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Evaluating the Role of the Fkpa Periplasmic Chaperone on the Secretion of the BrkA Autotransporter in *Escherichia coli* Strain K-12

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SUMMARY BrkA is a virulence factor of Bordetella pertussis, a causative agent of whooping cough, that can induce serum resistance and cell adhesion. As an autotransporter, BrkA is transported across the inner and outer membrane by using its N-terminal signaling sequence and C-terminal β-barrel, respectively. However, the detailed process of its translocation mechanism in the periplasm remains unknown. It has been observed that autotransporters keep their passenger domain in an unstable, non-native conformation while in the periplasm and only become stable once reaching the extracellular space. Studies have uncovered the interaction between periplasmic chaperones and autotransporters, suggesting they may play a role in their secretion across the periplasm. Current research has explored the roles of periplasmic chaperones like SurA, DegP, and Skp in the secretion of the BrkA autotransporter, but no available literature investigated the necessity of FkpA. FkpA interacts with the passenger domain of EspP, another T5SS autotransporter, suggesting that FkpA is necessary for BrkA secretion (1). We aimed to test this hypothesis by transforming wildtype (BW25113) and *fkpA* knockout (JW3309) *Escherichia coli* with pPALMB2 and pPALMC2 plasmids (2) and comparing the BrkA expression using western blot analysis. We captured a significant decrease in BrkA secretion from the western blot analysis, despite the presence of background noises, our data suggest that FkpA plays a role in BrkA secretion. Thus, we propose that FkpA is involved in facilitating the proper folding of the BrkA β-domain and maintaining a translocation-competent state.

INTRODUCTION

Bordetella serum-resistance killing protein A (BrkA) is a virulence factor of *Bordetella pertussis*, a gram-negative mucosal pathogen that causes whooping cough (3) and is capable of mediating cellular adherence and serum resistance (4). BrkA is an autotransporter that utilizes the Type V secretion system (T5SS) for its secretion and translocation across the inner and outer cell membranes. Compared to other bacterial secretion mechanisms, T5SS is relatively simple, using general transport mechanisms such as Secretion (Sec) translocase and β-barrel Assembly Machinery (BAM) that are commonly available in the cell (5). The BrkA autotransporter is 103 kDa in length and is processed into a 73 kDa N-terminal alpha domain containing the passenger and 30 kDa C-terminal β-domain in the periplasm. A signal sequence (25-50 AA long) is present at the N-terminus and is used to direct the secretion of the peptide across the inner membrane via Sec translocase. The 30 kDa β-domain, once cleaved from the peptide, forms a β-barrel translocation unit that inserts itself into the outer membrane, assisting the translocation of the 73 kDa passenger domain of BrkA to the cell surface (6). The passenger domain remains in an unstable, non-native conformation in the periplasm and becomes stable after translocation outside of the cell (7). Furthermore, it has been shown that once the BrkA autotransporter reaches the cell surface, it remains attached to the bacterium, which is characteristically distinct from other autotransporters (8). The processing mechanism of BrkA in the periplasm, however, remains unknown. Whether specific protein-based periplasmic chaperones are required for the secretion of BrkA autotransporters is yet to be thoroughly tested.

Research has suggested the involvement of chaperone proteins in autotransporter biogenesis (1). Perez et al. have demonstrated direct interactions of the chaperones SurA and

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DegP with the β- and passenger domain of autotransporters that are members of the T5SS (1), while Phan et al. revealed that DsbA, Skp, SurA, and DegP maintain as autotransporter intermediates in a translocation competent state during their time in the periplasm (9). Recent research underscores the importance of periplasmic chaperones on the secretion of autotransporters, of which three major biological functions have been discovered so far: i) stabilization of proteins' non-native conformation and facilitation of their folding (10). ii) catalyzation of rate-limiting steps (11). iii) degradation of misfolded proteins and prevention of protein aggregation (12). Yue J. tested whether some general periplasmic chaperones were necessary for BrkA secretion in *Escherichia coli* (13). Specifically, Yue J. uncovered that SurA was necessary while DegP and Skp were useful for BrkA autotransporter secretion (13). However, the necessity of FkpA was yet to be investigated. FkpA is another general periplasmic chaperone that was recently demonstrated to exhibit the prevention of premature folding and rate-limiting step catalyzation functions (1). It was found to interact with the passenger domain of EspP, another T5SS autotransporter, which suggests that it may be a candidate involved in autotransporter biogenesis (1). Given the structural similarity between EspP and BrkA, we suspect that knocking out the *fkpA* gene would impair the stability of BrkA protein structure and thus lead to protein degradation, inhibiting the BrkA from being secreted across the periplasm.

