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The DsbA Chaperone Protein May Be Necessary for Outer Membrane Secretion of BrkA

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SUMMARY Gram-negative bacteria utilize specialized secretion systems to transport proteins across their outer membrane and into the extracellular environment. Among these systems, autotransporter proteins, like BrkA in *Bordetella pertussis*, autonomously localize to the bacterial cell surface, increasing virulence and inhibiting bactericidal immune responses from hosts. While periplasmic chaperone proteins have been shown to be involved in autotransporter biogenesis, not all their specific roles in BrkA secretion, as it transits through the periplasm, are well understood. DsbA and SurA are two chaperone proteins found in the periplasm of Gram-negative bacteria. SurA has been previously implicated in the secretion of BrkA, while DsbA does not interact directly with BrkA, it's interactions with SurA and whether these aid in the secretion are unknown. We aimed to explore whether DsbA affects BrkA surface expression in *Escherichia coli* through loss-of-function experiments. Our results suggest that DsbA may play a role in BrkA secretion. The experimental outcomes indicate a possible role for DsbA in facilitating BrkA secretion, implicating a wider involvement of periplasmic factors in the complex orchestration of BrkA biogenesis and secretion.

INTRODUCTION

Gram-negative bacteria can utilize secretion systems to facilitate the transport of essential proteins and virulence factors to their outer membrane (OM) and into the extracellular space. One highly specialized method is the type V secretion system, where outer membrane proteins can autonomously localize to the surface of the bacteria (1). These secretion systems can also be referred to as autotransporters, as they are commonly expressed as a single polypeptide sequence. The domains of an autotransporter include an N-terminal signal sequence for translocation across the inner membrane, an N-terminal passenger domain (often responsible for the protein's activity), and a C-terminal β-barrel domain facilitating translocation across the OM (2, 3).

BrkA is a classical (Type Va) autotransporter found in *Bordetella pertussis*, the pathogenic bacterium responsible for whooping cough (4, 5). BrkA has an initial protein size of 103kDa before it is cleaved into 73kDa alpha passenger and 30kDa beta barrel functional domains which serve to enhance serum resistance, by inhibiting the bactericidal activity of complement, as well improving the adhesion of the bacterium to host cells (6). Within BrkA's alpha passenger domain, there is a conserved junction region (Glu601–Ala692) that contains two important subdomains (6). The first, called the hydrophobic secretion facilitator (HSF), is required for the secretion of the folding passenger domain (7, 8). The second is the autochaperone domain, who promotes passenger folding as it emerges from the cell (9). After secretion, BrkA is cleaved into its functional alpha and beta domains. The mechanism by which cleavage occurs remains unclear, however it has been suggested that this can be

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mediated by a general cell-surface protease or an autoproteolytic domain within BrkA (7). The BrkA passenger must make it through the periplasmic space in an unfolded or partially folded conformation (10, 11). As it translocates, periplasmic factors such as chaperone proteins have been shown to play a role in this process in other autotransporters (10). However, the interactions between periplasmic chaperone proteins and BrkA during its translocation through the periplasm have yet to be elucidated.

To uncover the role of periplasmic chaperone proteins in the successful secretion of BrkA, we aimed to investigate the role of the chaperone protein DsbA and its effect on successful BrkA surface expression. DsbA functions as a periplasmic chaperone protein by catalyzing the oxidation of disulphide bonds (12). It is a 21-kDa soluble protein and studied to be a primary source of disulphide bond formation in secreted proteins. Disulphide bonds are formed between the Cys-X-X-Cys motif, where X is representative of any amino acid. DsbA catalyzes a thiol-disulfide exchange (13). Previous studies have shown that this catalyzation of disulphide bond formation in classical autotransporters (e.g. Intimin), promotes the autotransporter's stability and, thus, secretion to the outer membrane (14). Interestingly, the amino acid sequence of BrkA only contains a single cysteine residue, therefore DsbA will not interact with BrkA through the formation of disulphide bonds. However, DsbA has been shown to have chaperone-like activity independent from its oxidoreductase activity, potentially due to its ability to bind peptides (15). It has been suggested that DsbA is responsible for the proper folding of various periplasmic chaperones such as PapD, MrpD, and PmfD via chaperone-like activity and disulphide bond formation (16). It has also been shown to be required for the secretion of Pertussis toxin by *Bordetella pertussis* (17). A previous literature review by *Denoncin et al.* also acknowledges the lack of understanding of DsbA's cooperation with other important periplasmic chaperones such as SurA and SkpA (18). Since DsbA's cooperation with SurA and other periplasmic chaperones is not well elucidated for the assembly of OM proteins, it could be predicted that the absence of DsbA may disrupt SurA's functional efficiency to release proteins for OM secretion. This may hinder the passenger stabilization of BrkA within the periplasm and decrease its surface expression. Due to the necessity of SurA and other periplasmic chaperones in BrkA secretion, investigating this gap may identify the significance of DsbA within the BrkA secretion pathway (7, 19).

