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Chaperone Protein BamB Directly Supports BrkA Translocation and Folding in *Escherichia coli*

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SUMMARY Autotransporters are virulence factors expressed on the surface of gram-negative bacteria as part of the type V secretion system. As the name suggests, the surface expression of an autotransporter is facilitated by passage through the protein's own translocation domain, rather than an additional channel. One such example is BrkA of *Bordetella pertussis,* a type V autotransporter responsible for adhesion, serum resistance, and cellular invasion. The assistance of chaperone proteins is required to translocate autotransporters across bacterial membranes and enable proper folding into functional structures on the cell surface. However, the exact chaperones required for BrkA surface expression are currently unclear. In this study, a panel of *Escherichia coli* chaperone knockout strains was transformed with a *brkA* expression vector to investigate the importance of these chaperones in stable autotransporter surface expression. Through trypsin accessibility assays and western blot analysis we found that BamB may be for the expression of cleaved BrkA on the outer membrane, and may directly interact with the protein during this process. We used cross-linking experiments and found that BamB may directly interact with BrkA autotransporter. This project aims to characterize the functional significance of a wide panel of chaperones with respect to BrkA. Insights derived from this study are relevant to immunotherapeutic and vaccine development, where BrkA is an attractive target due to its surface presentation and its role as a virulence factor.

INTRODUCTION

Autotransporters are the largest class of extracellular virulence proteins secreted by gram-negative bacteria (1, 2). Type V autotransporters commonly consist of a signal peptide, a passenger domain, and a translocation domain (3). *Bordetella* resistance to killing (BrkA) is a 103kDa type Va autotransporter protein in *Bordetella pertussis*, a gram-negative pathogen responsible for whooping cough (4, 5). BrkA inhibits the classical pathway of complement activation for bactericidal activity, allowing evasion of the innate immune system (6). With cytosolic ATPase Sec, the N-terminus signal sequence of BrkA permits translocation across the inner membrane (4). Within the periplasm, the 30kDa C-terminus translocation domain folds into a β-barrel to facilitate translocation of the N-terminus domain across the outer membrane (4). The N-terminus then undergoes autoproteolytic cleavage to produce the 73kDa passenger domain, which appears to remain membrane-bound via non-covalent association with the β-barrel (4). The exact mechanism by which periplasmic BrkA achieves folding and outer membrane translocation is an area of active study for the therapeutic design of small molecules against *B. pertussis* pathogenicity, in which chaperone proteins are of particular interest.

Chaperones are broadly defined as proteins that interact and assist in proper folding, stabilization, and transport of cellular proteins (7). Outer membrane proteins (OMPs) are

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dependent on periplasmic chaperones and outer membrane folding factors to ensure their function in the survival and virulence of gram-negative bacteria (8). To explore the role of chaperone proteins in the context of BrkA surface expression, a panel of chaperones associated with autotransporters and OMPs was identified: FkpA, SurA, DegP, Skp, DsbA, and BamB. Together this selection of proteins carryout a wide range of roles that are necessary for the expression of different OMPs, summarized in Table 1. Existing literature has demonstrated the necessity of these chaperones in the folding and activity of a wide range of OMPs. Skp, DegP, and SurA have been implicated in the correct surface expression and folding of OMPs such as OmpF, OmpA, OmpC, and LamB (15, 16). Moreover, some of these chaperones have already been implicated in the function of other type V autotransporters. FkpA, DegP, and SurA have been found to contribute autotransporter EspP passenger domain folding and secretion (23). Studies have also shown that loss of SurA in *Escherichia coli* was sufficient to result in loss of Ag43-mediated autoaggregation, a phenotype associated with virulence, due to abrogation of Ag43 passenger secretion (24). Another autotransporter, IcsA, has been found to require the activity of Skp to achieve native conformation and surface expression in *Shigella flexneri* (25). Despite the growing evidence that chaperones play important roles in mediating autotransporter function and surface expression, there has been a paucity in studies characterizing chaperone function in relation to BrkA.

TABLE. 1 Summary of protein panel used in this study, including known chaperone functions. Names provided are those most recently applied to the proteins as of April 2024.

Chaperone protein	Location	Function
FkpA	Periplasm	Periplasmic peptidyl-prolyl cis/trans isomerase (PPIase) activity. Suppression of defective protein periplasmic inclusion bodies (9-12).
SurA	Periplasm	Periplasmic peptidyl-prolyl cis/trans isomerase (PPIase) activity (10, 11).
DegP	Periplasm	Serine protease, degrades misfolded proteins. Chaperone, supports folding and OM insertion of periplasmic proteins (13, 14).
Skp	Periplasm	Adaptor chaperone, binds to misfolded DegP to prevent aggregate formation (15, 16).
DsbA	Periplasm	Periplasmic oxidoreductase, catalyzes protein folding by disulfide bridge (oxidation-dependent) and transient noncovalent bond (oxidation-independent) formation with substrate (17, 18).
BamB	Periplasmic lipoprotein at OM	Accessory protein modulating β-barrel assembly machinery (BAM) complex, mediates β-barrel assembly of OMPs (19-22).