In this study, we aimed to investigate the necessity of FkpA in the secretion of BrkA autotransporter by introducing pPALMC2 and pPALMB2 plasmids (2) that encode for BrkA into FkpA knockout (KO) JW3309 *E. coli* and wildtype (WT) BW25113 *E. coli*, and comparing their relative levels of BrkA expression. Since FkpA was reported to interact with some T5SS chaperones and is capable of stabilizing proteins' nonnative conformation (1, 10), we hypothesize that the FkpA periplasmic chaperone is necessary for BrkA autotransporter biogenesis.

We studied the necessity of FkpA on BrkA biogenesis by assessing the BrkA expression in the absence of FkpA in *E. coli*. By conducting whole cell lysate and running the lysate on western blot, we have found that knocking out the expression of FkpA periplasmic chaperones significantly impairs the biogenesis of BrkA autotransporter. This suggests that FkpA could serve as a potential therapeutic target when combating *B. pertussis*, while highlighting the significance of periplasmic chaperones in secreting T5SS autotransporters.

METHODS AND MATERIALS

***E. coli* K-12 knockout mutants – Keio collection.** The Keio collection consisted of *E. coli* K-12 mutants with in-frame, single-gene knockouts achieved through inactivating chromosomal genes using PCR products (14, 15). Specifically, for the JW3309 strain, a 70 bp-specific primer set was engineered with the kanamycin resistance cassette from pKD13 into the *fkpA* gene to generate the desired in-frame knockout strain (16). The BW25113 and JW3309 were used for our investigation of BrkA secretion (Table 1).

TABLE. 1 Strains and Genotype. Expected genotype of both BW25113 and JW3309 strain from *E. coli* K-12 Keio Collection with corresponding antibiotic resistance markers.

Strain	Genotype	Antibiotic Resistance Marker
BW25113	K-12 WT	N/A
JW3309-2	BW25113 K-12 with *ΔfkpA754::kan*	Kanamycin

Q5 Self-Directed Mutagenesis Kit. The Q5 Self-Directed Mutagenesis Kit (17) is a rapid and site-specific mutagenesis protocol tailored for synthesizing double-stranded plasmid DNA with high fidelity. This easy-to-use PCR kit was used in the preparation of pPALMC2 and pPALMB2, generously gifted by Loujain Bilal, Ayesha Lalani, Mairi MacAulay, and Parvin Malhi as found in *Chaperone DegP is not necessary for the polyhistidine tag-detected surface expression of autotransporter BrkA in E. coli BW25113*, as well as kanamycin resistance cassette detection PCR in knockout confirmation.

Polyhistidine tagged (6x His-tag) BrkA cloning vectors. 6x His-tag BrkA cloning vectors, pPALMB2, and pPALMC2 were generously gifted by Loujain Bilal, Ayesha Lalani, Mairi

MacAulay, and Parvin Malhi as found in *Chaperone DegP is not necessary for the polyhistidine tag-detected surface expression of autotransporter BrkA in Escherichia coli BW25113*, in progressing with our investigation into the dependency of BrkA autotransporter secretion with FkpA chaperones. The pPALMB2 and pPALMC2 cloning vectors were generated using the Q5 Site-directed Mutagenesis Kit, which incorporates 6x His-tag at the start of the passenger domain (Figure 1). The cloning vectors, pPALMB2, and pPALMC2 were then transformed into BW25113 and JW3309 in proceeding to western blot analysis.

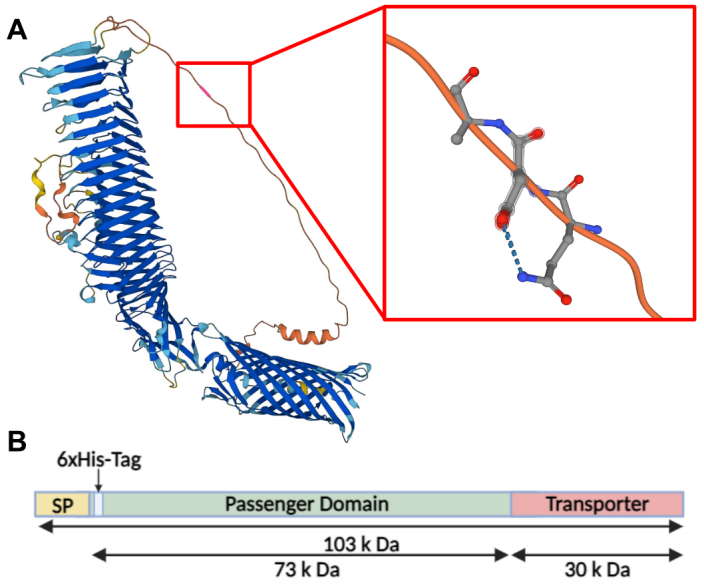

FIG. 1 6x His-tag in pPALMC2 and pPALMB2. According to 1alpha, both 6x His-tags are inserted in the same location in the passenger domain of the cloning vector. **(A)** Alpha-fold of 6x His-tag insertion. **(B)** 6x His-tag insertion in the protein sequence.

