (pink strand) appeared as though it might form a separate sub-domain from the rest of the junction. Figure adapted from [61].
NalP transporter domain shows a channel size of approximately 1 nm in width [23]. It seems that these channels are not large enough to accommodate either the pertactin or Hbp passenger in a folded conformation. Thus, this suggests that AT1 passengers are being secreted in an unfolded or partially folded translocation-competent form. If the passenger is being secreted in an unfolded state, then a mechanism likely exists to prevent degradation by periplasmic proteases. Perhaps the passenger is translocating through the periplasm in a folded state, and then unfolding to provide a conformation compatible with outer membrane translocation, before refolding on the surface. However, the energetics of folding and then unfolding to translocate through the pore are likely to be unfavourable. Alternatively, periplasmic chaperones such as DegP may have a role in autotransporter secretion during periplasmic transit, as suggested by the IcsA study [44].

We hypothesize that region 3 may function in BrkA secretion, perhaps by binding to a periplasmic chaperone, allowing maintenance of a linear, translocation-competent conformation while protecting against proteolysis in the periplasm.

3.2 Residues A$^{681}$-E$^{693}$ of Region 3 are important for BrkA secretion

To determine the role of region 3 in secretion, we constructed a BrkA mutant with deletions of the residues A$^{681}$-E$^{693}$ in region 3, and also a BrkA mutant with a deletion of the autochaperone domain (region 1 and 2) (Figure 3-2). A BrkA mutant with the entire junction region deleted was included for comparison [52]. All of the BrkA constructs were expressed from their natural promoter. The *E. coli* strain UT5600 [69], was used for expression of the BrkA constructs. This strain has a deletion in the outer membrane
Figure 3-2. BrkA junction deletion mutants used for study
A BrkA construct with a deletion of A<sup>681</sup>-E<sup>693</sup> was constructed to investigate the role of region 3 in secretion. This is not a complete deletion of region 3, as residues D<sup>694</sup>-L<sup>702</sup> of region 3 are intact. The BrkA junction Δ mutant [52] was included in the study, for comparison. Notably, this mutant includes the residues E<sup>693</sup>-L<sup>702</sup> of region 3. A BrkA autochaperone deletion mutant was also constructed, and contains the entire sequence of region 3. AC denotes the autochaperone domain and 3 denotes region 3. The hatched bar denotes the passenger, the grey bar represents the linker, and the orange bar represents the beta-core.
protease OmpT, allowing for analysis of BrkA surface expression independent of its folded state. Trypsin accessibility assays were performed to determine the effect of the deletion on BrkA secretion to the cell surface. In this experiment, proteins on the cell surface are degraded upon addition of trypsin, while intracellular proteins remain uncleaved. BrkA in the wild type strain has been shown to be present in unprocessed and processed forms. The cleavage event separates the 30 kDa transporter domain from the 73 kDa passenger domain, presumably after BrkA has reached the outer membrane. Samples were either treated or not treated with trypsin, and then processed in parallel. Antibody recognizing residues M\(^1\)E\(^{593}\) of the BrkA passenger was used in the Western blot to detect BrkA in the whole cell lysates. Wild type BrkA was present in the greatest abundance, with both processed and unprocessed forms present (Figure 3-3). The processed form of wild type was cleaved by trypsin, indicating its surface localization. The junction deletion and autochaperone mutants were expressed at similar levels, and were both present in lower amounts than wild type BrkA. Both these mutants showed processed and unprocessed forms, and were both secreted to the surface, as indicated by the accessibility to trypsin. The region 3 deletion mutant showed much lower levels of expression than both wild type and the other mutants. However, both processed and unprocessed forms of the region 3 mutant were surface expressed.

Immunofluorescence microscopy was used to examine surface expression of these mutants. Wild type and junction deletion mutants were both highly fluorescent, indicating that BrkA was expressed efficiently on the surface (Figure 3-4). The autochaperone deletion mutant was also fluorescent, although not to the same magnitude as wild type. In contrast, the region 3 deletion mutant showed very weak fluorescence
Trypsin accessibility assays were performed on *E. coli* UT5600 expressing various BrkA mutants. Cultures were then grown to OD$_{600}$ > 2, and then harvested. Samples were split into duplicates, with one sample trypsin treated at a concentration of 100ug/ml, and the other left untreated. The cells were then washed, resuspended in disruption buffer, and samples were loaded onto a 11% SDS-PAGE gel. The protein was transferred to a membrane, and western-blotted using rabbit anti-BrkA antisera as the primary antibody, and goat anti-rabbit HRP as secondary antibody. Maltose binding protein (MBP) was used as a loading control. The “U” denotes unprocessed BrkA, and “P” denotes processed BrkA. This result is representative of three experiments.

![Western blot image](image-url)

**Figure 3-3. The region 3 deletion mutant is deficient in surface expression.**

Trypsin accessibility assays were performed on *E. coli* UT5600 expressing various BrkA mutants. Cultures were then grown to OD$_{600}$ > 2, and then harvested. Samples were split into duplicates, with one sample trypsin treated at a concentration of 100ug/ml, and the other left untreated. The cells were then washed, resuspended in disruption buffer, and samples were loaded onto a 11% SDS-PAGE gel. The protein was transferred to a membrane, and western-blotted using rabbit anti-BrkA antisera as the primary antibody, and goat anti-rabbit HRP as secondary antibody. Maltose binding protein (MBP) was used as a loading control. The “U” denotes unprocessed BrkA, and “P” denotes processed BrkA. This result is representative of three experiments.
Figure 3-4. The region 3 deletion mutant is deficient in surface expression. Cultures were grown to an OD_{600} > 2. A volume of culture was incubated with a slide containing O-polylysine. The slide was then washed with 1% BSA in PBS, and incubated with anti-BrkA antibody. After washing, the slide was then incubated with goat-anti rabbit antibody conjugated to FITC. Another wash with 1% BSA was performed, and MOWIOL was added to the sample. The slides were viewed on a Zeiss Axioskop 2 MOT microscope, using the 100x oil immersion lens. Images were recorded with Eclipse version 6.0 (Empix Imaging Inc.). This result is representative of three experiments.
compared to wild type and the other mutants. The weak fluorescence suggests inefficient surface expression of the region 3 mutant, and is in agreement with our trypsin accessibility data (Figure 3-3). Together, the trypsin accessibility and immunofluorescence studies suggest that residues A$^{681}$-E$^{693}$ of region 3 are important for secretion of BrkA, in the presence of the autochaperone domain.

3.3 Residues A$^{681}$-E$^{693}$ of Region 3 are not required for in vivo folding

It was previously demonstrated that region 3 was not required for in vitro folding of BrkA (Figure 1-4). We decided to investigate whether region 3 would be required for folding in vivo. A low concentration of trypsin was used in a limited proteolysis assay to probe the tertiary structure of BrkA on the cell surface. The relative stability over time of the mutant constructs in this assay were compared to wild type BrkA. The processed form of wild type BrkA was stable over 15 minutes of exposure to trypsin (Figure 3-5). In contrast, most of the processed passenger of the junction and autochaperone mutants was degraded by 1 minute of trypsin treatment. This result was expected, as the junction mutant has been shown previously to be unfolded on the surface [52], and in vitro refolding assays have shown that deletion into the autochaperone domain prevents passenger folding (Figure 1-4). The region 3 mutant showed higher stability than the other mutants, as the processed form was stable at 1 minute, and partially stable at 5 minutes. This data suggest that residues A$^{681}$-E$^{693}$ of region 3 are dispensable for in vivo folding, while the autochaperone region is necessary for in vivo folding.
Figure 3-5. Residues A\(^{681-693}\) of region 3 are not required for in vivo folding of BrkA. A limited proteolysis assay was performed on wild type BrkA and BrkA mutant constructs to determine their folded state. Bacteria were grown to OD\(_{600}\) > 2 and harvested. Samples were treated with trypsin at a concentration of 0.01 mg/ml trypsin, and trypsin digestion was stopped at 1, 5, and 15 minute intervals by aliquotting volumes of sample into disruption buffer and boiling. A non-trypsin treated sample (C) was included as a negative control. The samples were loaded onto a 11% SDS-PAGE gel, and then immunoblotted with anti-BrkA antibody. "U" denotes unprocessed BrkA, while "P" denotes the processed BrkA passenger. This experiment is representative of two experiments. * The wild type blot was taken at a lower exposure time than the other blots for purposes of comparison.
3.4 Residues A<sup>681</sup>-E<sup>693</sup> of Region 3 are required for secretion of a folding-competent passenger

Taken together, the data from the trypsin accessibility assay and limited proteolysis assays suggest a conditional role of region 3 in secretion of BrkA. In the non-folding autochaperone mutant, the presence of region 3 did not seem to improve secretion, as the junction deletion mutant is secreted at similar levels. However, in the folding-competent region 3 deletion mutant, absence of residues A<sup>681</sup>-E<sup>693</sup> of region 3 results in a severe defect in secretion. Thus, we suggest that residues A<sup>681</sup>-E<sup>693</sup> of region 3 are required for secretion of a folding-competent BrkA construct. Region 3 might be associating with a chaperone to maintain an unfolded state compatible with secretion across the outer membrane. Alternatively, the BrkA passenger might be folding in the periplasm, and upon reaching the transporter, residues A<sup>681</sup>-E<sup>693</sup> of region 3 might act as a signal to transiently unfold the passenger for secretion.