Therefore, we hypothesized that DsbA is necessary for the OM secretion of BrkA. We aimed to test this hypothesis by using *E. coli* as a model and comparing the outer membrane secretion of BrkA in *E. coli* WT K-12 (BW25113) and DsbA knockout (JW3832**)** *in vivo* to observe the effect DsbA may have on BrkA surface expression. We found that DsbA may be necessary for BrkA secretion due to differing levels of BrkA expression following a trypsin time series availability assay analysis.

METHODS AND MATERIALS

Bacterial strains DH5α *E. coli* containing construct pDO6935, encoding BrkA and an ampicillin (Amp) resistance marker, was provided by Dr. David Oliver (9) from the University of British Columbia (UBC) and grown on LB agar plates containing ampicillin (100 µg/mL). The wildtype BW25113 parent *E. coli* strain and the *dsbA* knockout strain, JW3832, were obtained by the Microbiology and Immunology Department at UBC from the Keio collection (20) and grown on LB agar plates and LB plates with kanamycin (Kan) at 50 µg/mL, respectively.

Plasmid Extractions Plasmid DNA of bacterial strains, pDO6935 and the later designed pDO6935-6xHisTag constructs, were extracted using manufacturer-provided protocols in the BioBasic EZ-10 Spin Column Plasmid DNA Miniprep Kit (Cat. # BS413, BS414, BS614).

Preparation of competent DH5α, BW25113, JW3832 *E. coli* cells. DH5α, BW25113, and JW3832 *E. coli* strains were made competent utilizing the protocol described by *Chang et al.* (21). Cells stocks were stored at -70ºC.

Insertion of Hexa-histidine Tag into pDO6935. Primers were designed to insert a 6xHisTag into the BrkA sequence in pDO6935 at position Lysine108 with a HindIII cut site for

downstream screening. The primers were designed using NEBaseChanger (https://nebasechangerv1.neb.com/). The forward primer, Q5SDM_10/15/2023_F and the reverse primer, Q5SDM_10/15/2023_R were designed with two histidines and a HindIII cut site on the forward primer and four histidines on the reverse (Table S1.). The primers were obtained from Integrated DNA Technologies (IDT). pDO6935 was used as template DNA and was extracted from DH5α using the manufacturer-provided protocols in the BioBasic EZ-10 Spin Column DNA Miniprep Kit (Cat.#: ST82316).

Utilizing the protocol outlined within the Q5 site-directed Mutagenesis kit (New England Bio Labs, E0554), we constructed a potential 6xHisTag insertion into the pDO6935 plasmid and later transformed into target replicate cells. Two replicates were sent for whole plasmid sequencing by Plasmidsaurus.

Transformation of competent DH5α, BW25113, JW3832, C2987 *E. coli* cells. The transformation protocol was adapted from *Chang et al.* (21) and the pDO6935-6xHisTag construct was transformed into NEB 5-alpha Competent *E. coli* cells (NEB #C2987). Competent cells were thawed and 5µL of NEB KLD Enzyme Mix (NEB # M0554S) was added and gently mixed. The mixture was incubated on ice for 30 minutes, heat shocked at 42ºC for 30 seconds and placed on ice for 5 minutes. 950 µL of NEB Super Optimal broth with Catabolite repression Outgrowth Media (NEB #B9020S) was added and was incubated while shaking at 37ºC for 60 minutes. The cells were then spun down at 1000 rpm for 5 minutes. The pellet was resuspended in 100 µL of room temperature SOC media and plated onto LB ampicillin plates (100 µg/mL).

DH5α was used to create viable replicates of the pDO6935-6xHisTag (pENS) construct provided by *Goh et al.* BW25113 (WT) and JW3832 (KO) were also transformed with pENS using an adapted protocol by *Chang et al.* to obtain WT-pENS and KO-pENS strains (21, 22). These mixtures were incubated on ice for 30 minutes, and heat shocked at 42 ºC for 30 seconds before returning to ice for 2 minutes. 1 mL of warm liquid LB was added to each tube and incubated on a shaking incubator for 1 hour before plating on LB plates with the appropriate selection antibiotics.

Restriction Digest and Sanger Sequencing. Plasmids were extracted using the BioBasic EZ-10 Spin Column Plasmid DNA Miniprep Kit (Cat. # BS413, BS414, BS614). HindIII restriction digest was conducted following the New England BioLabs protocol, with heat inactivation at 80 ºC for 20 minutes (23). Gel electrophoresis was performed with a 1% agarose gel and was run for 49 minutes at 120V. Selected colonies were sent to GENEWIZ/Azenta Life Sciences (Seattle, WA) to perform Sanger sequencing using the 2seq primer designed by *Chen et al*. (24). (5'-GCATGGCTCGTGCCTGAT – 3'). Preparation for Sanger sequencing was done according to GENEWIZ sample submission guidelines.