Kanamycin resistance cassette detection primers. To authenticate if the strains ordered from the *E. coli* Keio knockout collection are true knockouts of FkpA (18), it is necessary to detect the presence of the kanamycin resistance cassette. Specifically, the BW25113 strain should possess an intact *fkpA* gene, whilst the JW3309 strain exhibits a disrupted *fkpA* gene with an inserted kanamycin resistance cassette (Table 1). In identifying the presence of intact or disrupted *fkpA gene* in BW25113 and JW3309 strains, a set of primers were designed to flank the *fkpA* region in the K-12 genomic DNA (Figure 2). PCR amplification using these primers (Table 2) would result in distinct products for each strain, with expected lengths of 861 bp for BW25113 and 1375 bp for JW3309, calculated by SnapGene (19).

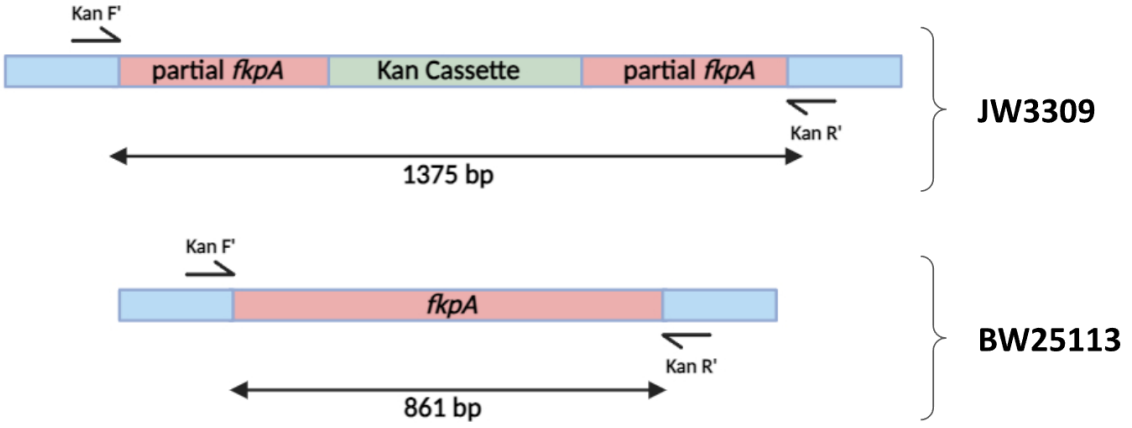

FIG. 2 Kanamycin Resistance Cassette Detection Primers. JW3309 contains a kanamycin resistance cassette engineered from pKD13, disrupting the *fkpA* genomic DNA. BW25113 contains an intact *fkpA* genomic DNA. Kanamycin resistance cassette detection primers, Kan F' and Kan R', flank the *fkpA* region. The expected length of the PCR product of JW3309 and BW25113 would be 1375bp and 861bp respectively.

TABLE. 2 Kanamycin Resistance Cassette Primer Sequence. Melting temperature (T_M) is calculated based on the NEB T_m Calculator (24), specifically tailored for the Q5 Self-Directed Mutagenesis Kit. Primer sequences are flanking the *fkpA* region in *E. coli* K-12 genomic DNA.

Gene	Primer	Sequence	Length	T_M (°C)
ΔfkpA754::kan	Kan F'	5' GTGAGATGCCCCGATCCT 3'	18bp	67
fkpA	Kan R'	5' CAGGCGGCGGTTCTTAATGCTTATT 3'	25bp	70
pUC19	193F'	5' GTGAAATACCGCACAGATGC 3'	20bp	64
	355R'	5' GGCGTTACCCAACTTAATCG 3'	20bp	63

To determine the length of kanamycin resistance cassette PCR products, 2% agarose gel DNA electrophoresis can resolve and visualize small DNA fragments sized between 0.2-2 kb with high resolution (20). Sanger sequencing of PCR products can offer additional confirmation to authenticate whether JW3309 was a true knockout (21).

