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Engineering an OmpT Cleavage Site in the BrkA Passenger Domain to Explore the Role of the Conserved Autochaperone Region

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SUMMARY The secretion of autotransporters stands as a distinctive mechanism employed by Gram-negative bacteria for the transportation of proteins to their cell surface. BrkA is an autotransporter protein in Bordetella pertussis which plays a pivotal role in conferring serum resistance and enhancing the bacterium's adherence to host cells. BrkA comprises three key domains: an N-terminal signal peptide domain, a 73 kDa passenger α-domain, and a 30 kDa translocator β-domain. BrkA secretion has been well studied, and it has been previously shown that the autochaperone region glutamate^{601}–alanine^{692} is essential for proper protein folding and protection against proteolysis. This region, however, is not necessary for translocation of the protein across the outer membrane. Previous literature has also shown that following translocation out of the cell, cleavage occurs at the asparagine^{731} – alanine^{732} site to dissociate the passenger from the translocator β-domain. However, despite cleavage at the cell surface, the BrkA passenger cannot be detected in the supernatant. It is thought that a non-covalent interaction may be responsible for this phenomenon by anchoring the passenger to the cell membrane but this process remains understudied. In this study, we sought to identify the specific region that may non-covalently interact with the β-barrel region by engineering a secondary OmpT cleavage site before glutamate^{601}. Two rounds of site-directed mutagenesis were employed to introduce a polyhistidine tag at the N-terminus of the BrkA passenger and then an OmpT cleavage site at glutamate 601 of the BrkA passenger. Western blot analysis of whole cell lysates of an OmpT-expressing strain of *E. coli* (UT2300) detected a band corresponding with the BrkA passenger (61 kDa) processed at the OmpT cleavage site, suggesting that it remains cell-bound after cleavage. A 61 kDa band was not observed in Western blots of whole cell lysates in an OmpT deficient strain of *E. coli* (UT5600). We did not detect a band of 61 kDa using Western blots of filtered culture supernatants in either strain. It is also possible that the concentration of the 61 kDa protein moiety was too low in supernatants for Western blot detection or that the cleaved BrkA passenger was unstable in the absence of the autochaperone region and degraded by extracellular proteases in the media.

INTRODUCTION

Type Va autotransporters are a class of polypeptides in Gram-negative bacteria consisting of 3 key domains (N-terminal signal peptide, C-terminal translocator β-domain, and a passenger domain) that dictate translocation across the double membrane (1). The N-terminal signal peptide guides translocation across the inner membrane (IM) via the Sec translocase (1). The C-terminal β-domain guides translocation of the α-domain (passenger region) across the outer membrane (OM) where it either remains anchored or released into the extracellular space (1). BrkA (Bordetella resistance to killing) is a type Va autotransporter which protects its host, *Bordetella pertusis*, from the bactericidal activity of serum complement proteins (1-3). BrkA has been proposed to have an integral β-barrel domain in the OM that threads the passenger through its pore (2). BrkA has been shown to require a conserved region of the amino acids glutamate^{601} – alanine^{692} to serve as an autochaperone for the passenger contained within BrkA (2). This region has been proposed to induce folding stability to the passenger protein and is necessary to prevent proteolysis by OM proteases OmpT and OmpP (2). This region, however, is not necessary for the translocation of the passenger across the OM (2). BrkA has also been proposed to contain a