This study aimed to assess the relationship between the autotransporter BrkA and our collection of six chaperones. Strains deficient in each chaperone were sourced from the *E. coli* BW25113 Keio strain collection, in which deletion is performed by targeted kanamycin cassette insertion (26). Knockout strains of each chaperone were transformed alongside a wildtype (WT) BW25113 strain with the pPALMC1 plasmid, which expresses BrkA with a detectable N-terminal 6xHis tag. BrkA expression, outer membrane translocation, and direct interaction with the aforementioned chaperone proteins were analyzed. It was hypothesized that the deletion of chaperone proteins that are necessary for BrkA expression and translocation would result in a loss of surface expression. The results indicated that BamB directly interacts with BrkA, and is necessary for its expression on the outer membrane surface. Other chaperones – Fkp, SurA, and DsbA – may also assist in BrkA expression but did not demonstrate necessity, supporting prior literature regarding their functional redundancy (23, 26). Despite the difficulty of elucidating chaperone-substrate interaction due to the transient nature of such complexes, our study identifies promising candidates for further research. Uncovering these chaperone-OMP interactions can provide key insights for the future development of vaccines and immunotherapeutics, both which are dependent on the correct expression of surface proteins such as BrkA in their native conformations.

METHODS AND MATERIALS

Overnight cultures. 5mL cultures of untransformed and pPALMC1-transformed BW25113 and Keio strains were grown at 37ºC with shaking for 16 hours. For pPALMC1 transformants, LB was supplemented with 100µg/mL ampicillin. All Keio strains were additionally supplemented with 50µg/mL of kanamycin. Cells were grown to a final OD_{600} of 1.0-2.0. For isopropyl β- d-1-thiogalactopyranoside (IPTG) induction, overnight cultures were grown for four hours in 1mM IPTG prior to cell harvest and protein extraction.

Preparation of chemically competent *E. coli* cells. Chemically competent *E. coli* cells were prepared using a protocol adapted from the Hancock lab (28). 50mL of LB media was inoculated with 500µL of overnight culture and incubated for four hours with shaking at 37ºC. Once the culture reached an OD_{600} of 0.4-0.6, cells were pelleted by centrifuging at 10,000RPM at 4ºC for five minutes, then washed in 10mL of 0.1M $CaCl_2$ and pelleted once more. Cells were then gently resuspended in 500µL of 0.1M $CaCl_2$ 10% v/v glycerol, and stored at -80ºC.

BrkA expression vectors. Two plasmids were utilized in this study, pENS and pPALMC1. Both pENS and pPALMC1 are derived from the pDO6935 plasmid, with the addition of a 6xHis-tag near the N-terminus of the BrkA passenger domain. In pENS, the 6xHis-tag is inserted between residues 59G and 60Q, whereas in pPALMC1 it is inserted between 57D and 58A (29, 30) While the molecular weight of full-length BrkA is reported to be 103kDa, surface-expressed BrkA from both plasmids is self-cleaved to produce a 30kDa β-barrel transporter domain and a 6xHis-tagged 73kDa passenger domain.

Plasmid extraction. All plasmids in this study were extracted from 5mL of overnight culture and purified using the Bio Basic plasmid DNA miniprep kit (Bio Basic, Cat. #BS414), eluting in 50µL of the manufacturer provided elution buffer. Plasmid quantity and quality were determined using Nanodrop $\lambda_{260}/\lambda_{280}$ and $\lambda_{260}/\lambda_{230}$ values.

Transformation of *E. coli* BW25113 and Keio chaperone knockout strains. Chemically competent cells were transformed with purified plasmids using a heat shock protocol. Cells were thawed on ice and 5µg of plasmid DNA were added. The DNA-plasmid mixture was incubated on ice for two hours. Cells were then heat shocked by warming cells at 42ºC for 30 seconds. Cells were then incubated on ice for 10 minutes before being diluted in 1mL of prewarmed LB media. Transformed cells were then incubated at 37ºC with shaking for one hour before plating on 100µg/mL ampicillin agar plates to select for transformants.

Growth curves. All overnight cultures were diluted to OD_{600} $2.00x10^{-2}$ (approximately $1.60x10^{7}$CFU/mL) in LB and kept on ice. The BW25113 pPALMC1 transformant was instead resuspended from frozen 25% glycerol stock. All transformant cultures were supplemented with 100µg/mL ampicillin. 200µL of each sample was added in four replicates alongside a negative control (LB media) to a 96 well clear bottom plate (Thermo Scientific, Cat. #165305). Growth was measured as OD_{600} sampled every 10 minutes across a 16-hour period by the BioTek Epoch2 Reader (Aligent, Cat. #EPOCH2NS).

Growth curve data analysis. Data analysis was performed in R using the Growthcurver package to fit the growth curve data to the logistic growth equation for population N at time t (31). This produced an estimate of carrying capacity (K) and intrinsic growth rate (r) for each strain. The estimated growth rates were then compared between strains with statistical analysis performed using one-way ANOVA tests.

EDTA minimum inhibitory concentration assay. Protocol adapted from the Hancock Lab (32). 100µL LB with 10mM ethylenediaminetetraacetic acid (EDTA) was added to the first column of a 96 well clear bottom plate (Thermo Scientific, Cat. #165305). Serial doubling dilutions were performed across the adjacent three columns to produce concentrations of 10mM, 5mM, 2.5mM, and 1.25mM EDTA in 100µL. A column without EDTA was included as a positive growth control. Wells were inoculated with 5µL overnight cultures diluted to

OD_{600} $1.25x10^{-4}$ in LB, alongside a sterile negative control without EDTA. All pPALMC1 transformant wells were supplemented with 100µg/mL ampicillin.