PCR amplification of pUC19 served as positive control for PCR, the expected length of pUC19 PCR product is 163 bp, which ran in parallel with kanamycin resistance cassette PCR products in indicating PCR master mix is sufficiently prepared.

Western blot methods for BrkA secretion detection: **TGX-Stain-Free FastCast Acrylamide Gel**. The FastCast kit was used to run in the standard Biorad Mini-PROTEAN (Bio-Rad®) style apparatus, casted four 1.0 mm stain-free 8% gels for SDS-PAGE. The casted gels were stored at 4°C for 4 weeks before the whole cell lysates were ready for SDS-PAGE.

Transform gifted pPALMC2 and pPALMB2 plasmid from 1alpha into BW25113 and JW3309 strains. Competent BW25113 WT and JW3309 *fkpA* knockout *E. coli* cells were transformed with pPALMC2 and pPALMB2 plasmids that were extracted using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic®). Single colonies of transformed BW25113 cells and JW3309 cells were selected and re-streaked on the respective selective plates. Transformed BW25113 cells were plated on ampicillin selective plates (100 ng/ml), whilst the transformed JW3309 *fkpA* KO cells were plated on ampicillin (100 ng/ml) and kanamycin (100 ng/ml) selective plates.

Whole-cell lysate for SDS-PAGE. 100 µl samples of (JW3309-pPALMB2 and JW3309-pPALMC2 for *fkpA* knockout cells; BW25113-pPALMB2 and BW25113-pPALMC2 for WT cells) were centrifuged for 2 min at 12000 rpm to separate the supernatant and cell pallets. Samples were then resuspended and washed with phosphate-buffered saline (PBS). 2x Laemmli sample buffer with β-Mercaptoethanol (BME) was added to each sample (20:1 dilution, 950 µl Laemmli sample and 50 µl BME). Finally, the solution was incubated under 95 °C for 12 minutes and centrifuged at 12000 rpm for another 15 minutes. The whole-cell lysates were stored at -20 °C prior to running SDS-PAGE electrophoresis.

SDS-PAGE electrophoresis and membrane transfer. We utilized the TGX-Stain-Free FastCast Acrylamide Gel (Bio-Rad®) for sample loading, added PageRuler (ThermoFisher Scientific®) #26621 ladder for western blot, Precision Plus Protein Unstained Standards Ladder (Bio-Rad®) for protein separation confirmation, and Chitinase C (ChiC) purified protein stained with Blue Juice serving as positive control with a length of 55 kDa. We then ran the gel at 150 V for 45 minutes. After the SDS-PAGE was completed, we used the Trans-Blot Turbo Midi Nitrocellulose Transfer Pack (Bio-Rad®) for membrane transfer.

Western blotting. The membrane was blocked using a 5% non-fat dry milk blocking buffer (1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent, skim milk powder). 6x His-tag monoclonal primary antibodies (ThermoFisher Scientific® Lot: YH376520) specific for

binding with 6x His-tag proteins (1:1000 dilution) were added to the blocking buffer aforementioned. After incubating for 1 hour at room temperature with agitation, we rinsed the membrane five times for 5 min with TBST (1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent). We prepared the horseradish peroxidase (HRP)-conjugated secondary antibody specific for the 6x His-tag monoclonal primary antibody and incubated it for an hour, then repeated the steps as primary antibody washing. We utilized the ChemiDoc MP Imaging System (Bio-Rad®) and captured the western blot image.

RESULTS

6x Histidine tagged (6 His-tag) BrkA cloning vectors. The 6x His-tag cloning vectors, pPALMC2 and pPALMB2, which are generously gifted by Loujain Bilal, Ayesha Lalani, Mairi MacAulay, and Parvin Malhi, are authors of the research paper *Chaperone DegP is not necessary for the polyhistidine tag-detected surface expression of autotransporter BrkA in Escherichia coli BW25113* (2). For detailed information on the insertion, location, and determination of the 6x His-tag cloning vector, please refer to this paper (2). Both pPALMC2 and pPALMB2 contain a 6x His-tag near the N-terminal signaling peptide of the *brkA* gene while also harboring an ampicillin resistance gene (Figure 1).