PCR and gel electrophoresis to confirm strain genotype. Internal and external primers were designed inside the kanamycin resistance cassette and within the downstream gene *yihF* of JW3832, respectively on Snapgene Viewer. Primers were obtained from Integrated DNA Technologies (Table S2). Primer F_K1 was designed by Narita and Peng (25). Overnight cultures of *E. coli* KO JW3832 and WT BW25113 were grown in 5 mL of liquid LB media with and without 50ug/mL kanamycin respectively. Genomic DNA was isolated from overnight cultures with Bio Basic™ One-4-All genomic DNA Mini-Preps Kit (cat no. BS88504). The purity and concentration of DNA were assessed using a NanoDrop 2000c spectrophotometer. PCR amplification of the kanamycin cassette inserted in the *dsbA* gene region was conducted using a protocol adapted from Hay et al. (26). PCR reactions were performed using Platinum *Taq* DNA polymerase (Invitrogen #11304-011), following the manufacturer's protocol. The PCR reactions were then run on the Bio-Rad T100™ Thermal Cycler, which was set to 95°C for 1 minute and 45 seconds for an initial denaturation, followed by 30 cycles of 95°C for 25 seconds, 61°C for 40 seconds, 68°C for 55 seconds and a final extension at 68°C for 5 minutes. PCR products were loaded alongside an Invitrogen 1 Kb ladder on a 1.2% agarose gel with RedSafe™ Nucleic Acid Staining Solution (FroggaBio #21141) in 1x TAE buffer. The gel was run at 110V for 40 minutes and visualized using a ChemiDoc™ MP Imaging System from Bio-Rad.

Trypsin Time Series Availability Assay. Overnight cultures of wildtype K-12 with pDO6935, wildtype K-12 cells with pENS (WT-pENS) and K-12 *dsbA* knockout cells (Keio Collection # JW3832) with pENS (Δ*dsbA*-pENS) were grown. Samples were normalized to an optical density at 600 nm (OD_{600}) of 10 in 200 µl of phosphate-buffered saline. The WT-pENS and Δ*dsbA*-pENS samples were split in half and 2 µl of 10 mg/ml bovine trypsin was added to 100 µl of each to yield a final trypsin concentration of 200 µg/ml. Samples were all incubated at 37°C. At 0, 5, 10, 20 and 60 minutes, 10 µl of each sample was taken out and immediately added to a 2x Laemmli sample buffer (BIO-RAD #161-0737) with 5% β-mercaptoethanol and then heated at 97°C for 5 minutes to stop the trypsin digestion. Samples were then centrifuged at 4,000 rpm for 4 minutes and put in the -20°C freezer. Once all samples had been collected, they were taken out of the freezer and SDS-PAGE was run using Mini-PROTEAN TGX Stain-Free Precast Gels (BIO-RAD # 456-8036) in the Mini-PROTEAN Tetra Cell apparatus (BIO-RAD #1658004EDU) following the manufacturer's protocol. PageRulerTM plus Prestained Protein 10 to 250 kDa Ladder (ThermoFisher #26619) and Precision Plus ProteinTM Unstained Protein Standards (BIO-RAD #1610363) were used with the ChiC protein (27), wildtype K-12 with pDO6935 and all time points from the WT-pENS and Δ*dsbA*-pENS samples. Images of the gel were then taken with the ChemiDoc MP Imaging system to visualize the gels.

Western. The BIO-RAD Trans-Blot Turbo Transfer System was used to transfer protein from SDS-PAGE onto Trans-Blot Turbo Mini 0.2 µm Nitrocellulose membranes (BIO-RAD #1704158) following the manufacturer's protocol. Membranes were blocked using 3% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Cat#161-0782) or 5% skim milk powder in 15 ml 1X Tris-Buffered Saline with 0.1% Tween®20 (TBS-T) for 1 hour at room temperature or overnight at 4°C on the orbital shaker. They were then washed 3 times with TBS-T. Next, they were incubated on the orbital shaker for 1 hour at room temperature or overnight at 4°C with the primary antibody, either 6x-His Tag monoclonal antibody (Invitrogen #MA1-21315) diluted to 1:1,000 or 1:2,000 in 15 ml of the 2.5% skim milk powder or 3% BSA or His-probe AD1.1.10 (Santa Cruz Biotech #SC_53073) diluted to 1:300 or 1:1200 in 2.5% skim milk powder. They were then washed 4 times in TBS-T and then incubated for 1 hour at room temperature with Goat anti-Mouse IgG (H+L) Secondary Antibody HRP Conjugate (Invitrogen cat # 31430), diluted to 1:100,000 in 2.5% skim milk powder. Membranes were then washed 3 times in TBS-T and incubated in 7 mL of Clarity Western ECL substrate reagents (BioRad, #170-5060) for 5 minutes. Images of the gel were then taken with the ChemiDoc MP Imaging system to visualize the membranes.