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cut site between the passenger and β-barrel region at asparagine^{731}–alanine^{732} (4), however, the cleavage mechanism is unknown (2). Furthermore, after autoproteolytic cleavage at asparagine^{731}-alanine^{732}, the passenger remains tightly associated with the OM indicating non-covalent interaction with the β-barrel region (2). With the given location of the asparagine^{731}–alanine^{732} cleavage site with respect to the β-barrel region and passenger region, it is proposed that the unknown mechanism of cleavage occurs within the channel of the β-barrel (2, 13). The extracellular region of the passenger, including the conserved and necessary glumate^{601}–alanine^{692} region, is located before this cleavage site (2). The glutamate^{601}–alanine^{692} region is also covalently attached to the rest of the BrkA passenger protein (2). These findings suggest that introducing a secondary cleavage site, before the unknown region non-covalently associating with the β-barrel, may allow the protein to be released from the OM and soluble in the extracellular space. From imaging through AlphaFold (Fig. 1D) we predict that BrkA loops through the translocation unit into the extracellular space, but remains attached to the β-barrel. The introduced secondary cleavage site should be located before the asparagine^{731} –alanine^{732} primary cut and also be located before the conserved glutamate^{601}–alanine^{692} autochaperone region to preserve the folding of the passenger. Therefore, we will introduce a secondary cleavage site before the BrkA glutamate^{601}–alanine^{732} by inserting an OmpT cut site. OmpT is an OM serine protease in Gram-negative bacteria as part of the omptin family (5). OmpT has a binding affinity for positively charged residue pairs, usually arginine-arginine, which it cleaves (5). Through the research by McCarter et al., a cut site can be artificially created to induce the proteolytic action of OmpT (5). Here, we hypothesized that the introduction of an OmpT cleavage site before the BrkA glutamate^{601}–alanine^{732} will result in a soluble protein protected from proteolysis by outer membrane proteases. We aimed to design and validate plasmid constructs introducing a 6xHis-Tag and OmpT cleavage site in the BrkA passenger to confirm the presence of secreted BrkA from our proposed model. After successful insertion of both the 6xHis-Tag and OmpT cleavage site into BrkA, a 61 kDa band was observed in the UT2300 protein lysate, implying that the passenger remains associated with the OM after OmpT cleavage.

METHODS AND MATERIALS

Primer Design. Primers were designed to bind to pDO6935 which was generously provided by Dr. Rachel C. Fernandez (University of British Columbia, Vancouver, Canada). HisInsBrkA-F and HisInsBrkA-R contained 5' ends that flanked residues Gly^{59} and Gln^{60} where the 6X His-tag was to be inserted. This insertion site was chosen to be close to the N-terminus, but 17 residues downstream of the signal peptide to avoid interference with Sec translocation, as indicated in Sun et al. OmpTcutInsBrkA-F and OmpTcutInsBrkA-R contained 5' ends that flanked residues Pro^{600} and Glu^{601} where the OmpT cut site consensus sequence (Arg-Trp-Ala-**Arg-Arg**-Val-Gly-Gly) was to be inserted. The OmpT cut site consensus sequence was designed by choosing the most frequent residues contained in each position as described in McCarter et al. These primers contained 5' overhangs that each contained half of their respective insertions, as indicated in the instructions of the Q5 Site-Directed Mutagenesis Kit (New England Biolabs). To determine primer lengths, sequences, annealing temperatures, and specificity, we used the NEBaseChanger online tool (New England Biolabs). The primer sequence is displayed in Supplemental Figure S2.

Site-directed mutagenesis. Site-directed mutagenesis was conducted using materials and instructions from the New England Biolabs Q5 Site-Directed Mutagenesis Kit (E0554).

Confirmation of successful sequence insertions. Isolated cloned colonies were inoculated in LB + ampicillin and incubated overnight at 37°C 200 RPM. Plasmid was isolated from cell cultures using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (BioBasic). Plasmids were digested with HindIII (New England Biolabs), according to manufacturer protocol. Digested plasmids were run on an an 1% agarose gel with RedSafe (FroggaBio) at 120V for 60 min, and visualized on a transilluminator. Upon visualizing a linearized plasmid at the predicted band size, undigested plasmids were sent to Plasmidsaurus for whole plasmid Nanopore sequencing following company instructions.