Kanamycin resistance cassette detection indicates JW3309 is a true *fkpA* knockout. The regions surrounding the *fkpA* gene were amplified using PCR to confirm that the Kan cassette was inserted at the correct location to disrupt *fkpA*, *ΔfkpA754::kan*, in the JW3309 strain (Table 1). The 2% DNA gel imaging indicated amplification of PCR products from BW25113 and JW3309 cells using the Kan F' and Kan R' primer sets in Lanes 2 and 3, respectively (Figure 3). The expected PCR product lengths were 861 bp for BW25113 and 1375 bp for JW3309 (Figure 2). However, both BW25113 and JW3309 in Lanes 2 and 3 had bands slightly below 1500 bp, aligning with both O'Generuler and the 100 bp DNA ladder, suggesting both PCR products had band lengths of 1375 bp (Figure 3). In Lane 4, the pUC19

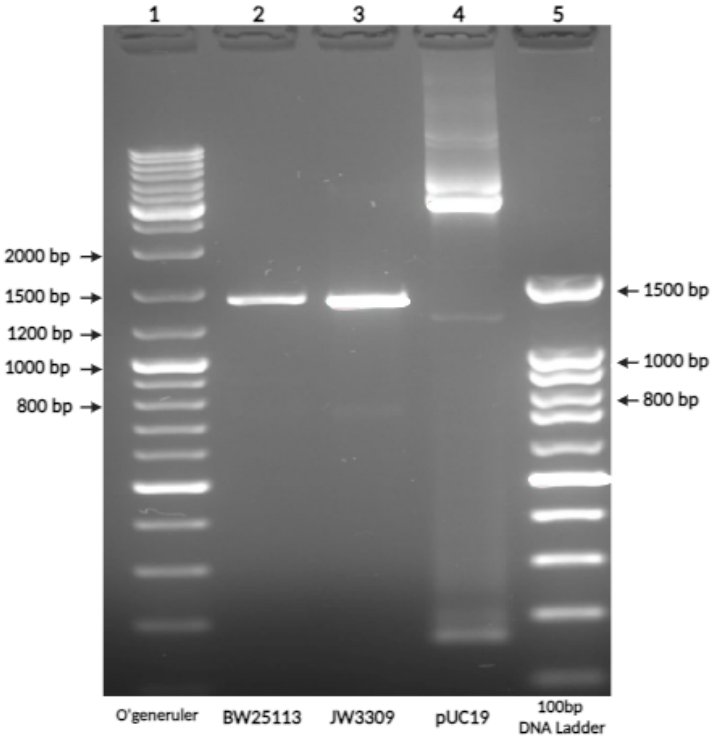

FIG. 3 JW3309 is an *fkpA* knockout. 2% DNA gel used in resolving small DNA fragments around 0.2–2kb. O'Generuler (Lane 1) and 100bp DNA ladder (Lane 5) used to confirm band length. BW25113 (Lane 2) and JW3309 (Lane 3) PCR products are shown to have band lengths slightly below 1500 bp, with JW3309 showing a faint and around 800 bp. pUC19 (Lane 4) served as positive control.

positive control displays the expected 163 bp band, a faint non-specific product band above 1200 bp, and a bright 2686 bp band representing the pUC19 cloning vector (Figure 3). The O'Generuler in Lane 1 and the 100 bp ladder in Lane 5 are provided as reference markers for the gel (Figure 3). Sanger sequencing results of both BW25113 and JW3309 PCR products indicate 99% identities as pECM1 and pECT3 with high quarry coverage, vectors that contain kanamycin resistance cassettes. The 1375 bp DNA gel band in Lane 3 and Sanger sequencing results confirmed that JW3309 cells are true knockouts of FkpA (Figure 3).

Western blot analysis indicates that FkpA is necessary for the proper folding of BrkA and plays a role in BrkA secretion. To determine the effects of periplasmic chaperone FkpA on the secretion of BrkA, we performed western blot analysis to compare the BrkA expression in WT BW25113 and *fkpA* KO JW3309 *E. coli* cells. Serving as reference markers, PageRuler #26621 Prestained Ladder and Precision Plus Unstained Standard Ladder were loaded in Lanes 5 and 6, respectively (Figure 4B). ChiC serves as a positive control in Lane 7, featuring purified protein with 6x His-tag insert (Figure 4). The thick smear in Lane 7 has