Samples were grown at 37ºC without shaking for 24 hours, after which the lowest EDTA concentration at which no turbidity was observed visually was recorded as the MIC. The experiment was repeated with a starting EDTA concentration of 2mM and doubling dilutions performed to final concentration of 0.25mM. If growth was recorded at all concentrations, the MIC was reported as [EDTA] >2mM.

SDS-PAGE and western blot. A volume equivalent to 1mL of OD_{600} 1.00 of each overnight culture was centrifuged at 10,000RPM for five minutes. Cell lysates for SDS-PAGE were prepared by resuspending pellets in 50µL of distilled water. Once resuspended, one part of 2x Laemmli buffer with 5% β-mercaptoethanol was added to the mixture and boiled at 95°C for five minutes. Lysates were centrifuged at 13,000RPM for 5 minutes and 10µL of the supernatant was loaded into the PROTEAN® TGX™ Precast Gel (Bio-Rad, Cat. #4568096). Electrophoresis was performed at a voltage of 200 V for 40 minutes. Proteins were transferred from the gel to a PVDF membrane using the Trans-Blot Turbo System (Bio-Rad Cat. #1704150EDU) running overnight at 25V and 4ºC. Whole protein staining was carried out by incubating the membrane in PoceauS for five minutes then destained using three five-minute washes in 1X Tris-Buffered Saline, 0.1% Tween (TBS-T). The membrane was then blocked for 30 minutes with 1% bovine serum albumin (BSA) in TBS-T, then washed thrice for five minutes with TBS-T. The monoclonal primary antibody against 6xHis tag (Invitrogen Cat. #MA1-21315) was diluted in 1:2500 TBS-T. The membrane was incubated overnight in the primary antibody at 4°C with shaking, then washed three times for five minutes with TBS-T. After 16 hours, the membrane was incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody diluted at 1:10000 in TBS-T for one hour at 4°C with shaking. The membrane was then washed using three 10-minute washes in TBS-T. To visualize the membrane, 6mL of Clarity Max™ Performance ECL western blotting substrate (Bio-Rad, Cat. #1705062) was added for five minutes in the dark and then imaged with ChemiDoc MP Imaging System (Bio-Rad, Cat. #12003154).

Trypsin accessibility assay. For each overnight culture, a volume equivalent to 1mL of OD_{600} 0.50 was centrifuged at 10,000RPM for five minutes. Cell pellets were then resuspended in 50µL of phosphate-buffered saline (PBS) and split evenly into two tubes. To one, 0.4µL of trypsin (15mg/mL) was added for a final concentration of 240µg/mL, this was repeated for each of the strains to generate one trypsin-treated and one negative control sample for each strain. The samples were immediately incubated at 37ºC for 10 minutes with shaking. At 10 minutes, 25µL of 2x Laemmli buffer with 5% β-mercaptoethanol was added to each tube and boiled at 95ºC for five minutes. Lysates were centrifuged at 13,000RPM for five minutes. Cell lysates were kept on ice until proceeding with the western blot protocol.

Formaldehyde crosslinking. For each overnight culture, a volume equivalent to 1mL of OD_{600} 0.5 was centrifuged at 10,000RPM for five minutes. Bacterial cells were then washed with 1mL of ice-cold 10 mM potassium phosphate (pH = 6.8) and pelleted by centrifugation at 10,000RPM for five minutes. Cells were then treated with 100µL 1% formaldehyde for one minute then immediately quenched using 65µL of 2.5M Tris. Fixed cells were then washed in 1mL of ice-cold 10mM potassium phosphate as described above. Each pellet was then resuspended in 25µL of deionized water before one part of 2x Laemmli buffer and 5% β-mercaptoethanol was added. Samples were then aliquoted between two tubes (25µL each), one of which was heated at 37ºC for 10 minutes to maintain crosslinks while the other tube was boiled for 20 minutes at 95ºC to reverse crosslinks. Lysates were chilled on ice for 10 minutes, then centrifuged at 13,000RPM to remove non-solubilized debris. 10µL of purified lysate was used for SDS-PAGE and western blotting.

RESULTS

K-12 *E. coli* BW25113 can express BrkA. To validate BrkA expression in WT *E. coli* K12 strain BW25113, a western blot was performed. Untransformed BW25113 was used as

a negative control alongside BW25113 transformed with either pPALMC1 or pENS. All strains were treated with and without 1mM IPTG to test for inductive effect.

Two distinct bands within the 75 and 150kDa range were identified in pPALMC1 transformants, corresponding to the cleaved (73kDa) and uncleaved (103kDa) BrkA protein (Fig. 1A). No signal was observed in the pENS-transformed strain (Fig. 1A). IPTG induction produced no observable differences in band patterns or intensities (Fig. 1A). Taken together, the presence of the 73 and 103kDa bands indicate intracellular and surface-expressed BrkA can be expressed in WT *E. coli* BW25113 using pPALMC1.