RESULTS

Unsuccessful Q5 Insertion of hexa-histidine tag but the HindIII restriction cut site was inserted correctly. To visualize the secretion of BrkA, a Q5 site-directed Mutagenesis kit (New England Bio Labs, E0554) was used to insert a hexahistidine tag into the BrkA found in pDO6935. 30 colonies appeared to be successfully transformed. However, following whole plasmid sequencing, the two analyzed replicates did not contain a hexahistidine tag at the anticipated Lysine108 insertion site. An additional Western blot screen for the 6xHisTag of 11 colonies was also unsuccessful. Previous literature indicated that a deletion of amino acid residues located at A52 to P600 does not impair BrkA's translocation and surface expression (6), therefore, theoretically, there are likely no issues with the desired location of insertion. However, following the initial screening, we performed a restriction digest of 11 separate colonies to identify if the HindIII restriction site was inserted correctly (Figure S1). Nine out of eleven colonies were successful in digestion and five samples were validated via Sanger sequencing. Interestingly, we identified a 6xHisTag in colony 7 to be found at the correct location but in the reverse 3' to 5' reading frame instead of the expected 5' to 3'. Because no BrkA would be expressed in the reverse reading frame, our insertion of the histidine tag was unsuccessful. With further analysis of the initial primer design, we found that the primers were designed against the complementary strand instead of the template strand. We believe this to be the cause of the unsuccessful insertion. All downstream applications utilizing a pDO6935-6xHisTag transformed into our model cells were obtained with a construct from

Goh et al. Δ*dsbA* JW3832 strains transformed with the plasmid from *Goh et al.* were named Δ*dsbA*-pENS and WT strains were named WT-pENS.

***E. coli* strains BW25113 and Δ*dsba* JW3832 carry the expected genotypes.** To confirm the genotype of our Δ*dsbA* JW3832 and wildtype (WT) BW25113 *E. coli*, we needed to establish the presence of a kanamycin resistance cassette in place of the *dsbA* gene of BW25113. To do this, we designed two forward primers on the kanamycin cassette (F_ProposalKan and F_K1) and one reverse primer (R_new_*yihF*) downstream in the *yihF* gene (Figure 1A). After extracting the genomic DNA from our ΔdsbA JW3832 and WT cells, we used these primers to amplify the kanamycin resistance cassette and the surrounding downstream gene region with PCR (Figure 1B). As a positive control, we amplified pUC-19 with its corresponding pUC19-193F and pUC19-355R primers and got an expected band at approximately 200 Bp (Figure 1B, (+) control). For our negative control, we ran distilled water in place of genomic DNA with our primer sets (Figure 1B, (-) control). No amplification was seen. As expected, the WT BW25113 showed no amplification with both primer sets (Figure 1B, *dsbA* WT). In contrast, the Δ*dsbA* JW3832 showed a band at approximately 1000 bp with our first forward primer (F_ProposalKan) and approximately 650 bp with our second forward primer (F_K1) when paired with our reverse primer (R_new_*yihF*) (Figure 1B, *dsbA* KO). This corresponds to our predicted expected sizes (Figure 1A) and establishes the presence of a kanamycin resistance cassette within the *dsbA* gene region of our Δ*dsbA* JW3832. Altogether, the results validate the expected genotypes. ‘