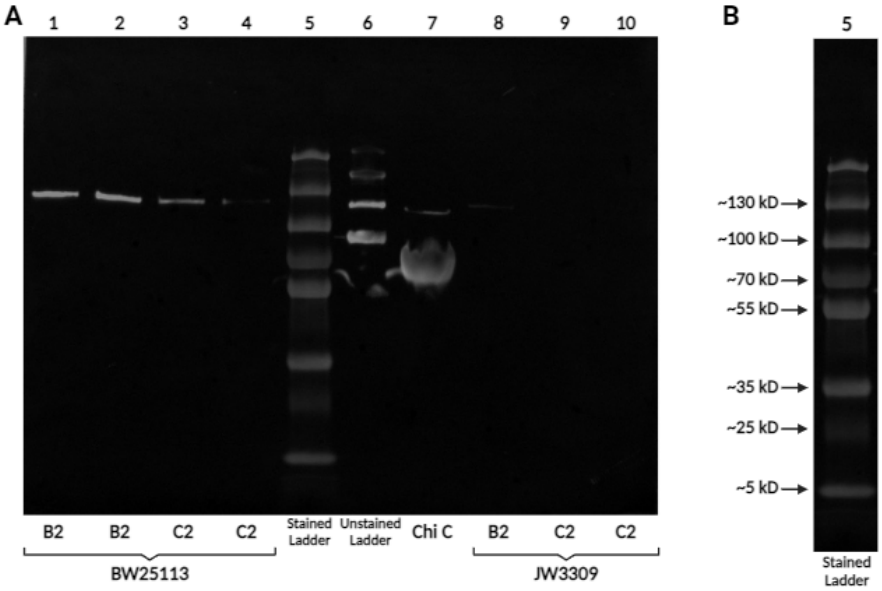

FIG. 4 FkpA is required for BrkA protein stability and maintenance. (A) Western blot detection of 6x His-tag on BrkA autotransporter via whole-cell lysates of transformed BW25113 and JW3309, using pPALMB2 (as B2) and pPALMC2 (as C2). Whole cell lysate of: BW25113 in Lanes 1-4, JW3309 in Lanes 8-10. PageRuler #26621 Pre-stained Ladder in Lane 5. Precision Plus Unstained Standard Ladder in Lane 6. Chi C positive control in Lane 7. **(B)** Labeled Pre-stained Ladder, corresponding with Lane 5.

a band length at around 55 kDa, suggesting the proper binding of the primary antibody to the 6x His-tag for ChiC (Figure 4A). Lanes 1 to 4 contain whole-cell lysates from transformed BW25113 cells that express pPALMB2 shown in Lanes 1 and 2, and pPALMC2 in Lanes 3 and 4 (Figure 4A). Notably, the intensity of BrkA protein bands observed from pPALMB2 is more intense compared to pPALMC2 (Figure 4A). The whole-cell lysates of BW25113 cells serve as the negative control, with the expression of the 103 kDa band correlating with the expected size of the unprocessed full-length BrkA protein (Figure 4A). Whole-cell lysates from transformed JW3309 with either pPALMB2 or pPALMC2 are shown in Lane 8 (pPALMB) and Lanes 9 to 10 (pPALMC) respectively (Figure 4). Although no bands were detected in Lanes 9 to 10, a faint 103 kDa band was observed in Lane 8, corresponding to the whole-cell lysate of JW3309 with the pPALMB2 vector (Figure 4). As indicated by full-length BrkA's presence in Lanes 1 to 4 of WT BW25113 whole-cell lysates and absence in Lanes 8-10 of FkpA knockout JW3309 lysates, the western blot analysis indicates the necessity of the periplasmic chaperone FkpA in facilitating proper folding and subsequent secretion of BrkA.

DISCUSSION

We first validated that JW3309 cells are indeed *fkpA* knockouts using PCR (Figure 3). The band in Lane 3 has a band of slightly below 1500 bp, near the expected length of 1375 bp of *ΔfkpA754::kan* PCR product (Figure 3) (14, 15). However, we observed the same results in Lane 2 for our negative control BW25113 cells, suggesting the PCR product also had a kanamycin resistance cassette inserted in the same location (Figure 3). We sent the PCR products for Sanger sequencing, indicating that the BW25113 and JW3309 strains contain kanamycin resistance cassette structure. This not only confirmed the JW3309 strain as a true FkpA knockout but also presented conflicting information about the BW25113 strain (Figure 3). As previously mentioned in the BW25113 strain construct, its genome should not contain kanamycin resistance cassettes within the *fkpA* gene. Thus, we suggest this was due to handling error and that Lane 2 may contain the JW3309 strains instead of the BW25113 strains (Figure 3).