3.5 Residues A<sup>681</sup>-E<sup>693</sup> of Region 3 and folding competence contribute to proteolysis resistance of BrkA in the periplasm

To investigate the function of region 3 in the periplasm during BrkA secretion, transporterless constructs of wild type and mutant BrkA were constructed. The transporter domain was deleted, forcing localization of the passenger domains to the periplasmic compartment. If any of the regions were required for association with a putative factor providing periplasmic stability, such as a chaperone, it was expected that a deletion mutant in that region would have low expression levels. The transporterless constructs were expressed in UT5600 and periplasmic extraction was performed on the
cells. Trypsin accessibility experiments showed that these constructs were not expressed on the surface (data not shown). Insoluble fractions representing intact cells, spheroplasts, and membranes were harvested, as well as the soluble fraction, which corresponds to the periplasm. Maltose-binding protein (MBP) was detected in the soluble fraction, showing that this fraction does contain periplasmic proteins. MBP was also observed in the insoluble fraction, and is probably contributed by intact cells. The transporterless constructs were observed to associate with the insoluble fraction, and were not detected in the soluble fraction (Figure 3-6A). This suggests that the transporterless constructs were probably forming aggregates and associating with the inner or outer membrane. In order to obtain transporterless constructs in the soluble fraction, this experiment was scaled up to utilize a larger culture volume (Figure 3-6B). With a larger amount of cells, transporterless constructs were detected in the soluble fraction. Wild type had highest amounts, followed by the region 3 mutant, and the autochaperone and junction mutants had similar low levels of the BrkA construct. Notably, the same profile was observed in the insoluble fraction of the small-scale periplasmic extraction experiment (Compare Figure 3-6A and 3-6B). It was expected that the wild type construct would have highest amounts in the periplasm, as it would possess the entire passenger sequence, allowing putative interactions with stabilizing factors. It is also possible that the wild type passenger might be folding inside the periplasm, providing protection against proteases. Both the junction deletion and autochaperone transporterless mutants were present at near undetectable levels. The region 3 mutant had higher levels than both the junction and autochaperone deletion mutants. These higher levels might be accounted for by the ability of the region 3 mutant to fold,
Figure 3-6. Residues A$^{681}$-E$^{693}$ of region 3 contribute to the steady-state levels of transporterless constructs in the periplasm.

Crude periplasmic extracts were prepared from cultures expressing transporterless constructs. Cultures were grown to OD$_{600}$ 1.5-2. The cultures were spun down, and washed in 500ul PBS. The pellet was then resuspended in 500 ul of periplasmic extraction buffer (50 mM Tris pH 7, 20% sucrose, protease inhibitors). Lysozyme and EDTA was then added to the cells to a final concentration of 10 mM EDTA + 100 ug/ml lysozyme. The sample was then incubated at room temperature for 10 minutes, before centrifugation at 10 000 rpm at 4°C. The soluble periplasmic extract was separated from the pellet, which represents unbroken cells and membranes. Insoluble and soluble fractions equivalent to 0.03 OD were loaded onto an 11% SDS-PAGE gel (A). The blots were probed with rabbit anti-BrkA antisera and also rabbit anti-MBP antisera to ensure that the periplasmic fraction was obtained. Cultures of 50 ml were used to scale up this experiment (B). Note: The insoluble fractions in (B) were not equalized for loading. The result in (A) is representative of three experiments. “AC” denotes the autochaperone domain.
providing resistance against proteolysis. Wild type transporterless construct had higher amounts than the region 3 deletion mutant, suggesting that residues $A^{681} - E^{693}$ are able to increase the passenger stability of a folding-competent passenger. Thus, these studies suggest that residues $A^{681} - E^{693}$ of region 3, and ability to fold seem to both contribute to periplasmic stability of a transporterless construct. The wild type construct might be obtaining high resistance to proteases by using residues $A^{681} - E^{693}$ to associate with a putative periplasmic chaperone, while assuming a folded or partially folded state. This interpretation of relative levels corresponding to construct stability is based on the assumption that similar levels of each construct are being produced in the cell. Pulse-chase studies are required to confirm that the relative amounts of each construct observed are due to stability. Taken together, the data tentatively suggests that for transporterless BrkA constructs, residues $A^{681} - E^{693}$ might be contributing to stability in the periplasm.

3.6 Discussion

Comparison of the sizes of the pertactin and Hbp passenger crystal structures with the estimated pore sizes of the IgA protease and PalA transporter domains, and the known pore size of the NalP transporter, suggests that an AT1 passenger passes through the channel in a folded, or partially unfolded state. The mechanism by which an AT1 passenger would be maintained in this translocation-competent state in the periplasm while resisting degradation by proteases is not known. Previously, it was shown that the conserved junction domain in BrkA was required for folding of the passenger [52]. ClustalW sequence alignment of this domain in a range of autotransporters showed that two conserved regions sandwiching a region of lower conservation (Figure 1-3).
Deletion of the C-terminal conserved region (region 3) had no effect on folding of BrkA
_**in vitro**_ (Figure 1-4). We hypothesized that this region might have a role in BrkA
secretion, perhaps by maintaining the passenger in a translocation-competent state during
secretion across the outer membrane. Trypsin accessibility assays and
immunofluorescence microscopy showed that deletion of residues A$^{681}$-E$^{693}$ of region 3
led to greatly reduced secretion (Figure 3-3,3-4). Using limited proteolysis studies, the
same residues were shown to be dispensable for folding _**in vivo**_ (Figure 3-5). Taken
together, this data suggest a role for residues A$^{681}$-E$^{693}$ of region 3 in secretion of a
folding-competent passenger (Figure 3-7). It is possible that A$^{681}$-E$^{693}$ might mediate
interaction with a periplasmic chaperone. This putative chaperone might maintain the
BrkA passenger in a linear-translocation competent conformation, while providing
resistance to proteolysis (Figure 3-7 A). Upon translocation across the outer membrane,
the wild type protein would fold on the surface. For the junction and autochaperone
deletion constructs, the presence or absence of residues A$^{681}$-E$^{693}$ does not affect
secretion, because they are not able to fold, allowing secretion since they assume a linear
form (Figure 3-7 C,D). The region 3 deletion mutant would fold in the periplasm, since it
cannot associate with the chaperone (Figure 3-7B). This would lead to inefficient
secretion, as the region 3 mutant would be too bulky to be translocated to the cell surface,
and would be degraded since it is trapped in the periplasm. Immunoprecipitation
utilizing transporterless BrkA, and periplasmic pull-downs using BrkA passenger coupled
to sepharose beads have failed to isolate this putative chaperone (data not shown).
Another possibility is that residues A$^{681}$-E$^{693}$ might interact with the translocation unit,
acting as a signal for unfolding of the passenger for secretion across the outer membrane.
Figure 3-7. Model explaining role of residues A^{681-693} of region 3 in BrkA secretion

Please refer to the discussion and conclusion section of Chapter 3 for explanation of each model. The thick line and thin lines represent the outer and inner leaflets of the outer membrane. The scribbled line represents a folded passenger, while the teal colored oval represents a putative chaperone. The hatched bar represents the passenger minus the junction domain, the blue and white bars are the autochaperone, the pink bar is region 3, the grey bar is the linker, and the orange bar is the beta-core.
In the future, pulse-chase studies on these constructs should be performed, to ensure that the observed secretion defects are not a result of decreased BrkA production.

As previously mentioned, the region 3 mutant has the residues D\textsuperscript{694}-L\textsuperscript{702} of region 3. In future studies, the role of this region 3 in BrkA secretion should be further examined by a complete deletion of region 3. It is possible that the entire region 3 sequence is necessary for secretion of a folded passenger, and elimination of any part of its sequence prevents its function. However, future deletion in this region should be made keeping in mind that the N-terminal limit of the BrkA translocation unit also lies within this sequence (E\textsuperscript{693}-L\textsuperscript{702}). This overlapping sequence might have the dual functions of secreting a folded passenger, while being part of the minimal translocation unit. Single point mutations in this sequence might be the best strategy, to prevent any major structural changes caused by a deletion.

To gain insight into the role of region 3 in the periplasm, the transporter was deleted from wild type and BrkA mutants, forcing localization to the periplasmic compartment. Periplasmic extraction was performed on the bacteria, and expression levels of the transporterless constructs were examined by Western blot. The relative amounts of wild type and BrkA mutants suggested that both A\textsuperscript{681}-E\textsuperscript{693} of region 3 and folding-competence might play a role in stability in the periplasm for the transporterless constructs (Figure 3-6B). However, pulse-chase analysis must be performed to confirm that the relative amounts observed can be attributed to stability, and not to differences in production. The relative expression profile of the transporterless constructs did not match the expression profile of their full-length counterparts. Notably, the expression of the non-folding constructs (junction and autochaperone deletion) was higher in
comparison to the region 3 mutant for the full-length constructs, but lower in the transporterless constructs. This data suggest that passenger translocation of the non-folding constructs occurs too rapidly for proteolysis to have a significant effect on surface expression.

We have not determined why the region 3 deletion mutant has such low surface expression. If our hypothesis is correct that the region 3 mutant is not secreted efficiently due to folding in the periplasm, it may be an activating signal for periplasmic stress response systems such as Cpx and sigmaE. The Cpx response system senses aberrant production of surface organelles, such as P pili [70] and Type IV pili [71], while the sigmaE system responds to misfolding of outer membrane proteins. By expressing wild type and mutant BrkA constructs in a CpxP-LacZ reporter strain, we can assess if the region 3 deletion mutant is causing a periplasmic stress response.