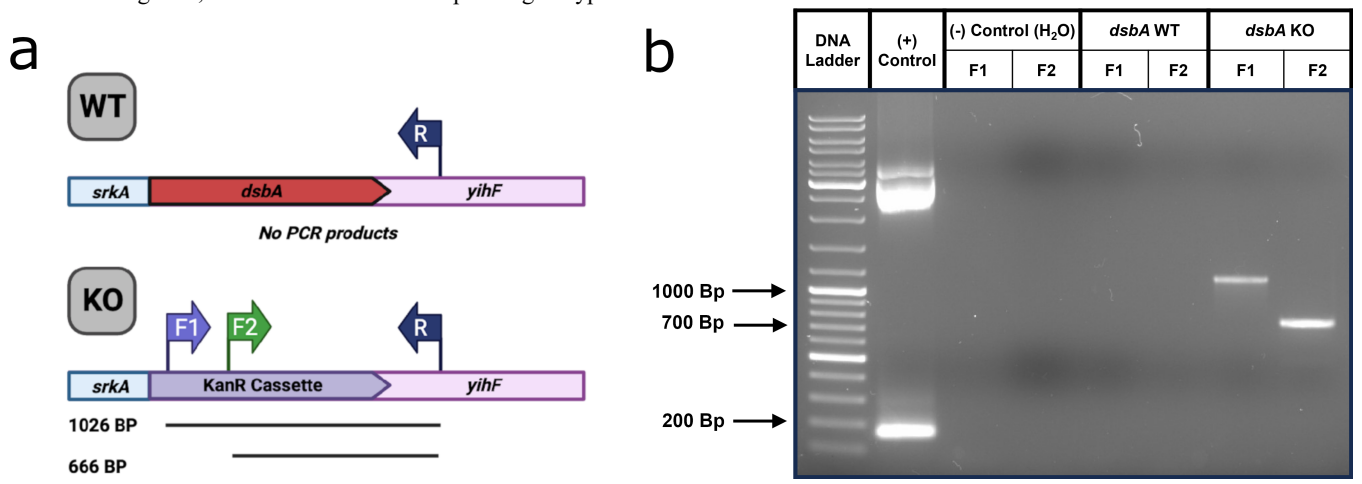

FIG. 1 DsbA Knockout Confirmation in both WT and KO Strains Utilizing PCR Amplification. (A) A representative diagram of the location of both our forward primers as F1 (F_ProposalKan) and F2 (F_K1) and our reverse primer as R (R_new_yihF) in the KanR cassette in the *dsbA* gene region of the genome of our KO and WT. The expected sizes of the PCR products from the primer pairings for the WT (no products) and KO (1026bp and 666bp) are shown. Created with BioRender.com. **(B)** PCR showing bands at approximately 1000 Bp and 650 Bp with our first and second forward primers respectively for our *dsbA* knockout, which was the expected size for amplification of the KanR cassette. No bands are seen with the WT. The positive control was pUC19 plasmids with their corresponding primers. The negative control was distilled water with our primer sets.

DsbA may be necessary for the secretion of BrkA. To determine the necessity of DsbA on the proper secretion of BrkA, we performed a trypsin accessibility assay using our Δ*dsbA* mutant to see if the passenger domain of BrkA was able to successfully translocate to the outer membrane, or if it was stuck in the periplasmic space. A band at 73 kDa would represent the cleaved passenger protein, which is thought to be secreted and therefore accessible to the trypsin, while a band at 103 kDa would represent the uncleaved passenger protein and is thought to be found in the periplasmic space and not accessible to the trypsin. Comparing these bands will help determine if BrkA is successfully secreted. After growing overnight cultures of Δ*dsbA*-pENS and WT-pENS and normalizing to an OD_{600} of 10, trypsin was added to each of the cultures. Samples were taken out at 0, 5, 10, 20 and 60 minutes to see how the trypsin was digesting the samples over time, with WT-pENS used to ensure that the trypsin was digesting as expected. The ChiC protein, previously isolated from pM3CRYY, a plasmid with a previously verified to contain a 6X Histidine tag, was used as a positive control to

ensure our antibodies were working (27). Wildtype *E. coli* containing the pENS parent plasmid pDO6935 (which lacks a 6xHis tag) was used as a negative control. Samples were then analyzed for BrkA expression and size using Western blot. Bands were visible at both 73 kDa and 103 kDa at the 5, 10 and 20-minute time points for Δ*dsbA*-pENS (Figure 2A). In contrast to WT-pENS, the bands for *dsbA*-pENS at 73 kDa did not change drastically over the time points in comparison to the band at 103 kDa. The results were then quantified with ImageJ and then graphed with GraphPad Prism software. The ratio of 73 kDa band intensity compared to the 103 kDa band intensity was calculated since this ratio gives a good indication of the deterioration of the 73 kDa band and takes into account the change compared to the 103 kDa band, which controls for loading differences in the sample amounts (Figure S2). The ratio of the 73 /103 kDa in Δ*dsbA*-pENS was found to increase over time, in contrast to the 73 /103 kDa ratio WT-pENS, which decreased over time (Figure 2B). The increase in the 73 /103 kDa ratio in Δ*dsbA*-pENSse indicates that the 73 kDa protein was not accessible to the trypsin which indicates that DsbA may be necessary for the secretion of BrkA.