Results from our western blot analysis demonstrated that BrkA is secreted in the WT BW25113 strain, whilst little to no secretion was observed in the FkpA knockout JW3309 strain (Figure 4, Supplemental Figure 1). Therefore, we suspect that periplasmic chaperone FkpA plays a role in BrkA autotransporter secretion by maintaining the passenger domain in a translocation-competent state during BrkA's transit in the periplasmic space. We also suspect that FkpA serves important functions in the proper folding of BrkA autotransporter. FkpA periplasmic chaperone facilitates and enhances the folding of outer membrane protein (OMP), thereby highlighting the chaperone's potential to mediate the folding of BrkA (22). Devlin et al. detailed that FkpA utilizes extensive chaperone interfaces to firmly bind unprocessed proteins, acting not as a folding catalyst to accelerate folding activity but rather enhancing the folding yield while decreasing the folding rate (14). FkpA binding to the passenger and β-domain of BrkA prevents premature folding, thus facilitating the processing of BrkA for proper conformation and subsequent secretion (1).

Plummer et al. suggest the possible co-regulated pathway of SurA and FkpA periplasmic chaperones activity in OMP folding and secretion, where both chaperones are necessary for the OMP processing (23). In the lanes containing whole-cell lysate of JW3309 strain, only the lysate, including the pPALMB2 vector, indicates a very faint band (Figure 4). This implies the FkpA chaperone is necessary for the folding and/or secretion of BrkA in the periplasmic chaperone network. The presence of the faint band in Lane 8 of the western blot may be explained by insufficient blocking and/or washing (Figure 4), leading to non-specific binding on the western blot analysis. Similarly, the unexpected presence of a fluorescently labeled unstained ladder contradicts our expectations, given that the conjugated secondary antibody exclusively binds to the primary anti-His Tag antibody, with potential non-specific binding issues possibly originating from the same issue as inadequate blocking during western blot preparation (Figure 4).

Finally, the BrkA protein expression levels are notably different between pPALMB2 and pPALMC2 vectors based on the thickness and brightness of the bands for the wildtype BW25113 lysates (Figure 4). Although the 6x His-tag insertion sites for both plasmids are identical according to (2), the alteration of the protein expression remains unclear and needs to be investigated further. We hypothesize that the alteration might be attributed to faulty cloning vector constructs, such as damaged promoter sequences, resulting in variability in expressed BrkA protein despite having the same 6x His-tag insertion location.

In our proposed model, FkpA plays a pivotal role in the BrkA secretion pathway, facilitating the translocation of BrkA through the periplasmic space, similar to the suggested use of SurA and FkpA in the autotransporter biogenesis of EspP (Figure 5) (1). We proposed

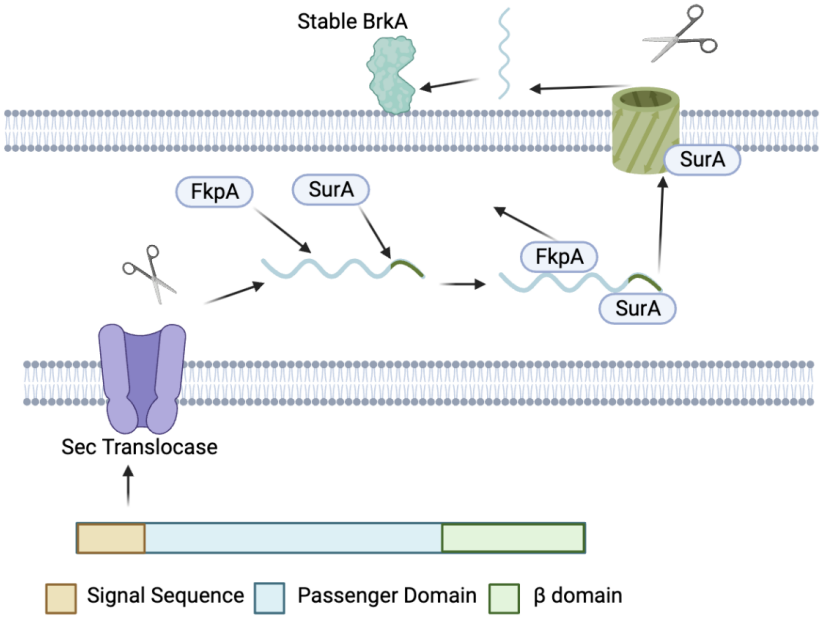

FIG. 5 Proposed mechanism of FkpA facilitating BrkA secretion. The N-terminal signal sequence is utilized to secrete BrkA passenger and β-domain through the inner membrane. The signal sequence is cleaved and FkpA is proposed to bind to the passenger domain in the periplasm and subsequently released before secretion through the outer membrane via the β-domain. Upon reaching the outer membrane, the β-domain undergoes cleavage, insertion, and formation of a transmembrane β-barrel that allows the passenger domain to reach the extracellular space.

that following cleavage from its signal peptide, FkpA binds to the passenger domain of BrkA in the periplasm, ensuring the structural stability of the passenger domain and preserving it in a translocation-competent state (Figure 5). Moreover, FkpA remains bound until the β-domain is cleaved and inserts itself as a β-barrel into the outer membrane, facilitating the secretion of the passenger domain (Figure 5). Subsequently, upon reaching the external environment, the passenger domain folds into its stable, native conformation and remains associated with the bacterial surface (Figure 5). In addition, we also propose that FkpA facilitates the folding and thus secures proper insertion of the β-domain into the outer membrane. This prospect is further supported by Ruiz-Perez et al., who, in their surface plasmid resonance analysis, demonstrated the interaction of FkpA with unfolded EspP-α autotransporters (1).