Interestingly, Velarde and Nataro discovered a domain containing a homologous sequence corresponding to region 3 in BrkA which was important for secretion of the AT1 autotransporter EspP [72]. This domain was termed the “hydrophobic secretion facilitation domain” (HSF). The HSF domain overlaps with both the autochaperone domain and region 3 of BrkA (Figure 3-8A, 3-8B). Their study was done with the intent to further define the “linker” region required for EspP secretion, which corresponds to both the autochaperone domain and alpha helical linker in BrkA. They found that with a small passenger, N to C terminal deletions into the linker led to progressively reduced secretion efficiency. Using a full length passenger, they found that certain point mutations within the linker led to decreases in secretion efficiency, in the range of 10-30% of wild type secretion [72]. The secretion defects were shown to be independent of
Figure 3-8. Comparison of the BrkA and EspP junction regions.
A. Domain architecture of BrkA and EspP. Shaded boxes, translocation unit (TU), which is made up of the linker region (dark grey) and the β-core (orange), passenger region (hatched box); signal peptide (SP). The Dotted box demarks relative position of junction region in BrkA and EspP. Red box represents the region necessary for folding of the BrkA passenger (autochaperone). Blue box represents the region of EspP shown to be required for efficient translocation of the full-length EspP passenger (HSF domain). B. ClustalW alignment of junction regions of autotransporters closely related to BrkA (pertactin, TibA) and EspP (Pet, Sat). (*) identity, (:) and (.) similarity) The sequences highlighted in yellow are predicted to be β-strands (Psi-Pred). The solid black line denotes the autochaperone domain. The dotted black line denotes the HSF domain of EspP. The solid pink line denotes region 3 of BrkA, of which residues A681-E693 are required for secretion of a folding-competent passenger. Hydrophobic residues shown to be important for efficient translocation of the EspP passenger are underlined [72]. C. Structure of the C-terminal region of the pertactin passenger (1DABA). Left: Pertactin residues G425-P573. Region corresponding to the autochaperone region (red) corresponding to the HSF domain (blue), region overlapping between the autochaperone and the HSF domain (purple); N-terminal region of pertactin passenger (green). Middle and Right: side and top views of pertactin residues H508-L666 showing side chains (yellow) of residues shown to be important for efficient EspP passenger secretion. Figure adapted from [61].
beta-barrel insertion, as wild type levels of EspP beta-barrel were detected in the outer membrane for the mutants.

This data must be analyzed with caution, since the host strain that they used, HB101, is a strain that has the outer membrane protease OmpT. To analyze secretion, they used an EspP construct with a Myc-His epitope cloned into the deleted serine protease motif, and ELISA was used to assay the amount of secreted protein. However, the Myc-His epitope tag contains a lysine residue. It is possible that if the EspP passenger is not properly folded, the epitope tag might be cleaved by OmpT, leading to lower levels of secreted protein. This possibility might account for the reason that certain point mutations in the linker that drastically reduce secretion efficiency overlap with the autochaperone region in BrkA (Figure 3-8B). Thus, due to the presence of OmpT, their system may not be optimal for analyzing secretion of EspP independent of its folded conformation.

Nevertheless, Velarde and Nataro observed that all of the residues in the linker domain that reduced secretion when mutated were hydrophobic or aromatic in nature. They termed the region containing these hydrophobic residues the hydrophobic secretion facilitation domain [72]. Interestingly, these same mutations had no effect on secretion of a truncated, non-folding EspP passenger. They selected hydrophilic and hydrophobic amino acids in the HSF, and made conservative and non-conservative mutations. Both non-conservative and conservative mutations to the hydrophilic residues were found to not affect EspP secretion. In contrast, mutation of the hydrophobic residues to aspartic acid residues reduced secretion levels to approximately 10-20% of wild type. The authors decided to examine the same secretion-deficient mutants in the strain UT5600,
which lacks OmpT. Use of this system would remove uncertainty of OmpT cleaving unfolded EspP on the surface. The secretion-deficient mutants were not detected on the cell surface, but were found in the periplasm. Thus, this data confirmed the importance of these hydrophobic residues in EspP secretion. Interestingly, five of these examined residues localize to region 3 of BrkA (Figure 3-8B). Two of these residues, F\textsuperscript{972} and I\textsuperscript{979} are located in the sequence of EspP homologous to the sequence A\textsuperscript{681}-E\textsuperscript{693} in BrkA, which was determined to be required for secretion of a folding-competent passenger. The importance of these two residues in secretion of EspP supports our finding that A\textsuperscript{681}-E\textsuperscript{693} is important for BrkA secretion. In accordance with their terminology, we will term the sequence A\textsuperscript{681}-E\textsuperscript{693} as the “hydrophobic secretion facilitation” (HSF) of BrkA; the domain required for secretion of a folding-competent passenger. The limits of the BrkA HSF remain to be experimentally determined. Three residues of the EspP HSF domain lie in region 3 of BrkA (H\textsuperscript{698}, W\textsuperscript{700}, L\textsuperscript{702} in BrkA) C-terminal to the BrkA HSF domain. It is likely that the C-terminal limit of the BrkA HSF domain extends into this region. However, this is also the area of the N-terminal limit of the BrkA translocation unit. The boundary between the autochaperone and HSF domain in BrkA should also be determined in the future (Figure 3-9). Further, from the EspP study [72], it is impossible to determine which point mutations in the EspP HSF domain have decreased the secretion efficiency due to mutations affecting folding, leading to degradation by surface proteases (OmpT\textsuperscript{+} strain used) and which mutations are decreasing secretion efficiency by preventing secretion of a folded passenger. A set of C\rightarrow N terminal deletions starting from the C-terminal limit of the HSF domain and extending towards the autochaperone domain would give insight into the autochaperone/HSF boundary in BrkA. It is possible
Figure 3-9. Boundaries of the Autochaperone and Hydrophobic Secretion Facilitation domain in BrkA.
The current sequence of the BrkA hydrophobic secretion facilitation domain (HSF) is from $A^{681}-E^{693}$.
The C-terminal limit of the BrkA HSF borders on the N-terminal limit of the linker (grey box) of the BrkA translocation unit. The boundary between the BrkA HSF and linker has not been determined. Also, the boundary between the autochaperone (AC) and HSF domains remains to be experimentally determined. The "TU" denotes the translocation unit. Figure adapted from [61].
that the autochaperone and HSF domains of BrkA may partially overlap. This is currently under investigation. It is interesting that all of the residues in the EspP HSF domain that were found to be critical for secretion were hydrophobic in nature. PapD is a periplasmic chaperone functioning in the assembly of the Pap pilus in uropathogenic *E. coli* [73-75]. Pap pilus assembly occurs by the chaperone-usher pathway, in which each pilus subunit has an immunoglobulin-like fold, but are missing a strand in the C-terminus, resulting in a hydrophobic groove. The PapD chaperone caps the hydrophobic groove of the chaperone subunit [14, 76], supplying a strand to complete the fold of the subunit, while shielding the hydrophobic core of the subunit from the periplasm. This chaperone-subunit complex then shuttles to the outer membrane usher, where the subunit is incorporated into the growing pilus structure. The crystal structure of PapD in complex with PapG, the adhesin subunit, showed contacts between hydrophobic residues in the interacting surfaces of PapD and PapG, which might stabilize the association [77]. It was also discovered that single point mutations in conserved hydrophobic residues in the hydrophobic groove of PapG prevented its association with the PapD chaperone [14]. The importance of hydrophobic residues for association with PapD parallels the finding that hydrophobic residues are required for EspP secretion. It is possible that these residues of EspP are required for association with a periplasmic chaperone, and that a non-conservative mutation to a single hydrophobic residue abrogates this interaction, as seen with the PapG-PapD complex. When modeled on the pertactin crystal structure, some of the residues important for EspP secretion (Figure 3-8C blue strand) seem to orient towards one face of the protein, perhaps forming a domain to interact with a chaperone. One of the isoleucine residues and the phenylalanine residue fall within the
homologous region of the BrkA HSF, which contains several conserved hydrophobic residues. Thus, the hydrophobic residues in the BrkA HSF might form a surface for chaperone interaction. However, in the periplasm, which is the putative site of chaperone interaction, this region of the protein might have a different fold, with the residues orienting towards different positions than as shown in the crystal structure.

Future studies should involve mutagenesis of single hydrophobic residues in the autochaperone and HSF domains of BrkA to polar or charged residues to determine their importance in secretion or folding. If the point mutations in the BrkA HSF have affect secretion, then these same point mutations can be made in the autochaperone mutant, to determine whether mutation of these residues has an effect on secretion of a non-folding BrkA construct. We expect the point mutations to have no effect on secretion of this mutant, if our hypothesis is correct that the BrkA HSF (A\textsuperscript{681}-E\textsuperscript{693}) is only required in the presence of folding-competence.