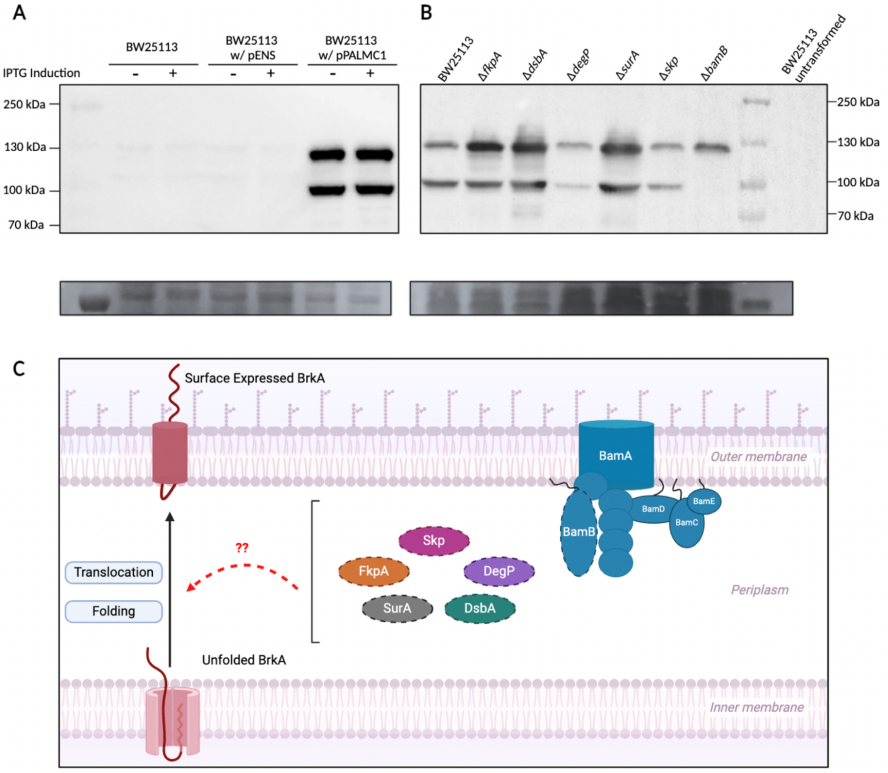


FIG. 1 BrkA is detectable in pPALMC1-transformed *E. coli* strains. (A) Western blot with anti-6xHis-tag performed against untransformed BW25113, as well as BW25113 transformed with pENS and pPALMC1 plasmids. IPTG induction (1μM, 4h) is indicated with (+/-). (B) Western blot with anti-6xHis-tag performed against pPALMC1-transformed *E. coli* WT BW25113, and chaperone knockout Keio strains. Figure made in Biorender. (A-B) The bottom blot indicates total loading control stained with Ponceau S. (C) Schematic representation of potential chaperone protein interactions for the translocation and folding of BrkA. Chaperones with dashed borders represent Keio mutant strains.

Chaperone proteins play a role in the translocation and autoproteolysis of BrkA. To investigate whether chaperones are necessary for the expression of uncleaved and cleaved BrkA, a panel of six chaperone proteins was identified: FkpA, DsbA, DegP, SurA, Skp, and BamB. Keio single knockout strains of each chaperone were successfully transformed with pPALMC1 and all demonstrated BrkA expression identified by western blot (Fig. 1B). Untransformed and pPALMC1 transformed BW25113 WT were used as negative and positive controls, respectively. The *ΔdegP* and *Δskp* strains exhibited similar levels of cleaved and uncleaved protein to WT expression patterns (Fig. 1B). In contrast, *ΔfkpA*, *ΔdsbA,* and *ΔsurA* showed a darker uncleaved BrkA band (Fig. 1B). For *ΔdegP,* the band corresponding to cleaved BrkA was very faint. However, for *ΔbamB,* despite WT levels of uncleaved BrkA expression, no band corresponding to the cleaved BrkA product was detected (Fig. 1B).

Knockout of BamB, Skp, and DegP exposes uncleaved BrkA to proteolytic trypsin degradation. The role of chaperone proteins on the surface expression of BrkA was investigated using a trypsin accessibility assay. Intact cell pellets corresponding to pPALMC1-transformed WT and knockout BW25113 strains were treated with trypsin, alongside PBS treatment as a negative control. Because the outer membrane excludes trypsin, only surface-presented proteins will be accessible to trypsin digestion. The 6xHis-tagged BrkA in each Keio strain was visualized with SDS-PAGE and western blotting (Fig. 2).

The negative PBS-treated BW25113 control showed bands at 103 and 73kDa, representing uncleaved and cleaved forms of BrkA respectively (Fig. 2). Following trypsin treatment, the BW25113 strain did not show a 73kDa band, suggesting that it was digested by trypsin.

When comparing the panel of pPALMC1-transformed chaperone knockout strains to WT cells, *ΔfkpA, ΔdsbA,* and *ΔsurA* strains showed similar results. When untreated, all three knockouts showed a 103 and 73kDa BrkA band but lost the 73kDa band when trypsinized