Preparation of heat-shock competent *Escherichia coli* strains UT2300 and UT5600. *Escherichia coli* strains UT5600 and UT2300 were generously provided by Dr. Rachel C. Fernandez (University of British Columbia, Vancouver, Canada). After overnight incubation at 37°C 200 RPM, strains were diluted 100 fold and incubated at 37°C 200 RPM. Upon reaching 0.3-0.4 OD600, cells were immediately placed on ice for 10 min. 50 mL of cell culture was then pelleted at 4000 RPM at 4°C for 10 mins, resuspended in 20 mL ice-cold 0.1M $CaCl_2$, and incubated on ice for 30 min. Cells were then pelleted again at 4000 RPM at 4°C for 10 mins, resuspended in 2mL ice-cold 0.1M $CaCl_2$ with 15% glycerol, and incubated at 4°C for 16 hours. 200 µL aliquots of competent cells were stored at -70°C until use.

Transformation of pENS and pOCS into *Escherichia coli* strains UT2300 and UT5600. 200 µL aliquots of heat-shock competent cells, stored at -70°C, were thawed on ice. 2uL of pENS and pOCS were added to respective aliquots and mixed by flicking 5 times. Mixtures were then incubated on ice for 30 minutes, heat-shocked at 42°C for 30 sec, then incubated on ice for another 2 min. 1 mL of pre-warmed SOC media was added to each mixture. Mixtures were then incubated at 37°C 200 RPM for 1 hour. 50 uL of cell mixtures were then spread on 1% LB + ampicillin plates and incubated overnight at 37°C. Transformed colonies from incubated plates were used for further study.

Cell Lysis and Protein Extraction. Induced *E. coli* (NEB 5α, UT2300, UT5600) cultures were split into 1 mL fractions and pelleted by centrifugation. Supernatants were collected and passed through 0.45 µm sterile syringe filters (Pall), then centrifuged at 10,000 rpm in 3 kDa Pierce™ Protein Concentrators PES (Thermo Scientific) until an A280 protein concentration reading of ≥ 3.5 µg/µL was achieved on a NanoDrop™ 2000 spectrophotometer (Thermo Scientific). Cell pellets were resuspended in 1 mL of TBS with 1X cOmplete™ Mini Protease Inhibitor Cocktail (SigmaAldrich) and lysed using a Bead Beater Homogenizer (MP FastPrep®-24) with 0.1g of 0.1 mm glass beads (BioSpec Products, Inc) at 6 m/s for 60 seconds. Cell lysates were collected and centrifuged at 13,000 rpm for 10 minutes, and the supernatant was collected. A280 protein concentration readings were collected for each sample, and concentrations were normalized by dilution using TBS with 1X protease inhibitor cocktail. Samples were stored at -20°C for subsequent analysis by SDS-PAGE.

SDS-PAGE. Protein samples were diluted in a 1:1 ratio using 2X Laemmli Sample Buffer with 5% BME (Bio-Rad), then denatured at 95°C for 5 minutes and cooled on ice for 2 minutes. Each sample was loaded at a volume of 15 µL into 4–20% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad), and proteins were separated by SDS-PAGE using the Bio-Rad protein electrophoresis chamber system. 5-10 µL of Precision Plus Protein™ Unstained Protein Standards Ladder (Bio-Rad) and PageRuler™ Plus Prestained Protein Ladder (Thermo Scientific) were also loaded onto the gel. Gels were run at 120 V for 40 minutes with 1X TRIS-base/Glycine/SDS running buffer. SDS-PAGE gels were imaged using the ChemiDoc Imaging System (Bio-Rad).