Another possibility for the function of the BrkA HSF is the stabilization or insertion of the BrkA transporter domain in the outer membrane. If A\textsuperscript{681}-E\textsuperscript{693} is involved in this stage of secretion, then its deletion might inhibit BrkA surface expression by reducing the amount of functional transporter in the outer membrane. Near UV spectroscopy thermal denaturation studies on the beta-domain of the autotransporter AIDA-1 suggest that a homologous region to which the BrkA HSF localizes might provide stability to the AIDA-1 beta-barrel transporter in vitro [78]. Like many other autotransporters, the AIDA-1 protein undergoes processing to form alpha and beta-domains. The beta-domain was found to contain a surface expressed region (beta-1), and a protease resistant beta-barrel (beta-2). The beta-2 domain seems to correspond
functionally to the BrkA translocation unit, as deletions into this region prevented surface expression of a heterologous CtxB subunit [49]. Near UV analysis during thermal denaturation showed that a recombinant AIDA-1 beta construct missing beta-1 led to a construct that was fully denatured at 80 °C, compared to the 100 °C required to denature a full length AIDA-1 beta construct [78]. This study suggests an in vitro role for beta-1 in stabilizing the translocation unit of AIDA-1. However, the role of beta-1 domain in stabilization of beta-2 for AIDA-1 in vivo has not been determined. The C-terminal residues of the beta-1 domain correspond with the HSF in BrkA. Thus, it is possible that region 3 of BrkA might function in stabilization of the translocation unit. I have created a BrkA construct with a His-tag at the C-terminus of the translocation unit (data not shown). This construct is surface expressed, and the processed transporter can be detected by an anti-His tag antibody. Creation of a BrkA HSF deletion mutant with a C-terminally fused His-tag will facilitate the future investigation of whether the HSF is involved in transporter domain stabilization or insertion.
4. **SurA mediates secretion of BrkA in *E. coli***

4.1 **Introduction and Rationale**

An important question in autotransporter secretion is the state of the protein in the periplasm. The mechanism by which autotransporters are able to avoid aggregation, misfolding, and degradation in the periplasm is not known. Studies on the AT1 member IcsA from *Shigella flexneri* have suggested that the periplasmic intermediate in autotransporter secretion is highly transient [42]. This may allow the autotransporter to escape degradation by periplasmic proteases. Comparison of the pore size of various AT1 translocation units with the size of the pertactin and Hbp crystal structures suggests that they would have to translocate in an unfolded form [23, 28, 29, 43, 46, 47]. Periplasmic chaperones might keep the passenger unfolded, while providing protection against proteases.

It was recently demonstrated that DegP, a periplasmic chaperone with temperature regulated protease activity [79], was required for the efficient surface expression and localization of IcsA, an autotransporter protein in *S. flexneri* [44]. The authors showed that the chaperone activity of DegP was responsible for efficient secretion of IcsA. This was the first evidence that a periplasmic chaperone was shown to be involved in secretion of a native autotransporter.

Since DegP was found to be important for IcsA secretion, we decided to investigate whether DegP or other periplasmic chaperones might have a role in BrkA secretion. We used the secretion of BrkA in *E. coli* as a model to study the role of periplasmic chaperones in autotransporter secretion. A number of periplasmic proteins in *E. coli* have been shown to have chaperone activity, such as SurA, DegP, and Skp. DegP,
a periplasmic protease, was demonstrated to also possess chaperone activity [79]. It was shown that a DegP mutant defective in protease activity was able to bind to a mutant form of OmpC in the periplasm, preventing lethality [80]. Skp has been shown to assist the folding of the outer membrane protein OmpA into lipid membranes in vitro. Skp has been demonstrated to interact with OmpA [81] and PhoE [82] close to the plasma membrane, suggesting that it functions in maintaining OmpA solubility in the periplasm. SurA is a member of the peptidyl-prolyl isomerases, a group of proteins that are able to catalyze cis-trans isomerization of proline bonds [83]. A role in the secretion of outer membrane proteins such as OmpA, OmpF, OmpC, and LamB has been demonstrated for SurA. In a SurA knockout, levels of these proteins are reduced in the outer membrane [83]. SurA was found to be required for the formation of an assembly intermediate in the LamB trimer [83]. We hypothesize that one of these periplasmic chaperones plays a role in the secretion of BrkA.

### 4.2 SurA is required for secretion of BrkA in E. coli

To examine the requirement of Skp, DegP, and SurA on BrkA secretion, knockout strains were created using P1 phage transduction. Skp, DegP, and SurA knockout alleles were transduced using P1 bacteriophage into UT5600, an *E. coli* strain that lacks the outer membrane protease OmpT. OmpT has been shown to degrade unfolded BrkA that is localized on the cell surface [52]. By performing these studies in an OmpT" strain, we can investigate the effect of periplasmic chaperone knockouts on the surface expression of BrkA independent of its folded state on the cell surface. Surface expression of BrkA in the wild type and chaperone knockout strains was studied using
trypsin accessibility assays. Anti-BrkA antibody, which recognizes the passenger domain, was used to detect BrkA in the Western blot. Very low amounts of processed BrkA passenger was observed in the SurA knockout strain, and this was expressed on the surface, since it was trypsin accessible (Figure 4-1). A small amount of unprocessed BrkA was also detectable in the SurA knockout strain. In comparison, the DegP knockout strain had slightly lower amounts of processed BrkA than in the wild type strain, while the Skp knockout strain had similar levels of processed BrkA as the wild type strain (Figure 4-1). The processed BrkA passenger was surface expressed in both the DegP and Skp knockout strains, since they were found to be trypsin accessible. This suggests that SurA has a role in the secretion or biogenesis of BrkA in *E. coli*. DegP and Skp seem to be dispensable for secretion of BrkA in *E. coli*. 
Figure 4-1. **SurA is required for BrkA secretion.**

Trypsin accessibility assay to determine the surface expression of BrkA in the wild type and periplasmic chaperone knockout strains with pDO6935 which encodes BrkA. The trypsin accessibility assay was performed as described in the text. Anti-BrkA antibody was used for detection of BrkA in the Western blot. The "U" denotes unprocessed BrkA and "P" denotes processed BrkA. This result is representative of three experiments.
4.3 The BrkA secretion defect in the SurA knockout strain can be complemented

Since SurA seemed to be involved in BrkA secretion, we wished to study whether expression of SurA from a plasmid in the knockout strain would complement the BrkA secretion defect. SurA and BrkA were coexpressed from different plasmids in the SurA knockout mutant. The pBBR1 series of vectors [84] were used for BrkA expression, instead of the pBS SK+ vectors, to allow for use of ampicillin resistance plasmids encoding for SurA. Trypsin accessibility experiments were performed, and whole cell lysates were run on an SDS-PAGE gel, and then transferred to a PVDF membrane. Membranes were immunoblotted with either anti-BrkA antibody (Figure 4-2A) or anti-SurA antibody (Figure 4-2B). BrkA secretion in the SurA knockout mutant was partially complemented by expression of SurA from the plasmid (Figure 4-2A). Processed BrkA in the complemented SurA knockout mutant was expressed on the surface, as it was cleaved by trypsin. SurA was detected in the lysate of the wild type strain, but not in the lysate of the uncomplemented SurA knockout strain (Figure 4-2B). This confirmed that SurA was not being expressed in the SurA knockout mutant. SurA was detected in the complemented SurA knockout, along with a number of smaller bands (Figure 4-2B). The smaller bands probably represent degradation products from the overexpression of SurA. This experiment showed that the BrkA secretion defect in a SurA knockout mutant of *E. coli* can be complemented by providing a copy of SurA on a plasmid. These results confirm the requirement of SurA for secretion of BrkA in *E. coli*. 
Figure 4-2. The BrkA secretion defect in a SurA knockout strain can be complemented with SurA from a plasmid. Trypsin accessibility experiment on SurA knockout strains with and without complementation with SurA on a plasmid to determine BrkA surface expression. Anti-BrkA (A) and anti-SurA antibody (B) was used for detection in the Western blot. The "P" denotes processed BrkA. This result is representative of three experiments.
4.4 SurA is required for stability or insertion of the BrkA C-terminus into the outer membrane

SurA is required for the secretion of the outer membrane proteins OmpC, OmpF, OmpA, and LamB [83], which have a beta-barrel structure [85-88]. Additionally, peptide binding studies on SurA have shown that it has strong affinity for the aromatic-x-aromatic tripeptide motif, that is prevalent in integral outer membrane proteins [89]. This evidence suggests that SurA mediates secretion of beta-barrel proteins. Recently the crystal structure of the transporter domain of the autotransporter NalP was solved and found to be 12-stranded beta-barrel [23]. The transporter domain of BrkA is predicted to have a beta-barrel structure. Since SurA is important for the secretion of several outer membrane proteins with a beta-barrel structure, we hypothesize that SurA mediates secretion of BrkA by facilitating the secretion of the transporter domain of BrkA to the outer membrane.

To address this possibility, we decided to examine the levels of BrkA transporter domain in the wild type strain compared to a SurA knockout strain. A trypsin accessibility experiment was performed on wild type and SurA knockout strains expressing wild type BrkA and control vector. Protein from the SDS-PAGE gel was then transferred overnight onto a PVDF membrane. After transfer, the gel was stained with Coomassie brilliant blue, to detect protein that had remained on the gel. Proteins with a high isoelectric point, such as the BrkA C-terminus (pI = 9.78) would be expected to remain on the gel after transfer. As expected, the SurA knockout strain surface expressed lower amounts of BrkA than the wild type strain (Figure 4-3B), and SurA was not detected in the SurA knockout (Figure 4-3C). The BrkA C-terminus was detected on the
**Figure 4-3. SurA interacts with the BrkA transporter to facilitate secretion.**

Trypsin accessibility experiment on wild type and surA knockout strains with control or BrkA plasmids to determine surface expression. The gel used for transfer was stained with Coomassie brilliant blue (A) to detect the BrkA C-terminus. The membranes were probed with either anti-BrkA antibody (B) or anti-SurA antibody (C). The arrows indicate the BrkA C-terminus in the whole cell lysate of wild type *E. coli* expressing BrkA. The “U” denotes unprocessed BrkA and the “P” denotes processed BrkA. This result is representative of three experiments.
stained gel in the wild type strain expressing BrkA, but appeared as a very faint band in the SurA knockout strain (Figure 4-3A). This data suggests that SurA is required for stability of the transporter domain in the periplasm, or for insertion of the transporter domain of BrkA into the outer membrane.