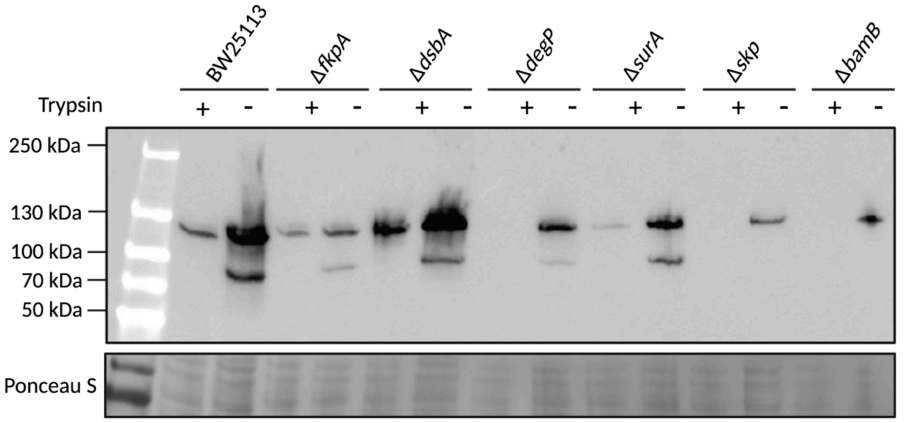


FIG. 2 Uncleaved BrkA is susceptible to trypsin in Δ*bamB*, Δ*skp* and Δ*degP* mutants. Western blot of pPALMC1-transformed *E. coli* BW25113 and chaperone knockout Keio strains treated with (+) and without (-) trypsin (240 µg/mL). Ponceau S protein loading control is shown below.

(Fig. 2). The *ΔdegP* mutant resulted in 73 and 103kDa bands when untreated with trypsin, both of which were lost upon trypsin digestion (Fig. 2). Similarly, un-trypsinized pPALMC1-transformed *Δskp* and *ΔbamB* showed a single 103kDa band which was lost upon trypsinization. These results suggest that the 103kDa uncleaved BrkA protein is accessible to trypsin in mutants lacking one of Skp, BamB, or DegP.

Deletion of BamB results in permeabilization of the *E. coli* membrane. To investigate whether the loss of the 103kDa band was due to membrane destabilization, an MIC assay for EDTA was performed. If membrane stability was compromised, a lower MIC was anticipated as EDTA permeabilizes the membrane (31). Cells were grown in doubling dilutions of EDTA alongside a positive growth control without EDTA.

The starting broad range of EDTA concentrations demonstrated MIC values of 5mM and 2.5mM for the untransformed WT and chaperone knockout strains, respectively (Table 2). *ΔbamB* failed to grow and was thus excluded. No pPALMC1-transformed strains grew within this range, reported as an MIC of 1.25mM (Table 2). To improve resolution between transformed strains, the experiment was repeated with EDTA concentrations of 2mM, 1mM, 0.5mM and 0.25mM (Tables 3, 4).

TABL. 2 MIC of EDTA is higher in WT *E. coli* than chaperone protein knockout strains, and is decreased by BrkA expression. 1x10^4 CFU of WT and chaperone knockout *E. coli* strains were grown overnight at 37ºC in a 100µL volume of LB. Media contained 100µg/mL ampicillin for pPALMC1 transformants. Cells were treated with EDTA in doubling dilutions from 10mM to 1.25mM. The lowest concentration of EDTA at which no growth was observed is reported as the MIC.

Genotype	EDTA MIC (mM)	
	Untransformed	Transformed
WT	5.0	1.25
ΔdegP	2.5	1.25
ΔdsbA	2.5	1.25
ΔsurA	2.5	1.25
Δskp	2.5	1.25
ΔfkpA	2.5	1.25

TABLE. 3MIC of EDTA in *E. coli* strains is decreased by BrkA expression, enhanced by chaperone knockout. As described in Table 1, WT and chaperone-deficient *E. coli* were assayed for MIC of EDTA using a lower concentration range of 2mM to 0.25mM. The lowest concentration of EDTA at which no growth was observed is reported as the MIC. Strains which grew at all concentrations are listed as MIC of >2mM and those which showed no growth are listed as *NG.*

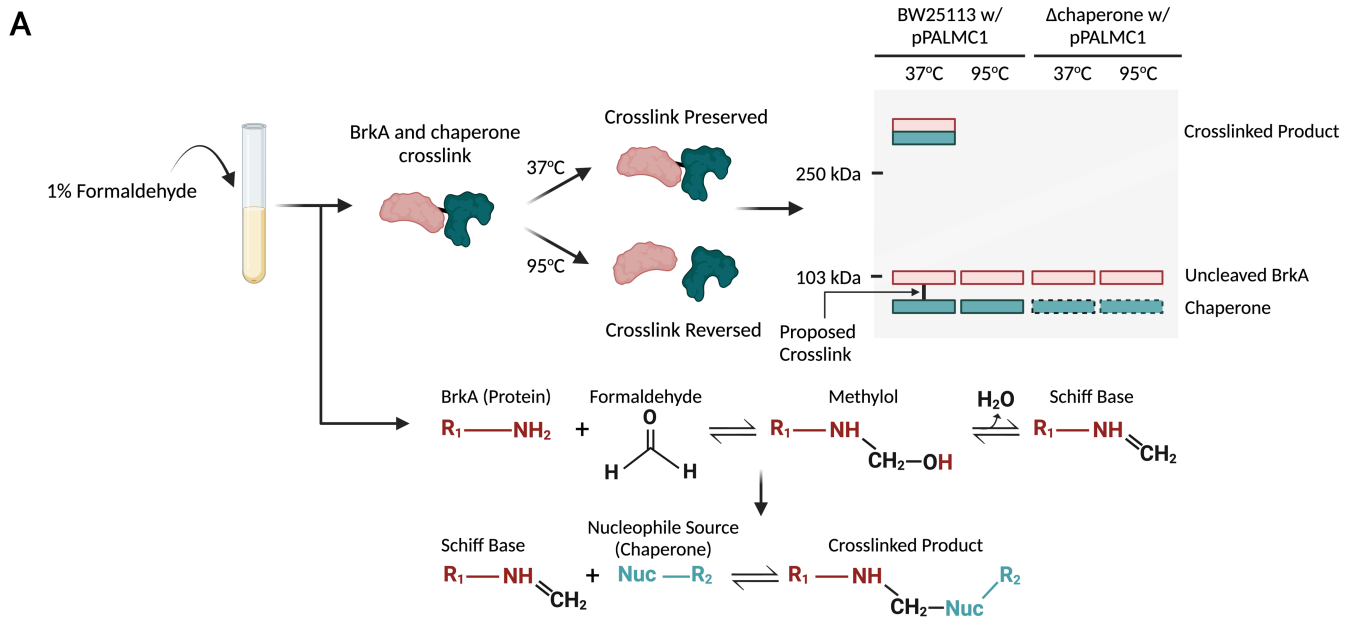
Genotype	EDTA MIC (mM)	
	Untransformed	Transformed
WT	>2.0	2.0
ΔdegP	>2.0	0.5
ΔfkpA	>2.0	0.5
ΔsurA	>2.0	1.0
ΔbamB	1.0	*NG.*