Western Blot. Resolved proteins were transferred from the SDS-PAGE gel to a PVDF membrane using the Trans-Blot Turbo Transfer Pack (Bio-Rad). Blocking was performed using TBS-T with 3% BSA (Sigma-Aldrich) for 1 hour at room temperature, followed by 3 washes using TBS-T. The membrane was then incubated overnight at 4°C on a shaker, with primary 6X His-Tag antibody (Invitrogen) diluted 1:2000 in blocking buffer. Three washes were performed using TBS-T, followed by 2 hours of incubation with goat-anti-mouse-IgG HRP-conjugated secondary antibody diluted 1:10,000 in 5% skim milk, and 3 additional TBS-T washes. The membrane was then incubated with Clarity Western ECL substrate reagents (Bio-Rad) for 5 minutes, and imaged using the ChemiDoc (Bio-Rad).

RESULTS

Construction of a BrkA protein epitope. To detect BrkA in cell culture, we cloned a 6X His-tag near the N-terminus of BrkA in the expression plasmid pDO6935, generously gifted by Dr. Rachel Fernandez (The University of British Columbia, Vancouver, Canada), for anti-His antibody detection. We used site-directed mutagenesis, with overlapping primers

containing the 6X His-tag, to develop the pENS (Epitope N-Terminal Sequence) plasmid (Figure 1A). We inserted the 6X His-tag 17 residues downstream of the signal peptide to ensure that the 6X His-tag did not interfere with signal peptide (SP) cleavage efficiency, as described in Sun et al. (Figure 1A, 1C; Supplemental Figure S1). The 6X His-tag was also inserted into a predicted non-folding region of BrkA, to ensure protein function was unaffected, located in the extracellular space after translocation (Figure 1D). Whole plasmid Nanopore sequencing (Plasmidsaurus) revealed the successful insertion of the 6X His-tag into pDO6935.