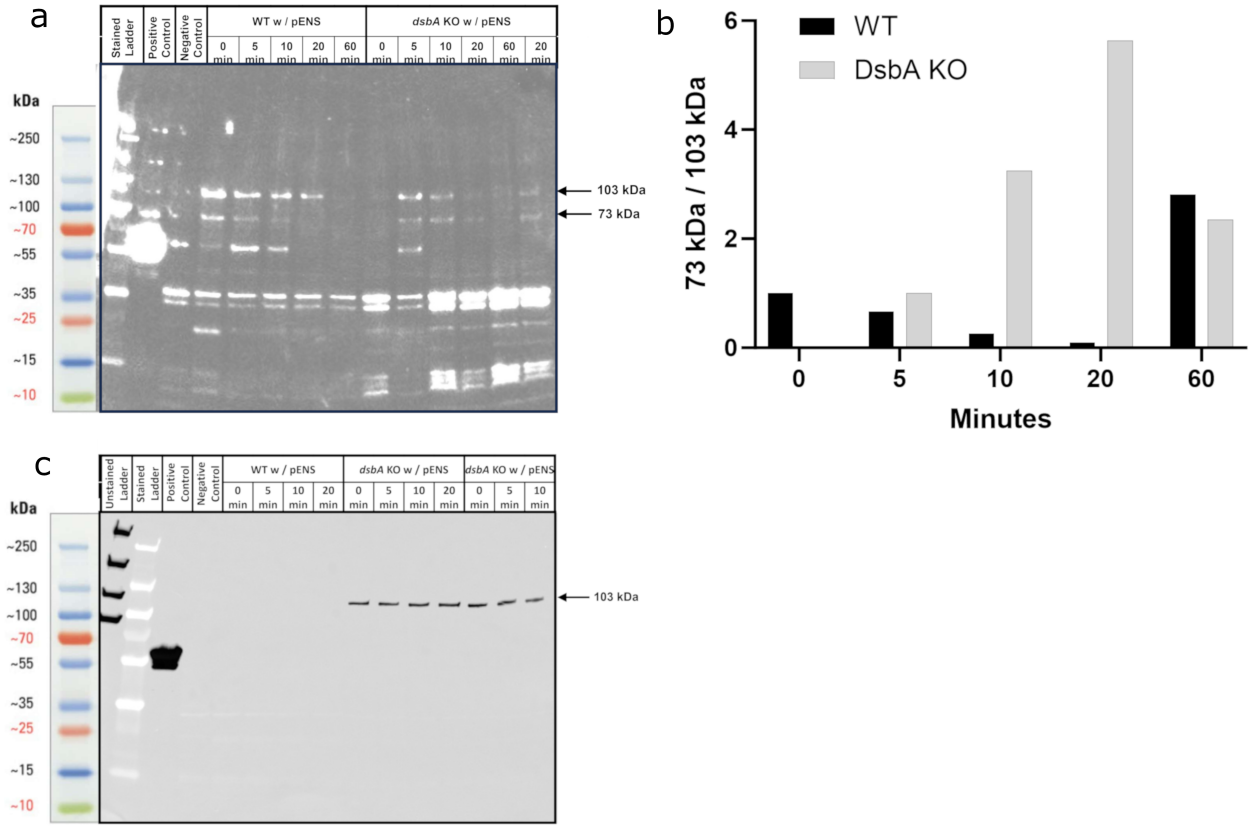

FIG. 2 DsbA may be necessary for the secretion of BrkA. (A) WT-pENS and Δ*dbsA*-pENS cells that were treated with 200 µg/ml of bovine trypsin for 0, 5, 10, 20 and 60 minutes and BrkA was subsequently detected via Western blot. Note: After loading all of the Δ*dbsA*-pENS time points, it was thought that there was less added to the 20 min lane, so a 2nd 20 min lane was added at the end. **(B)** Densitometry analysis of the Western blot image in (A) was performed using ImageJ to quantify the intensity of the bands. Changes in the ratio of the 73 kDa band intensity compared to the 103 kDa band intensity at 0, 5, 10, 20 and 60 minutes following a trypsin accessibility assay for WT-pENS and *ΔdbsA*-pENS cells (n=1). The 2nd 20 min *ΔdbsA*-pENS was used in the quantification. **(C)** WT-pENS and Δ*dbsA*-pENS cells that were treated with 200 µg/ml of bovine trypsin for 0, 5, 10 and 20 minutes and BrkA was subsequently detected via Western blot.

Due to a high level of background in the Western blot, we repeated the experiment. This time, however, we performed the trypsin availability assay at 0, 5, 10 and 20-minute time points. In this second experiment, we observed that BrkA was only detected at 103 kDa, indicating that the passenger protein was not cleaved or secreted (Figure 2C). These data, while unable to replicate the 73 kDa BrkA band observed in Figure 2B, still indicate that DsbA may be necessary for BrkA secretion because there was no protein accessible to the trypsin.