TABLE. 4 MIC of EDTA in *E. coli* is decreased by BrkA expression. Experimental setup described in Table 2 was repeated for the *E. coli* chaperone knockouts *Δskp* and *ΔdsbA*. The lowest concentration of EDTA at which no growth was observed is reported as the MIC. Strains which grew at all concentrations are listed as MIC of >2mM and those which showed no growth are listed as *NG*.

Genotype	EDTA MIC (mM)	
	Untransformed	Transformed
WT	>2.0	1.0
Δskp	>2.0	0.25
ΔdsbA	>2.0	0.25

Reflecting the MIC values obtained in the higher concentration assay, all untransformed strains except *ΔbamB* grew at all concentrations of EDTA, reported as MIC >2mM (Tables 3, 4). In contrast *ΔbamB* showed an MIC of 1mM EDTA. Transformation with pPALMC1 resulted in increased susceptibility to EDTA in all strains (Tables 3, 4). The most severe increase in susceptibility was again observed in *ΔbamB* cells, which showed no growth even without EDTA added. Transformed *Δskp* and *ΔdsbA* showed an MIC of 0.25mM (Table 4). *ΔdegP* and *ΔfkpA* showed an MIC of 0.5mM, and *ΔsurA* had the highest MIC of all transformed knockouts at 1mM (Table 3). These data indicate that the knockout of chaperone proteins – in particular, BamB – resulted in increased membrane permeability, which increased further upon transformation with pPALMC1.

BamB and FkpA chaperones may directly interact with BrkA. To investigate direct interactions between BrkA and the chaperone proteins, a formaldehyde crosslinking experiment was conducted. Crosslinked pPALMC1-transformed BW25113 WT cells retained the 103 and 73kDa bands, and gained two additional bands with molecular weights greater than 250kDa (Fig. 3B). When boiled at 95°C both bands, which moving forward will be referred to as the "heavy" and "light" bands, were no longer detected (Fig. 3B). Absence of these bands in untransformed BW25113 WT cells and across all boiled lysates suggested they may be crosslinked complexes containing BrkA. Both heavy and light bands were present in treated lysates of *ΔdsbA*, *ΔdegP*, *ΔsurA*, and *Δskp.* (Fig. 3B). Crosslinked *ΔbamB* samples exhibited a complete loss of signal from the "heavy" crosslinked band. Crosslinked *ΔfkpA* also showed a partial loss of signal in the "light" band and a complete loss of signal from the middle band. These data suggest that BrkA interacts directly with two proteins and that deletion of *fkpA* or *bamB* results in a loss of these interactions.