4.5 SurA does not interact with the BrkA HSF to mediate secretion

Although we found that SurA facilitates secretion of the transporter domain of BrkA to the outer membrane, the possibility of SurA interaction with the passenger to mediate secretion should be considered. In Chapter 3, the significance of the BrkA HSF domain for secretion of a folded passenger was discussed. When modeled on pertactin, the hydrophobic residues in the BrkA HSF domain oriented to one face, perhaps forming a surface for interaction with a chaperone. The possibility of interaction between SurA and the HSF for BrkA secretion was investigated.

To test the interaction of SurA with the BrkA HSF to facilitate secretion, wild type BrkA, autochaperone mutant, junction mutant, and HSF (region 3) mutant were expressed in wild type and SurA knockout backgrounds. Trypsin accessibility assays were performed, and BrkA was detected in the Western using anti-BrkA antibody. In all cases, the processed passenger was expressed on the surface (data not shown). The non-trypsin treated samples were run on the same gel to compare the expression level (Figure 4-4). In the wild type strain, the relative expression level of the processed passenger of each construct was consistent with our earlier results (compare “P” in Figure 4-4 A or B with Figure 3-3). The HSF mutant had lowest expression, the autochaperone and junction mutants had similar levels of higher expression, while the wild type construct
Figure 4-4. SurA does not require the Hydrophobic Secretion Facilitation domain to mediate BrkA secretion.
The relative expression level of wild type BrkA and mutants with deletions in the junction, region 3, or autochaperone was compared in wild type strain and SurA knockout strain backgrounds by Western blot. BrkA was detected using anti-BrkA antibody. The "U" denotes unprocessed BrkA, and the "P" denotes processed BrkA.
A. The same exposure time was used. B. * This blot was exposed for 5 minutes, while the blot on the right was exposed for 30 minutes.
was expressed in larger amounts than any of the mutants. Each construct in the SurA knockout was expressed at lower levels than in the wild type strain (Figure 4-4A). However, the relative expression profile of wild type BrkA and mutant constructs in the SurA knockout strain was similar to the profile in wild type (Figure 4-4B). As in the wild type strain, the HSF mutant had lowest levels of processed passenger. The autochaperone mutant had slightly higher levels of processed passenger than the junction mutant, but both these constructs had higher expression levels than the region 3 mutant. As in the wild type strain, wild type BrkA showed highest levels of processed passenger in the SurA knockout strain. The similarity of the relative expression profile of these constructs in both the wild type and SurA knockout (Figure 4-4B) suggest that none of the deleted regions in the mutants interact with SurA to facilitate secretion. If SurA interaction with the BrkA HSF was required for secretion, than the amount of processed passenger of wild type and HSF mutant BrkA would be expected to be similar in the SurA knockout. However, wild type and HSF mutant BrkA still have the same relative expression profile in the wild type and SurA knockout strain (Figure 4-4 B). Thus, we conclude that interaction of SurA with the BrkA HSF domain is not required for BrkA secretion.
4.6 The periplasmic PPIases PpiD and PpiA are not required for BrkA secretion

SurA is a peptidyl-prolyl isomerase (PPIase) [83] that belongs to the parvulin family of PPIases [90]. Thus far, three families of periplasmic PPIases have been discovered in *E. coli*, the rotamase, parvulin, and FKBP families [91]. Since SurA possesses PPIase activity, we wanted to investigate whether another periplasmic PPIase would be required for BrkA secretion. To answer this question, we created a PpiA knockout strain in UT5600, using P1 phage transduction. PpiA is a member of the rotamase family of PPIases. BrkA was expressed in the PpiA knockout strain, and a trypsin accessibility assay was performed to determine surface expression of BrkA. The PpiA knockout strain expressed slightly higher amounts of processed BrkA compared to the wild type strain (Figure 4-5). The processed BrkA band in the PpiA knockout was cleaved by trypsin treatment, indicating that it was on the surface. This evidence shows that PpiA does not play a role in secretion of BrkA in *E. coli*.

Another PPIase that might play a role in BrkA secretion is PpiD, a member of the parvulin family of PPIases. PpiD was isolated as a multicopy suppressor of the detergent sensitivity and membrane defects exhibited by a SurA knockout mutant [92]. Interestingly, mutations in the PPIase active site of PpiD resulted in inability to complement detergent sensitivity in the SurA knockout strain, suggesting that PPIase activity is important for its biological function. A PpiD knockout mutant showed a defect in secretion of OmpF, OmpC, and OmpA, although the phenotype was not as severe as in a SurA knockout strain [92]. The similar outer membrane protein secretion defect of SurA and PpiD knockout mutants suggests the possibility that they might function together in a complex. The authors were unable to isolate SurA and PpiD double
Figure 4-5. The peptidyl-prolyl isomerases PpiA and PpiD are not required for BrkA secretion.

Trypsin accessibility assays were performed on *ppiA*, *ppiD*, and *ppiDsurA* knockout strains to determine surface expression of BrkA. BrkA was also expressed in wild type and *surA* knockout strains for comparison. The "U" denotes unprocessed BrkA and "P" denotes the processed BrkA passenger. This result is representative of three experiments.
knockout mutants, and suggested that this might indicate redundancy of these proteins in a function essential to viability. Taken together, this evidence suggests that SurA and PpiD might have a redundant function in outer membrane protein secretion. However, another group has recently been able to create a SurA PpiD double knockout mutant [93]. To investigate the role of PpiD in BrkA secretion, we created a PpiD knockout strain of *E. coli* UT5600 using the one-step chromosomal inactivation protocol, which relies on the lambda red recombinase [64]. PCR of an internal fragment of the *ppiD* gene that should have been deleted in the PpiD knockout strain was used to confirm the gene knockout (Appendix 1A). The internal fragment of *ppiD* of approximately 1.1 kbp was amplified from the wild type strain, while the potential PpiD knockout strain showed no amplification of this fragment (Appendix 1B), confirming that *ppiD* was indeed deleted in the strain. We were also successful in generating a PpiD SurA knockout mutant, in agreement with Dr. Silhavy's results [94], and in contrast with Raina and Dartigalongue's results [92]. BrkA was expressed in the PpiD knockout strain, and a trypsin accessibility assay was performed. We found that the PpiD knockout strain showed slightly lower levels of secreted processed passenger than the wild type strain (Figure 4-5), suggesting that PpiD does not have a major role in BrkA secretion. This result was not unexpected, because the outer membrane protein secretion defect in a PpiD knockout strain is much less severe than the defect observed in a SurA knockout [92]. PpiD may have a minor role in outer membrane protein secretion in comparison with the necessity for SurA, accounting for why BrkA secretion is not greatly affected by absence of PpiD. The SurA PpiD double knockout strain, showed the similar low levels of BrkA processed passenger secretion as observed in the SurA knockout strain (Figure 4-5), supporting our finding...
that SurA is the essential factor for BrkA secretion in *E. coli*.

### 4.7 The peptidyl-prolyl isomerase activity of SurA is not required for secretion of BrkA

Since SurA is a PPIase, we decided to investigate whether this activity was important for BrkA secretion. Structure-function analyses have shown that SurA has chaperone activity and PPIase activity, which are localized to separate domains [66, 83]. SurA is composed of an N-terminal non-conserved domain, followed by two conserved parvulin domains, with a C-terminal tail domain that is required for stability of the protein (Figure 4-6). The second parvulin domain was shown to be responsible for the peptidyl-prolyl isomerase activity of SurA [83], while the N-terminal domain coupled with the C-terminal tail, and missing both parvulin domains, were shown to have chaperone activity [66]. To determine whether the peptidyl-prolyl isomerase activity of SurA was required for secretion of BrkA, an active-site SurA mutant deficient in PPIase activity (SurA-2A), and also an N-terminal SurA mutant missing both parvulin domains but with the C-terminal tail (SurA-NCt) were separately coexpressed with BrkA in *E. coli* UT5600. The active-site SurA mutant provided partial complementation of BrkA secretion at similar levels to complementation by wild type SurA (Figure 4-7A). In contrast, the SurA-NCt mutant was unable to complement BrkA secretion (Figure 4-7A). The anti-SurA antibody could not detect SurA-NCt (Figure 4-7B), because it does not recognize this construct [66]. However, the SurA-NCt mutant was shown to be expressed through blotting with anti-His antibody, since the construct contains a C-terminal His tag (Figure 4-7C). Interestingly, the Coomassie stain of whole cell lysates
Figure 4-6. The domain architecture and crystal structure of SurA.
A. The crystal structure of SurA [108] colored by domain. SurA is composed of a signal peptide, an N-terminal domain, 2 parvulin domains (P1 and P2), and a C-terminal tail. The second parvulin domain (P2) is responsible for the in vitro PPIase activity of SurA. The in vivo and in vitro chaperone activity of SurA has been localized to the N-terminal domain [66]. The C-domain is necessary for stability of SurA [66]. B. The helices of SurA that interact with the peptide of a neighbouring SurA molecule in the SurA crystal [108] are colored light purple. The His^{376} and Ile^{378} residues found to be necessary for in vitro PPIase activity of SurA [66] are indicated in (A) and (B) by the dark purple side chains.
Figure 4-7. The PPIase activity of SurA is dispensable for BrkA secretion.
Trypsin accessibility experiment on SurA knockout strains uncomplemented (pQE60 is the control vector), or complemented with Wild type SurA (pSurA), SurA active site mutant (pSurA-2A), or an N-terminal SurA mutant (pSurA N-Ct). Anti-BrkA antibody (A) was used to detect BrkA in the Western blot. Anti-SurA (B) and anti-His-tag antibody (C) was used to detect the SurA constructs in the Western blot. A separate SDS-PAGE gel was run and stained with Coomassie brilliant blue to detect outer membrane proteins (D). “U” denotes unprocessed BrkA and “P” denotes processed BrkA. “a” denotes LamB monomer and “b” denotes OmpC/OmpF according to [83]. This result is representative of three experiments.
showed that the SurA-NCt mutant complemented the secretion defect of outer membrane proteins (identified as OmpF/OmpC and LamB by comparison with Rouviere and Gross's data [83]) in the SurA knockout strain (Figure 4-7D). This data suggests that the N-terminal domain and both parvulin domains, but not the PPIase activity, is required for secretion of BrkA in *E. coli*. Thus, the chaperone activity of SurA may be mediating secretion of BrkA.