DISCUSSION

We set out to determine if DsbA is necessary for the secretion of BrkA. Our results suggested that DsbA may be important for successful BrkA surface expression as a loss of DsbA results in the reduced surface expression of BrkA. To account for the variable protein concentration between samples in our first Western blot, we normalized the band intensity of the 73 kDa cleaved passenger in relation to the 103 kDa uncleaved passenger-beta-barrel polypeptide within each condition to investigate the differences in passenger secretion (Figure 2B). Comparisons of the 73/103 kDa ratios between wildtype and *dsbA* knockout provided insight into the changes in BrkA passenger secretion due to the presence or absence of DsbA. The wildtype bacteria generally show a decrease in 73/103 kDa ratios with increasing exposure to trypsin digestion from 0 min to 20 min (Figure 2A), indicating more cleavage of the surface-expressed passenger domain with increasing exposure to trypsin. As time goes on, levels of the 73 kDa passenger decrease while the unsecreted 103 kDa protein within the periplasm remains protected from trypsin. The apparent increase of the 73/103 kDa ratio at the 60-minute time point is an artifact, as there is little to no presence of the 103 kDa band. This loss of the 103kDa band may be due to overexposure to trypsin resulting in trypsin entering the periplasm and digesting 103 kDa polypeptide. On the other hand, the *dsbA* knockout cells exhibit an increase in the 73/103 kDa ratio over time, indicating a higher amount of cleaved passenger protein compared to passenger-beta-barrel polypeptide (Figure 2B). The increased 73/103 kDa ratio suggests that BrkA passenger is being cleaved but does not reach the cell surface, therefore, avoiding digestion by trypsin. This seems to support our hypothesis that DsbA may directly or indirectly assist with BrkA surface expression. The second Western blot showed different results as there was no detectable 73 kDa band in our Δ*dsbA* mutants, but similar conclusions could be drawn, that DsbA is necessary for the processing (cleavage) and surface expression of the BrkA passenger. Additionally, in our second Western blot, our WT conditions did not have any BrkA expression, independent of trypsin presence. Since the second Western blot was done a week later using the same transformed cells as the first, there may have been a stress response in the cells due to the addition of pENS. This stress response may have caused mutations or changes in expression of the pENS plasmid in the wildtype cells, which may have impacted BrkA expression, resulting in no signal in the wildtype lanes of the Western blot (Figure 2C). DsbA may play a stimulating role that amplifies the proposed stress response which may explain why BrkA expression was not as affected in DsbA knockout cells. Plasmids transformed into cells can divert resources from the cell and interfere with growth resulting in mutations to plasmid as the cells divide (28). As the cells continue to divide the mutated cells can outcompete the cells with the initial transformation and can lower the expression of the plasmid. This may have resulted in mutations in WT-pENS that led to the failure of cells to produce BrkA.

Current Model (Figure 3A). One of the current models for wildtype BrkA secretion suggests that the HSF domain of BrkA interacts with putative periplasmic factors to inhibit the activity of the nearby autochaperone. The inhibition of the autochaperone may prevent premature folding and allow for the translocation of the unfolded or semi-folded passenger through the narrow beta-barrel and to the outer membrane. The protein might then be cleaved at the linker region by the proposed autoprotease within the beta-barrel.

Working Model #1 (Figure 3B). DsbA may interact with the HSF region of BrkA. By knocking out DsbA, the interaction at the HSF region may have been interrupted and the early folding of the passenger could have been initiated by the autochaperone. It is possible that BrkA begins translocation by inserting into the beta-barrel but is unable to fit through due to the bulkiness caused by the premature folding of the passenger. The passenger then remains in the periplasm but cleavage may still occur within the short segment that is inside the beta-barrel. This would explain the presence of 73 and 103 kDa bands despite the passenger not reaching the surface (Figure 2A).

Working Model #2 (Figure 3C). This model, similar to Working Model #1, proposes that DsbA is necessary for the processing of the BrkA protein. The main difference here is that cleavage of BrkA does not occur at the linker sequence and the BrkA polypeptide remains intact, which could explain the predominant presence of the 103 kDa protein but a lack of the 73 kDa protein.