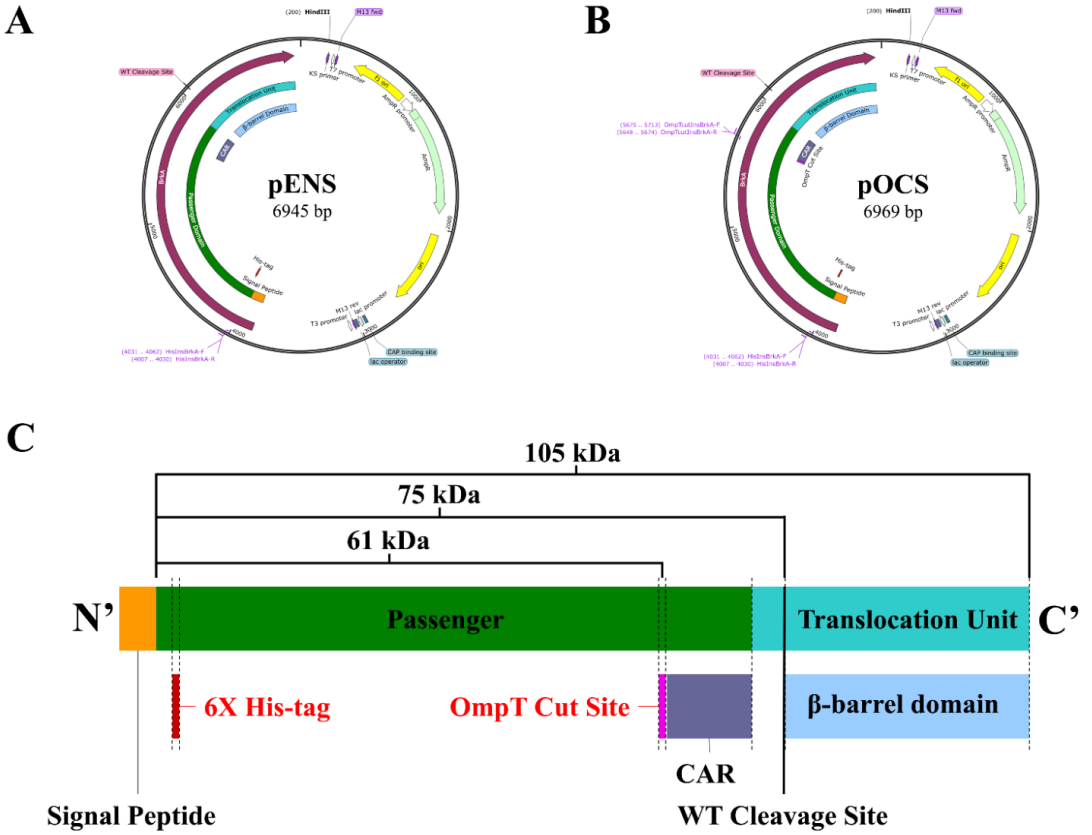

FIG. 1 Construction of Epitope N-Terminus Sequence (pENS) plasmid and OmpT Cut Site (pOCS) plasmid. **A.** Vector map of pENS plasmid. **B.** Vector map of pOCS plasmid. **C.** BrkA domain organization in final pOCS construct. Signal peptide (residues 1-42); passenger domain (residues 43-706); translocation unit (residues 707-1024); conserved auto chaperone region (CAR) (residues 615-706); β-barrel domain (residues 746-1024); WT cleavage site (residues 745-746); 6X His-tag (residues 60-65); OmpT cut site (residues 607-614). The wild-type domains (black) and insertions (red) of BrkA are expressed by pOCS. Predicted protein masses (kDA) after cleavage displayed. **D.** Predicted BrkA folding (AlphaFold). 6X His-tag and OmpT Cut Site insertion locations identified in unfolded regions.

Construction of a BrkA cleavage site. We inserted a consensus OmpT cut site in the passenger region of BrkA in pENS. Using site-directed mutagenesis, with overlapping primers containing the OmpT cut site consensus sequence, to create the pOCS (OmpT Cut Site) plasmid (Figure 1B, 1C). We analyzed the amino acid residue frequency of OmpT cut sites in various bacteria, as described in McCarter et al., and engineered a high-efficiency OmpT consensus sequence based on the highest frequency residues: Arg-Trp-Ala-**Arg-Arg**-Val-Gly-Gly (cleavage occurs between the two bolded residues). As previously described by Oliver et al., the conserved autochaperone region is essential to the proper folding and protection of BrkA from extracellular proteolysis. Therefore, we inserted the consensus OmpT cut site directly adjacent to and upstream of the autochaperone (Figure 1C). We also inserted the OmpT cut site consensus sequence into a predicted non-folding region of BrkA to ensure protein function was unaffected (Figure 1D). With successful OmpT cleavage at this cut site, the BrkA passenger domain may be released into the extracellular space based on the predicted protein structure (Figure 1C, 1D). After translocation across the inner membrane (IM) via signal peptide cleavage, we expect three proteins to be present in expressing cells. The 6X His-tag detectable protein will be 105 kDA after signal peptide cleavage, 75 kDA after cleavage at the WT cut site, and 61 kDA after predicted cleavage at the inserted OmpT cut site (Figure 1C). The 61 kDA protein will potentially be soluble in the extracellular space. Whole plasmid Nanopore sequencing (Plasmidsaurus) revealed the successful insertion of the OmpT cut site consensus sequence into pENS.

Successful detection of pENS BrkA 6xHis-tag insertion. To verify the expression of the 6xHis-tag in the cloned BrkA autotransporter, we conducted a western blot, blotting for our inserted 6X Histidine tag in BrkA. Protein lysates were isolated from pENS-expressing *E. coli* NEB-5*α* using bead-beating lysis and diluted to varying concentrations to confirm the specificity of the expected bands. Protein lysates from pDO6935-expressing *E. coli* NEB-5*α* served as a negative control. A single band was observed at 90 kDa in all three experimental lanes, falling between the expected sizes of uncleaved 105 kDa BrkA and the 75 kDa cleaved passenger domain (Figure 2). While bands appeared in the negative control lanes, these are likely attributed to protein ladder spillover. Despite the single-band observation, successful 6xHis-tag insertion was confirmed. However, this result necessitated further investigation and was repeated in Figure 3.