4.8 Discussion

Few studies have addressed the periplasmic phase of autotransporter secretion. Pulse-chase studies have revealed that the periplasmic state is highly transient [42]. The chaperone activity of DegP has been implicated in the secretion of IcsA in *S. flexneri* [44], suggesting that autotransporters might require periplasmic chaperones for secretion through the periplasm. To investigate the requirement of periplasmic chaperones in the secretion of autotransporters, we used BrkA secretion in *E. coli* as a model. We used P1 phage to transduce periplasmic chaperone knockout alleles into UT5600, an *E. coli* strain deficient in the outer membrane protease OmpT. OmpT has been shown to degraded unfolded mutants of BrkA [52]. The absence of OmpT in this strain allowed us to study surface expression independent of the final folded state of BrkA. The Skp knockout showed similar levels of BrkA secretion as the wild type, while the DegP knockout had slightly lower levels of BrkA surface expression than wild type, suggesting that Skp and DegP are not essential for BrkA secretion in *E. coli*. In contrast, a SurA knockout resulted in much lower surface expression of the BrkA passenger. These results suggest that SurA is necessary for BrkA secretion in *E. coli*. 
To confirm the requirement of SurA for secretion of BrkA, a copy of wild type SurA in a plasmid was coexpressed with BrkA in the SurA knockout strain. There was partial complementation of BrkA secretion in a SurA knockout strain with the SurA plasmid. SurA was detected by Western blot in the lysate of the complemented SurA knockout strain, as well as a number of degradation products. This degradation might be a consequence of the high level of production of SurA. SurA was not detected in the whole cell lysate of the uncomplemented SurA knockout strain. Thus, the SurA knockout BrkA secretion defect can be complemented, providing further evidence that SurA is important for Brk secretion in *E. coli*.

SurA has been found to be important for the secretion of the outer membrane proteins LamB, OmpA, OmpC, and OmpF [83], all of which have the characteristic beta-barrel structure of integral outer membrane proteins [85-88]. The transporter domain of BrkA is predicted to be a beta-barrel structure. Thus, the requirement of SurA for BrkA secretion may be due to SurA facilitating secretion of the transporter domain to the outer membrane. We found that DegP and Skp did not play a role in BrkA secretion. There are studies that suggest that DegP and Skp are involved in outer membrane protein secretion. A DegP mutant with chaperone but not protease activity has been shown to sequester secretion incompetent mutants of OmpF [80] and OmpC [95] in the periplasm, preventing lethality. *In vivo* studies have demonstrated that Skp interacts with the outer membrane protein OmpA at the inner membrane, and is necessary for its release [81]. *In vitro*, Skp has been shown to assist the insertion of OmpA into phospholipid bilayers [96]. These studies support a role of Skp and DegP in outer membrane protein secretion. Despite this evidence, wild type levels of outer membrane proteins are observed in the
DegP knockout strain. The Skp knockout strain has been shown to have a defect in outer membrane protein secretion [97]. In our whole cell lysates, we observe a slight reduction in levels of outer membrane proteins in the Skp knockout, but the defect in the outer membrane protein profile is much more severe for a SurA knockout (data not shown). This suggests that in our strain, SurA is the critical determinant for secretion of outer membrane proteins, with Skp playing a less prominent role. It is not surprising then, that SurA is required for BrkA secretion, while DegP and Skp are not essential.

Interestingly, DegP is required for efficient secretion and polar localization of the autotransporter IcsA in *Shigella flexneri* [44]. Whether other AT1 members also require SurA or DegP for secretion remains to be seen. SurA homologues have been predicted in several Gram negative bacteria, such as *Burkholderia, Caulobacter crescentus, Salmonella typhimurium, Shigella. flexneri, B. pertussis*, and *Yersinia pestis*. There is 31% sequence identity and 50% similarity between the SurA homologues in *E. coli* and *B. pertussis*. We hypothesize that SurA in *B. pertussis* is also necessary for secretion of BrkA. The requirement of BrkA for a conserved chaperone explains why it can be secreted in *E. coli*. If other AT1 autotransporters also utilize highly conserved periplasmic chaperones for secretion, this would account for the ability of autotransporters from a range of different bacteria to be heterologously secreted in *E. coli*.

We hypothesized that SurA facilitates surface expression of the BrkA passenger by mediating secretion of the transporter domain to the outer membrane. An assay was developed which utilized the high isoelectric point of the BrkA transporter domain (pI = 9.78). For transfer of proteins to the membrane, transfer buffer was used. After
transfer, the gel was stained with Coomassie brilliant blue to detect the BrkA transporter, which should remain in the gel due to its high isoelectric point. In the SurA knockout, lower levels of the BrkA transporter domain were observed compared to the wild type strain. The transporter domain is likely to be in the outer membrane, assuming that processing of BrkA occurs after outer membrane insertion. This suggests that SurA may be required for stability of the BrkA transporter in the periplasm. Alternatively, SurA may play a role in outer membrane insertion of the transporter. Either of these possibilities would lead to the observed defect in surface expression of the BrkA passenger in the SurA knockout strain. However, SurA might also interact with the BrkA passenger domain to facilitate secretion.

The HSF domain of BrkA was shown to be required for secretion of a folding-competent passenger (please refer to Chapter 3). To investigate whether SurA might interact with the HSF domain to facilitate secretion, BrkA mutants were expressed in both wild type and SurA knockout strains. The SurA knockout strain showed lower amounts of processed passenger of wild type BrkA and mutant constructs, but the relative abundance was similar to the profile observed in the wild type strain. This data suggested that SurA does not interact with HSF to mediate BrkA secretion. However, SurA might still be facilitating BrkA secretion through interaction with another sequence of the passenger separate from the HSF. There is also the possibility that another periplasmic chaperone is required by the HSF to facilitate BrkA secretion. A possible candidate is DsbG, a disulfide-bond isomerase with chaperone activity [98] which is conserved in _B. pertussis_.

74
SurA belongs to a family of proteins known as peptidyl-prolyl isomerases. The characteristic activity of these proteins is the cis-trans isomerization of proline bonds. The isomerization of proline bonds often determines the rate of protein folding [91]. SurA may mediate secretion of BrkA through its PPIase activity. Thus, we chose to investigate whether other periplasmic PPIases in *E. coli* would be required for BrkA secretion. To test this, a PpiA, PpiD, and a SurAPpiD double knockout strain were created. BrkA secretion levels were not significantly reduced in the PpiA and PpiD knockout strains, suggesting that they did not have a role in BrkA secretion. Our success in creating a SurAPpiD double knockout strain was a surprise, as previous data has supported that SurA and PpiD have redundancy in a function essential for cell viability [92]. There may be a difference in the strain background accounting for our ability to generate a SurAPpiD double mutant. The SurAPpiD double mutant showed a similar BrkA secretion defect as in the SurA mutant, suggesting that SurA is the necessary factor for BrkA secretion.