This overall pathway suggests that FkpA directly interacts with the passenger domain of BrkA and acts in accordance with SurA, similar to DegP and SurA in EspP autotransporter biogenesis (1). However, it is possible that FkpA can act indirectly or in tandem with other periplasmic chaperones. Future studies can validate this mechanism by exploring additional interactions of FkpA with BrkA and other chaperones through methods like surface plasmon resonance spectroscopy that can directly confirm the interaction between FkpA and BrkA (18).

Limitations While PCR confirmation has helped to verify the knockout of the FkpA periplasmic chaperone in JW3309 *E. coli* strain via successful amplification of a 1375 bp PCR product, it remains unclear why a band at the 1375 bp position is present in the negative control. According to our primer design, we expected the PCR amplicon of our BW25113 negative control to be 861 bp in length (14). It was most likely due to labeling or handling errors resulting in mismatched bands and sequencing data of BW25113 when conducting the PCR for kanamycin resistance cassette detection. Redoing the PCR using a fresh BW25113 colony picked from the starter plate would confirm the wild type has the intact *fkpA* gene.

Furthermore, due to technical issues involved in preparing and loading samples onto the SDS-PAGE gel, the ChiC positive control lane had a notable smeared band and seemed contaminated. The ladders for the SDS-PAGE electrophoresis also exhibited an aberrant pattern obscuring the result for our western blot (Figure 4A). As for Lanes 3 and 4 on the western blot membrane, although both came from whole cell lysates using the pPALMC2 plasmid transformed BW25113, the lanes demonstrate distinct patterns in terms of 6x His-tag protein quantity. This inconsistency of protein loading might also have induced a false negative for samples in the FkpA lane, and a bicinchoninic acid (BCA) protein assay should be used in the future to standardize the amount of protein loaded. Moreover, we did not include the trypsin digestion prior to the whole-cell lysate assay in the western blot experimental design. The use of trypsin would tell us more about FkpA's impact on BrkA's surface expression since trypsin digest would theoretically cleave BrkA's extracellular component, separating the passenger domain from β-barrel and producing a result in the manifestation of two distinct bands in a western blot analysis if FkpA is indeed influential in BrkA surface expression.

Conclusions This study investigated the role of FkpA periplasmic chaperone on the secretion of the BrkA autotransporter across the inner and outer membrane of *E. coli.* To determine FkpA's importance in secretion, we transformed a 6x His-tagged BrkA vector into *fkpA* KO *E. coli* cells and assessed protein secretion via western blot analysis. Although confirmation of the kanamycin resistance cassette location yielded an unexpected negative control result and technical issues were present in our western blot results, we were able to determine that FkpA is required for BrkA secretion to the outer membrane, therefore, defining its necessity in autotransporter secretion. In every replicate, the FkpA knockout significantly impaired the secretion of BrkA. Finally, further studies can be performed to understand the interaction between BrkA and FkpA to elucidate the fate of BrkA secretion in the periplasm. Building on the foundation of this study, similar research can be done on other periplasmic chaperones for a more comprehensive understanding of the BrkA secretion pathway. This study furthers the research on BrkA autotransporters by suggesting the importance of FkpA on BrkA biogenesis and improves the understanding of the BrkA processing mechanism in the

periplasm. BrkA autotransporter's ability to secrete itself across the inner and outer membrane makes it a very potent drug delivery tool, and further characterization of BrkA can have valuable therapeutic implications.

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CONTRIBUTIONS

All authors contributed to laboratory experiments and data collection and conducted thorough background research. JB was responsible for writing and editing the abstract, introduction, discussion, limitations, conclusions, and acknowledgments. IC was responsible for writing and editing the material and methods, results, figures, tables, and discussion and directing daily laboratory experimentation workflow. JS was responsible for writing the abstract, results, discussion, and contributed to the figures. RX was responsible for writing the material and methods, results, discussion, figures, and editing the conclusion and future directions.

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