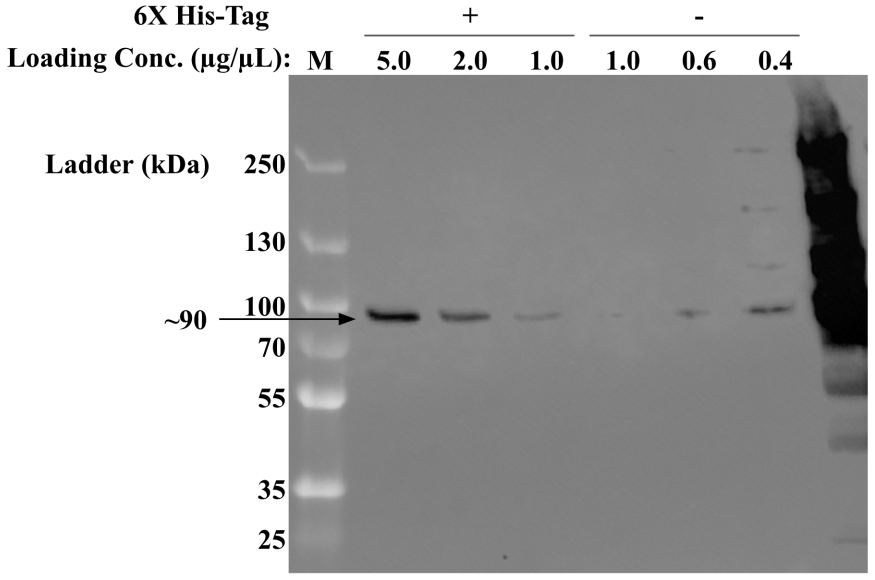

FIG. 2 Successful insertion of 6X His-Tag into BrkA. *E. coli* NEB 5α were transformed with pENS containing BrkA with an N-terminal 6X His-Tag, or pDO6935 containing untagged BrkA. Cell lysates from the induced bacteria were analyzed at multiple concentrations (5.0, 2.0, 1.0, 0.6, and 0.4 µg/µL) by Western blot.

Detection of 61 kDa band in protein lysate via Western blot suggests OmpT cleavage of BrkA passenger at the engineered cut site but does not indicate complete dissociation from the OM. After Nanopore sequencing revealed successful insertion of the OmpT cut site in pOCS, we aimed to validate the hypothesized BrkA passenger cleavage into the supernatant. We conducted another Western blot against 6xHis using pOCS and pENS transformed into OmpT-expressing UT2300 and OmpT-deficient UT5600 *E. coli* strains from the Keio collection. Along with protein lysates, supernatant proteins were extracted through