The next question was whether the peptidyl-prolyl isomerase activity of SurA was required for secretion of BrkA. SurA might specifically recognize BrkA, allowing it to readily bind and catalyze isomerization of proline bonds in BrkA. PPIases consist of three subfamilies, the Fk506 binding proteins, cyclophilins, and the parvulins, of which SurA is a member [91]. The smallest member of the parvulin family is parvulin, a 92 amino acid protein with PPIase activity [90]. The other members of this family have at least one parvulin-like domain, in addition to other domains [91]. SurA is a modular protein, with a signal peptide, an N-terminal segment with no significantly homology to any other proteins, two parvulin domains, and a C-terminal tail domain required for
stability [66]. The PPIase activity of SurA has been previously shown to localize to the second of two parvulin-like domains [83]. To investigate the requirement of this activity for BrkA secretion, a SurA active site mutant was coexpressed from a plasmid, along with BrkA in a SurA knockout strain. The active site mutant was able to partially complement BrkA secretion, at levels similar to wild type SurA, suggesting that the PPIase activity of SurA is not necessary for BrkA secretion in *E. coli*. This result was expected, because the PPIase activity of SurA was shown to be dispensable in the secretion of outer membrane proteins such as OmpC, OmpF, LamB, and OmpA [66]. Since PPIase activity is not required for BrkA secretion, we wanted to determine whether the N-terminal domain of SurA was the essential determinant for BrkA secretion. An N-terminal SurA construct missing both parvulin domains, but flanked with the C-terminal tail, was coexpressed with BrkA on separate plasmids in the SurA knockout strain. No complementation of BrkA secretion was observed. An SDS-PAGE gel stained with Coomassie brilliant blue showed that the secretion defects of OmpF/OmpC and LamB were complemented by the N-terminal SurA construct. The complementation of secretion defects of these proteins by the N-terminal construct is consistent with earlier studies [66]. Our data suggests that the parvulin domains of SurA are not required for secretion of OmpF/OmpC and LamB, but they are required for BrkA secretion. BrkA secretion in *E. coli* requires the N-terminal and parvulin domains of the SurA protein, but does not require the PPIase activity of SurA.

*In vitro* thermal aggregation and renaturation studies with citrate synthase have shown that SurA can act as a chaperone [66]. SurA binds preferentially to outer membrane proteins in *in vitro* studies [66]. This *in vitro* data coupled with the major
defect in outer membrane protein secretion in a SurA knockout strain suggest that SurA acts as a chaperone. The in vitro chaperone activity of SurA has been shown to localize to the N-terminal domain [66]. In addition, the N-terminal SurA construct is sufficient to complement the outer membrane protein secretion defects and detergent sensitivity in the SurA knockout strain [66], suggesting that the N-terminal domain is responsible for the in vivo function of SurA. We propose that the chaperone activity in the N-terminus of SurA mediates the secretion of BrkA in E. coli, while the PPIase activity is dispensible. The parvulin domains also provide some unknown function independent of its PPIase activity that is necessary for secretion of BrkA in E. coli.

The requirement of the entire SurA protein for secretion of BrkA, but not the PPIase activity, is reminiscent of the structure-function studies on PrsA. The PrsA protein of Bacillus subtilis is a PPIase from the parvulin family that is required for viability, as well as secretion of AmyQ, an amylase. Interestingly, the PPIase activity of PrsA was not required for in vivo function, but the parvulin domain was required [99]. Thus, both SurA and PrsA require an unidentified function of the parvulin domain that is separate from the PPIase activity for secretion of BrkA and AmyQ, respectively.

We found that levels of BrkA transporter were lower in the SurA knockout mutant. Assuming that the processing of BrkA into passenger and transporter domains occurs after insertion into the outer membrane, this would suggest that the transporter was localized to the outer membrane. The low levels of transporter in the outer membrane of the SurA knockout suggest that the observed BrkA secretion deficiency occurs at either the step of transporter insertion into the membrane or stability of the transporter in the outer membrane, or at an earlier secretion step. We suggest that the
role of SurA in BrkA secretion might be to 1) facilitate insertion or stability of the transporter domain in the outer membrane, or 2) to stabilize the transporter in the periplasm. The end result of SurA mediating these putative functions is higher levels of BrkA transporter reaching the outer membrane, allowing greater levels of passenger translocation to the surface. There is evidence that supports a role for SurA in facilitating folding of the outer membrane protein LamB. SurA was discovered to be involved in the formation of an assembly intermediate of the outer membrane protein LamB, which is a trimer. Specifically, SurA was required for folding of LamB monomer into f-monomer (folded monomer), which was an intermediate for trimerization into mature LamB. [83]. SurA may facilitate a similar folding reaction with the BrkA transporter in the periplasm, resulting in a protease-resistant folded transporter that can insert into the outer membrane to allow passenger translocation. In the absence of SurA, the BrkA transporter transits through the periplasm in an unfolded state, and is more susceptible to proteases, resulting in lower levels of transporter reaching the outer membrane, and thus less surface expression of the passenger.

In addition the model proposed above, there are other possibilities that may account for the BrkA secretion defect in the SurA knockout strain. Absence of SurA might be indirectly causing the BrkA secretion defect, perhaps by downregulation of BrkA production. The 4-5 fold decrease in LamB secretion in a SurA knockout mutant was shown to be partially due to decreased synthesis [83]. However, pulse-chase analyses showed that downregulation of LamB only contributed to half of the overall LamB secretion defect, while the rest could be attributed to defect in assembly [83]. Assuming BrkA is also downregulated to similar levels as LamB in the SurA knockout,
this would account for only half of the severe BrkA secretion defect that was observed. The rest of the events causing the BrkA secretion defect would occur at other steps after translation.

SurA is a member of the sigma E regulon [100]. The sigma E system senses periplasmic stress and outer membrane pertubations caused by misfolding of outer membrane proteins [101]. To resolve the periplasmic stress, the sigma E system upregulates proteases such as DegP [102] to degrade non-productively folded proteins. Chaperones such as Skp and SurA are also upregulated as part of the sigma E response [101], and function in facilitating folding of misfolded proteins in the periplasm. The sigma E regulon is induced in SurA knockout mutants compared to wild type [101]. This is presumably due to the periplasmic stress caused by the leaky outer membranes of SurA knockout mutants. SurA knockout strains have been demonstrated to have higher levels of DegP protease than wild type [83]. The BrkA secretion defect may be due to degradation by higher amounts of DegP in a SurA knockout. However, we found that the level of surface expressed BrkA was slightly lower than wild type in a DegP knockout strain (Figure 4-1). If DegP was degrading BrkA, we would expect an increase in BrkA secretion in the absence of DegP expression. Therefore, the low levels of secreted BrkA in the SurA knockout strain are probably not due to degradation by high levels of DegP.
5. DISCUSSION AND CONCLUSION

The junction domain was initially identified in the \textit{S. marcescens} autotransporter PrtS \[60], and was hypothesized to be required for folding of the passenger. The junction domain was then identified in BrkA and was necessary for folding \[52]. ClustalW sequence alignment of the junction in a range of autotransporters revealed a highly conserved subdomain (region 3) that seemed to form its own domain when examined in the pertactin passenger crystal structure. Comparison of the pertactin and Hbp passenger crystal structures with the size of the NalP pore, and estimated pore sizes of transporter domains of PalA and IgA protease suggested that the passenger might go through the channel in an unfolded or partially folded translocation-competent state. We hypothesized that region 3 was involved in the maintenance of this state, possibly by binding to a chaperone in the periplasm. The involvement of periplasmic chaperones in BrkA secretion was investigated in parallel. We attempted to test these two hypotheses in this thesis.

What are the boundaries of the AC/HSF domains in BrkA?

The junction domain of BrkA was shown to have two distinct highly conserved subdomains; the autochaperone region (AC) that is necessary for passenger folding \[52], and the hydrophobic secretion facilitation domain (HSF) that is required for secretion of a folding-competent BrkA passenger (Figure 3-9). The current boundaries of the BrkA HSF were determined to be $A^{681-693}$. In future studies, the boundaries between the autochaperone domain and the HSF, and between the HSF and the minimal translocation unit should be determined. In the autotransporter EspP, hydrophobic residues in a region
called the hydrophobic secretion facilitation domain (HSF) domain were shown to be critical for secretion [72]. The EspP HSF shares overlap with both the autochaperone domain and the HSF of BrkA. A few of the residues in the EspP HSF that significantly reduced secretion upon mutation are conserved in the BrkA autochaperone domain. The *E. coli* strain used in the EspP study was HB101, which is an OmpT+ strain. Thus, it is possible that some of the residues that they mutated affected folding, leading to degradation by OmpT at the cell surface, and resulting in the observed secretion deficiency. We predict that in future studies, the EspP HSF domain will be revealed to have two separate domains, with one mediating folding, and the other mediating secretion of a folding-competent passenger. However, it is probable that in both BrkA and EspP that the HSF and autochaperone domains overlap, with the overlapping region mediating both activities. The high conservation of the junction domain in several AT1 members (Figure 1-3) suggests that this two domain functional architecture may apply to other AT1 autotransporters.

**How does the BrkA HSF influence levels of transporterless constructs in the periplasm?**

The mechanism by which the BrkA HSF mediates secretion of a folded passenger was not determined. Studies using transporterless BrkA constructs trapped in the periplasmic space suggested that the BrkA HSF, along with folding-competence, might play a role in providing stability in the periplasm (Figure 3-6B). We make the assumption that the relative levels of transporterless constructs in the periplasm reflect differences in stability, and not rate of production. Of course, pulse-chase studies will be
required to confirm that stability is the factor leading to these differences in steady-state levels. However the results from this system might be artificial, as autotransporters probably pass through the periplasm quickly [42]. When comparing the periplasmic levels of the transporterless constructs with the surface expression of full-length constructs, the profile of the wild type and mutants are different. The autochaperone and junction mutants have lower expression than the region 3 mutant in the transporterless study (Figure 3-6B), but higher expression in the full-length study (Figure 3-3). Thus, periplasmic stability is probably not a major factor in determining the efficiency of surface expression. The periplasmic stability provided by region 3 in the transporterless constructs may only be relevant to the system, and not actual autotransporter secretion to the surface.