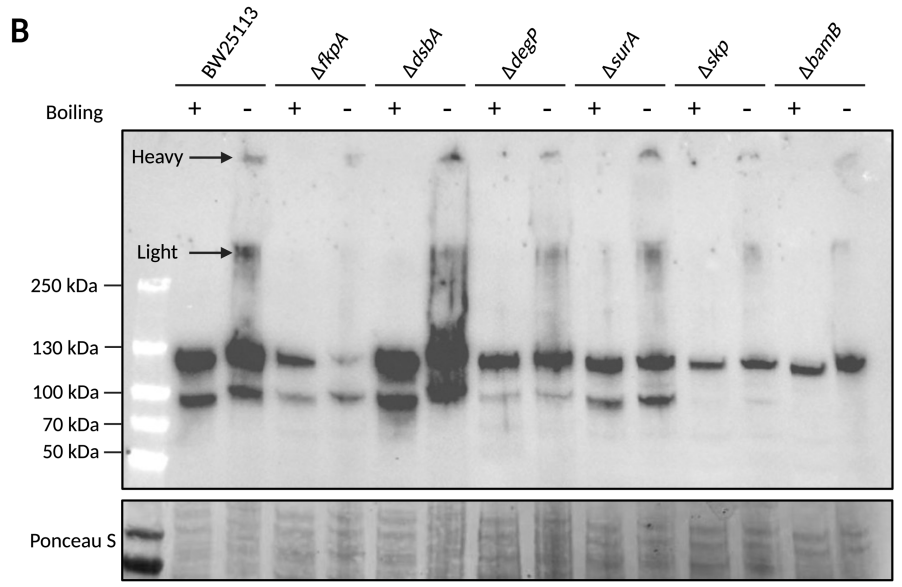


FIG. 3 BrkA-containing crosslinked complexes are absent in Δ*bamB* and Δ*fkpA*. (A) Schematic representation of formaldehyde crosslinking experiment and expected results. In the presence of a crosslinked product, no band will be shown in both the boiled and unboiled conditions. (B) Western blot of pPALMC1-transformed *E. coli* BW25113 and chaperone knockout Keio strains treated with formaldehyde and heated at 95°C or 37°C. "Heavy" and "Light" bands correspond to protein complexes of higher and lower molecular weight. Ponceau S protein loading control is shown below.

DISCUSSION

This study sought to explore the role of six chaperone proteins in the secretion process of autotransporter BrkA. Previous studies have individually investigated the necessity of DegP, FkpA, and DsbA; however, these led to inconclusive results (29, 33-35). Here, a novel investigation into how six different chaperones individually contribute to the folding and surface presentation of BrkA is presented. Specifically, the potential role of chaperones FkpA, DsbA, DegP, SurA, Skp, and BamB in the translocation, folding, and autoproteolysis of BrkA was studied.

To validate expression of BrkA in K-12 *E. coli* BW25113, two BrkA-expressing plasmid constructs were used to transform BW25113. Only BW25113 transformed with pPALMC1 resulted in highly detectable bands of the expected 103kDa and 73kDa proteins (Fig. 1A). The remaining lanes produced faint bands of the uncleaved protein but the 73 kDa cleaved product could not be detected. Traces of the uncleaved protein may be a result of non-specific binding to proteins of similar molecular weight since only the pPALMC1-transformed resulted in thick distinct bands. Thus, cleaved and uncleaved BrkA can be expressed in *E. coli* BW25113 following pPALMC1 transformation and all subsequent experiments were performed using pPALMC1. BrkA band intensities remained consistent between IPTG-treated and untreated samples, suggesting that while the *lac* operon is upstream, transcription of brkA in pPALMC1 is not under lac regulation (Fig. 1A). This is consistent with previous reported uses of pPALMC1 (37).

Once BrkA expression was validated in WT, a panel of chaperone knockout strains were transformed with pPALMC1 and their ability to express cleaved and uncleaved BrkA was characterized. All mutant strains except for the *ΔbamB* cells demonstrated a WT BrkA expression phenotype, suggesting they may not be essential in BrkA folding, translocation, or autoproteolysis (Fig. 1B). The UniProt structure of BrkA suggests it does not form disulphide bonds, further supporting the notion that DsbA is not necessary for BrkA folding and function (38). In the context of FkpA, DegP, SurA, and Skp, this observation may be explained by functional redundancy of folding activity as observed in prior literature (27, 39).

ΔbamB, on the other hand, did not show the 73kDa band indicative of BrkA cleavage (Fig. 1B). As part of the BAM complex, BamB has been implicated in the surface expression of other β-barrel OMPs including OmpA, OmpF, and LamB (40, 41). The data therefore

demonstrate that the BAM complex, including BamB, may be necessary for successful BrkA autoproteolysis.

To investigate whether BamB or other chaperones were necessary for successful BrkA translocation across the outer membrane, a trypsin accessibility assay was performed. Only secreted proteins should be accessible to trypsin digestion. In WT transformants trypsin digestion resulted in the loss of the cleaved 73kDa band while the uncleaved 103kDa band was still present (Fig. 2). This suggests that uncleaved BrkA was protected from trypsin by intracellular localization. This WT phenotype was also displayed in *Δfkp, ΔdsbA*, and *ΔsurA* cells, supporting the above inference that they are not individually necessary for BrkA secretion (Fig. 2).

On the other hand, the trypsin-treated *ΔbamB* mutant lost the 103kDa band, suggesting that the uncleaved BrkA protein was accessible to trypsin (Fig. 2). It is possible that the loss of BamB results in reduced throughput of the BAM complex, and hence membrane destabilization caused by periplasmic protein accumulation (40). This could in turn permit trypsin access to the periplasmic space, leading to the digestion of the 103kDa protein. This destabilization hypothesis is supported by the high sensitivity to EDTA demonstrated by *ΔbamB* cells (Table 3). Together, the data supports that BamB deletion disrupts the outer membrane and periplasmic contents.

We observed similar trypsinization phenotypes in the *ΔdegP* and *Δskp* mutants, but no distinct corresponding increase in EDTA sensitivity (Fig. 2; Table 3). From this, we postulate that the loss of DegP or Skp may weaken but not permeabilize the membrane. Instead, the trypsinization of the intracellular 103kDa protein might be attributed to mechanical damage to this weakened membrane during the trypsin protocol, though this hypothesis requires further study. Additional tests for membrane permeability could be performed, such as flow cytometry run on cells treated with propidium iodide.