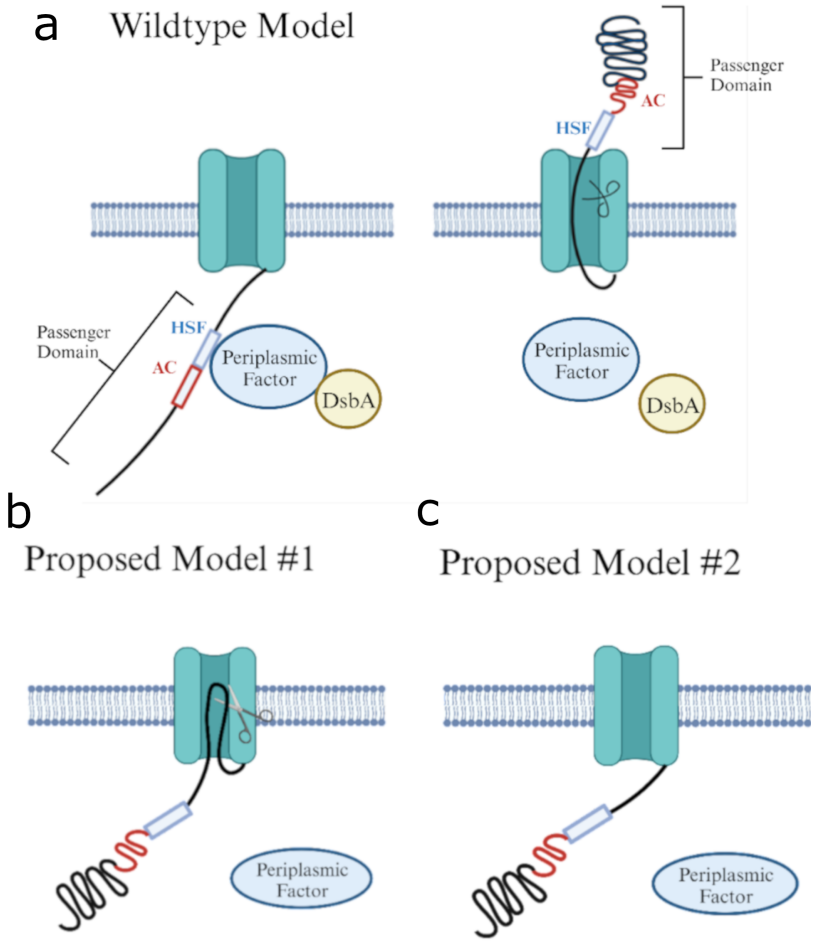

FIG. 3 Adaptation of the current BrkA secretion method and proposed models for BrkA secretion in the absence of DsbA. (A) We hypothesized that DsbA interacts with putative periplasmic factors which in turn bind to the hydrophobic secretion facilitator region (HSF) within the passenger domain. The association between HSF and periplasmic factors may inhibit folding induced by the juxtaposed autochaperone region (AC). The conservation of the unfolded or partially folded passenger in the periplasm may then allow translocation through the beta-barrel which results in BrkA surface expression. **(B)** In the absence of DsbA, periplasmic factors may not be able to be associated with the HSF region. In turn, premature folding of the BrkA passenger initiated by the autochaperone region may occur and inhibit full translocation. The possibility of partial translocation could account for proposed auto protease activity within the beta-barrel resulting in cleavage of the passenger without surface expression. **(C)** In the absence of DsbA, premature folding of the passenger may completely prevent translocation. BrkA polypeptide remains uncleaved either as a soluble protein in the periplasm or integrated into the OM via beta-barrel while the passenger remains in the periplasmic space. Created with BioRender.com.

Limitations Although our study suggests that DsbA may be necessary for the secretion of the BrkA passenger, a few important limitations should be addressed to verify the reproducibility of our results. In our first Western blot (Figure 2A), there was a significant amount of background, therefore, to visualize distinct bands, the Western blot had to be overexposed when imaging. This was partly due to insufficient blocking by skim milk and was later resolved during our second Western blot when blocked with BSA. Additionally, protein levels were not standardized between lanes (Figure S2), although normalizing the intensity of the 73 kDa band to the 103 kDa band indicated that the 73 kDa band was not getting digested by the trypsin. When performing the second trypsin accessibility assay, the same bacterial stock that had been previously transformed 8 days prior was used, which may have resulted in a stress-induced response that resulted in no visible signal on the Western blot due to BrkA not being expressed in the WT-pENS cells via unknown mechanism.

Since DsbA plays a role in the formation of disulphide bonds in a wide variety of proteins, using a *dsbA* knockout mutant strain of *e. coli* may have resulted in other proteins not being folded properly which may have affected the results. Possibly DsbA is not directly involved in the secretion of the BrkA, but it is involved in folding other proteins that somehow help with the secretion of BrkA.

Conclusions This study highlights a potential role for DsbA within the secretion pathway of BrkA where DsbA may be necessary for the successful secretion of A to the outer membrane. We performed two trypsin digestions followed by a Western blot in a time series to identify whether the 73 kDa and 103 kDa components would be present on the blot. Within our first Western, we found that the 73/103 kDa ratio in the KO DsbA model does not decrease over time relative to the WT.

Future Directions This study provides novel insight into the potential role of DsbA in the secretion of the BrkA autotransporter. Future studies should investigate the reproducibility of our results and further understand how different conditions may affect BrkA secretion. The addition of a secretion-incompetent version of BrkA as a negative control could be used to compare to the results of Δ*dsbA* and could strengthen the evidence suggesting DsbA's role in the secretion of BrkA. Further studies could also look at the effects of varying ages of transformed cells and blocking solutions, further investigate whether BrkA and DsbA directly interact, or if DsbA is necessary for the function of other periplasmic factors necessary for secretion such as SurA (14). Similarly, investigating additional placements of the histidine tag within the passenger sequence of BrkA would also offer a more comprehensive evaluation of our findings, confirming that the chosen tag location did not inadvertently influence BrkA's secretion pathway. This could involve systematically varying the 6xHisTag position and assessing its impact on BrkA's surface expression and secretion efficiency.

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CONTRIBUTIONS

RC, DL, LL and MY all collaborated in completing and executing laboratory experiments within this study. RC worked on the methods, results, discussion, and figures. DL worked on the results, discussion, and figures. LL worked on the abstract, introduction, methods, results, discussion, and figures. MY worked on the abstract, introduction, methods, results, discussion, and figures. All team members revised the paper and figures as a whole and contributed equally to the final manuscript and project as a whole. Co-authorship should therefore be granted equally to all four members.

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