**Does SurA interact with the BrkA HSF to mediate BrkA secretion?**

One possibility for the mechanism of function for the BrkA HSF domain is that it might be associating with a chaperone to facilitate secretion of the passenger. When the hydrophobic residues critical to EspP secretion in the HSF domain were modeled on pertactin, which has high sequence conservation with BrkA, they seemed to orient towards one face (Figure 3-8C). The phenylalanine and one of the isoleucine residues modeled localizes to the BrkA HSF domain, which contains a number of conserved hydrophobic residues. The hydrophobic residues of the BrkA HSF domain might be forming a hydrophobic surface for association with a chaperone, similar to the hydrophobic/hydrophobic interactions formed between the PapD chaperone and pilin subunits [14].
Interestingly, the periplasmic chaperone SurA was discovered to be important for BrkA secretion in *E. coli* (Chapter 4). SurA was found to facilitate BrkA surface expression by mediating secretion of the BrkA transporter to the outer membrane (Figure 4-3A). However, SurA did not require interaction with the HSF domain to mediate BrkA secretion (Figure 4-4). This does not exclude the possibility that SurA might interact with another sequence in the BrkA passenger, or that another periplasmic chaperone might associate with the BrkA HSF to mediate secretion of a folding-competent passenger.

**Is the region 3 mutant jamming due to inefficient translocation across the outer membrane?**

If our hypothesis is correct that the BrkA HSF mediates secretion of a folding-competent passenger, then we expect expression of the region 3 mutant to cause periplasmic stress. We speculate that since the HSF is missing in this mutant, it is not able to associate with a chaperone to remain unfolded, and thus folds in the periplasm (Figure 3-7B). This prevents translocation, and the region 3 mutant accumulates in the periplasm, perhaps activating the Cpx system which responds to stress signals such as accumulation of surface organelles like P pilin subunits in the periplasmic compartment [14]. Currently, experiments using a CpxP-LacZ reporter strain are being performed to determine whether the region 3 mutant causes an elevated Cpx response compared to expression of wild type BrkA.
What is the common feature shared by the proteins that require SurA for secretion?

SurA was shown to be required for secretion of BrkA in *E. coli* (Chapter 4). This is the first time that a peptidyl-prolyl isomerase has been implicated in autotransporter secretion. Interestingly, SurA is involved in secretion of the outer membrane proteins OmpA, OmpC, OmpF, and LamB in *E. coli* [83, 103]. OmpA is a monomeric protein that has an N-terminal domain that forms an eight-stranded beta-barrel [86], with a C-terminal periplasmic domain that binds to peptidoglycan [104]. The external loops of the transmembrane domain of OmpA are involved in F-plasmid conjugation [105]. OmpC and OmpF are porins that function in osmoregulation. The crystal structure of OmpF consists of a homotrimer, with each monomer consisting of a 16-stranded beta-barrel [88]. OmpK36, an osmoporin of *Klebsiella pneumoniae* has 87% sequence identity to OmpC in *E. coli*, and is also a homotrimer of 16-stranded beta-barrels [85]. *E. coli* LamB is a trimer composed of 18-stranded beta-barrels, each forming a channel [87]. LamB acts as a porin to mediate passive diffusion of maltodextrin across the outer membrane. The common feature shared between these outer membrane proteins and BrkA is that they all possess an anti-parallel beta-barrel as part of their structure. It was not surprising then, that SurA was found to facilitate outer membrane localization of the BrkA transporter domain, which is predicted to be a beta-barrel. The finding that SurA mediates secretion of the BrkA transporter to the outer membrane might have implications not only for secretion of other AT1 autotransporters, but also for other surface organelles that contain a beta-barrel as part of their structure. Both the AT2 and AT3 family of autotransporters are predicted to have transporter domains that are beta-barrels. Whether other AT1 members, the AT2, and AT3 subfamilies share a similar
requirement of SurA for secretion remains to be seen. It is also intriguing to speculate that SurA might be necessary for secretion in TPS systems as well, as the cognate transporter in that system is a beta-barrel.

**What is a possible mechanism by which SurA facilitates secretion of BrkA?**

SurA plays a role in the secretion of both monomeric (OmpA), and trimeric (LamB, OmpC, OmpF) outer membrane proteins in *E. coli* [83, 101]. Studies on LamB expression in a SurA knockout showed that there was a defect in the formation of an assembly intermediate in trimer assembly called f-monomer (folded monomer) [83]. The LamB assembly pathway is thought to proceed from monomer, to folded monomer, to meta-stable trimer, which then forms the stable trimer in the outer membrane [106]. SurA might be facilitating folding of the monomer to f-monomer, allowing the LamB intermediate to assume a protease-resistant conformation. In the SurA knockout, the inability of the LamB monomers to fold might lead to proteolysis, resulting in the observed deficiency in trimer assembly [83]. The finding that SurA possesses chaperone activity [66] coincides with its ability to fold the LamB monomer. Interestingly, *in vitro* studies have suggested the existence of a folded monomeric species of OmpF [107]. Whether this species is relevant to the *in vivo* secretion of OmpF, or the requirement of SurA for formation of this species *in vivo* has not been determined. The LamB assembly study suggests that SurA might play a role in outer membrane secretion by generating a folded species that resists degradation in the periplasm, resulting in higher levels of secretion. This mechanism of action would aid secretion whether the outer membrane beta-barrel protein was monomeric or oligomeric. We suggest that SurA facilitates
folding of the BrkA transporter, allowing it to assume a protease-resistant state. Thus, higher levels of the transporter would reach the outer membrane, increasing passenger translocation to the cell surface.

**What is a possible structural basis for SurA binding to the BrkA transporter domain?**

The crystallographic structure of *E. coli* SurA has been solved, and is an asymmetric dumbbell with the N, P1, and C domains forming a core module, with the P2 domain tethered as a satellite domain 3 nm from the core module (Figure 4-6A) [108]. Within a SurA crystal, the alpha-helix from one SurA molecule binds within a groove in the N-terminal domain formed in an adjacent SurA molecule (Figure 4-6B). Interestingly, the core domain of SurA has been shown to bind heptapeptides containing the aromatic-random-aromatic motifs common in integral outer membrane proteins [89], and is also present in the transporter domain of BrkA at the C-terminus (YSF). It remains to be seen whether this site (Figure 4-6B) is actually involved in binding of SurA to porins and the BrkA transporter domain to mediate its chaperone function.

**Why are the parvulin domains necessary for secretion of BrkA, but not for porin or OmpA secretion?**

Structure-function studies on SurA in *E. coli* suggested that the chaperone activity of the N-terminal domain was required for secretion of OmpF, OmpC, LamB, and OmpA [66]. The two C-terminal parvulin domains and the PPIase activity were dispensable in the secretion of those outer membrane proteins [66]. Our SurA complementation studies
showed that the N-terminal domain, and both parvulin domains, but not the PPIase activity, were required for BrkA secretion. BrkA and the outer membrane proteins share a similar requirement of the SurA N-terminal domain, and also dispensability of the PPIase activity of SurA for secretion. However, BrkA requires both parvulin domains for secretion, whereas OmpF, OmpC, LamB, and OmpA do not [66]. The major feature that distinguishes BrkA from these outer membrane proteins is the presence of the surface expressed passenger. It is possible that the SurA parvulin domains provide some unknown function independent of PPIase activity, that assists secretion of BrkA by interaction with the passenger. We can only speculate that the chaperone activity of the N-terminal domain, and some unknown determinant provided by the parvulin domains, are necessary for secretion of BrkA in *E. coli*.

**Autotransporter: Is a name change overdue?**

Recent studies on autotransporter secretion have revealed that other proteins are required for their surface expression. In this thesis, SurA was shown to be required for secretion of BrkA in *E. coli*. A highly conserved outer membrane protein among Gram negative bacteria called Omp85 was shown to be necessary for secretion of IgA protease in *N. meningitidis* [51]. Based on the requirement of IgA protease on Omp85 for secretion, Oomen *et al.* pondered that if Omp85 formed a channel for secretion of an autotransporter, then the designation “autotransporter” is no longer appropriate [23]. We offer an alternative viewpoint on the dependence of autotransporter secretion on other proteins. If the definition of an autotransporter is a protein that provides all of the specialized determinants for secretion of the passenger to the surface, then the
"autotransporter" label is still valid. The specialized secretion determinant of an autotransporter would be the translocation unit, which is necessary for passenger secretion. Omp85 was not only important for the secretion of IgA protease, but was also necessary for secretion of a wide range of other proteins such as porins, a secretin involved in type IV pili formation, and siderophore receptors [51]. Similarly, SurA was required not only for secretion of BrkA in *E. coli*, but for several porins and OmpA as well [83, 103]. SurA and Omp85 are not specialized, but general determinants of secretion of autotransporter proteins, as they are also essential for secretion of other classes of proteins. Therefore, we believe that the designation "autotransporter" is not a misnomer, even if other general determinants required for autotransporter secretion are discovered.
REFERENCES


APPENDIX 1. Confirmation of the *ppiD* knockout

A. The expected outcome from PCR amplification of the ~1.1 kb internal PpiD fragment from the chromosome of wild type and PpiDA mutant (JY665). The dotted bar represents the kanamycin resistance cassette, and the white bars represent the ppiD gene or parts of the ppiD gene (in the case of the ppiD Δmutant) Primers are indicated by the arrows, and the vertical lines indicate primer binding to the chromosome. This diagram is not drawn to scale.

B. PCR amplification was done from two different chromosomal preps from separate colonies of wild type and PpiDA mutant (JY665). The primers used for confirmation of the knockout bound to 461559-461583 and 462591-462618 of the *E. coli* chromosome.