Taken together, the above findings support the necessity of BamB in BrkA expression. However, they do not discern a distinct role of any other chaperone protein, an issue that we hypothesized might be due to functional redundancy. Additionally, loss of one chaperone may impact the translocation, folding, and expression of other chaperone proteins. This limits our ability to draw a causal link between the knockout phenotypes and the chaperone function. Thus, a crosslinking experiment was performed to identify direct chaperone-BrkA interactions. When crosslinking was performed in pPALMC1-transformed BW25113 WT, two crosslinked protein bands appeared upstream of BrkA (Fig. 3B). When performed on transformed chaperone knockout strains, the “light” crosslink band was not present in the *ΔfkpA* mutant while the “heavy” band was lost in the *ΔbamB* mutant, suggesting BamB may directly interact with BrkA. FkpA has a molecular mass of 29kDa and often functions as a dimer, suggesting a total weight of 161kDa when crosslinked with uncleaved BrkA (42). The BAM complex (including BamB) has a molecular mass of 200kDa, suggesting a crosslinked mass of 303kDa (43). These masses may correspond to the two bands observed, although exact mass interpretation is hampered by an inaccurate protein ladder with a range that does not exceed 250kDa. Another consideration could be that BamB may be acting independently of the BAM complex, suggesting an additional function of BamB that has not yet been explored. However, this possibility requires further investigation as the identity of these bands cannot be directly concluded without performing direct staining for BamB and FkpA or conducting a co-immunoprecipitation experiment.

Limitations and Future Directions This study represents a broad investigation into how different chaperone proteins contribute to the proper folding and surface expression of BrkA. We investigated the morphology, growth rate and carrying capacity, membrane integrity, and BrkA expression patterns of six different chaperone-deficient *E. coli* cells. Given the broad scope covered, many findings still require validation and further investigation.

Due to time and resource constraints, the knockout genotype of Keio strains used was not verified using conventional PCR gels meaning the chaperone protein knockout was not confirmed. In order to validate the correct insertion site of the kanamycin cassette, future work needs to validate each of the strains. Additionally, all western blots were based on a single biological replicate, which may be a source of error as the comparison of BrkA expression patterns across experiments showed inconsistencies in the detection of cleaved

BrkA products. Therefore, future western blots should be repeated as they are currently based on a single biological. Notably, *Δskp* showed a 73kDa cleaved product in the western blot, but not in the control conditions of subsequent trypsin accessibility and crosslinking blots. As a result, our findings should be reproduced by other teams before moving forward.

Additionally, while trypsin accessibility offers some insights into the surface expression status of BrkA, it offers little insight into the 3D conformation of the protein. Because proper folding is necessary for protein function, future studies should investigate how chaperones contribute to BrkA native conformation using spectroscopic methods, limited proteolysis assays, or native gel electrophoresis.

This project attempted to establish direct chaperone-BrkA contact using nonspecific formaldehyde crosslinking, where the absence of a band in knockout strains suggests a loss of interaction. In the future, teams should further optimize formaldehyde crosslinking conditions to better capture these interactions. Additionally, the gradient gel used was not optimal for the range of molecular weights generated after crosslinking, due to the anticipated high molecular weight associated with protein-protein crosslinking. Future assays may benefit from lower percentage gels with a protein ladder that spans higher molecular weights. If crosslinking conditions are optimized, affinity purification and mass spectrometry can be used to confirm the identity of the BrkA interactors in a high throughput manner.

Finally, this study does not address the possibility of functional redundancy existing across chaperone proteins. As a result, exploring BrkA expression in *E. coli* strains deficient in multiple chaperones may be required, which can be generated using plasmid-encoded antisense RNAs expressed in a Keio knockout background to allow controllable expression.

Conclusions In this study, the role of FkpA, DsbA, SurA, Skp, DegP, and BamB chaperone proteins in the translocation and folding of autotransporter BrkA was explored. Using the pPALMC1 plasmid, BrkA surface expression in *E. coli* BW25113 and Keio chaperone knockout strains was characterized. The results demonstrated that BamB was necessary for the expression of cleaved, surface-expressed BrkA. To characterize translocation differences in our mutant strains, the deletion of BamB was found to permeabilize the outer membrane. Finally, formaldehyde crosslinking suggested that direct interactions may occur between BamB and BrkA.

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CONTRIBUTIONS

FB, JC, IP, and KV contributed equally to the overall project and should be considered equal co-authors of the manuscript. FB carried out final transformations with pENS, pPALMC1, and pBAD. FB carried out optimization and final iterations of Fig1A and Fig1B SDS-PAGE/western blot, and Fig3A crosslinking SDS-PAGE/western blot. JC attempted the Keio knockout-verification via PCR. JC also carried out the trypsin accessibility protocol design and experimentation. IP investigated the effect of chaperone knockout and BrkA expression on membrane permeability, the composition of cross-streak analyses and the EDTA permeability assay. IP also performed data analysis for the cross-streak assay (not shown) and fitting of growth curves to the logarithmic growth equation. KV performed the growth curve analysis for untransformed and transformed strains with pPALMC1 and pENS, imaged the colony morphology of all strains, and finalized all figures for the manuscript. Optimization of strain transformation, SDS-PAGE, general lab upkeep, reagent preparation, and western blotting was equally contributed to by all members of the team. Each member wrote their own methods and results section corresponding to their main project. All authors contributed equally to the writing of abstract, introduction, and discussion, and provided feedback throughout the process. All members contributed to the final edit and review.

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