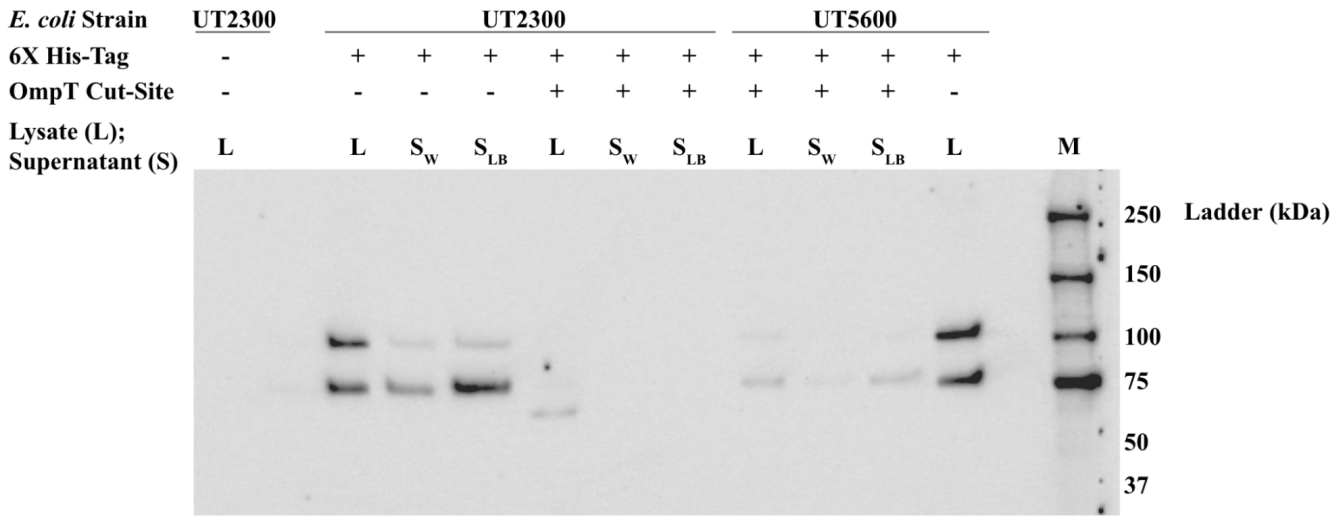

FIG. 3 Detection of 61kDa band in lysate and absence in supernatant indicates association of BrkA passenger with cell membrane after cleavage by OmpT at engineered cut site. *E. coli* UT2300 were transformed with pDO6935 (BrkA), pENS (BrkA with a 6X His tag), or pOCS (BrkA with a 6X His tag and OmpT cut site). OmpT-deficient *E. coli* UT5600 were transformed with pENS or pOCS. Cell lysates were collected from every culture, and culture supernatants were collected from pENS-expressing UT2300, pOCS-expressing UT2300, and pOCS-expressing UT5600. The culture supernatants were concentrated using centrifugal filters, and A280 protein concentrations were measured using either water or LB broth as a blank (S_{LB}, S_W). Sample concentrations were normalized and analyzed by Western blot.

initial filtration with a 0.45µm filter, followed by concentration using centrifugal filtering. Supernatant proteins were diluted based on NanoDrop spectrophotometer estimations blanked with LB broth (S_{LB}) or water (S_W). Both dilutions were run for Western blot analysis (Figure 3). 75 and 105 kDa bands were observed in pENS lysates in both UT2300 and UT5600 (lanes 3 & 12) as previously expected in Figure 2. A faint 61 kDa band was detected in the pOCS UT2300 lysate but was absent in the supernatant lanes. No 61 kDa bands were observed in the UT5600 or pENS-expressing UT2300 control lanes. However, 75 kDa and 105 kDa bands were detected in the supernatant of these conditions, suggesting potential contamination of the supernatant. Taken together, these results indicate BrkA cleavage by OmpT at the inserted cut site was successful, but is not sufficient to dissociate the passenger from the outer membrane.

DISCUSSION

In this study, we aimed to determine the region of the BrkA autotransporter passenger domain that non-covalently associates with the B-barrel after WT cleavage during translocation. Based on a previous study by Oliver et al. (2), we hypothesized that BrkA Glu^{601}-Asn^{731} contains this region and cleavage before Glu^{601} will result in a soluble, extracellular protein protected from proteolysis. Two plasmid constructs (pENS and pOCS) expressing an N-terminal His-tag and engineered OmpT cut site were created and functionality was verified through Western blot. The expected cleaved band in pOCS expressing *E. coli* UT2300 post-OmpT cleavage was detected in the lysates and not in the supernatant as anticipated, suggesting that BrkA undergoes cleavage but likely remains associated with the OM.

In the first Western blot analysis comparing pENS and pDO6935 (control) expressing *E. coli* NEB5-α, a singular band at 90 kDa was observed (figure 2). This was contrary to our expected band sizes of 105 kDa and 75 kDa—which are the reported sizes of BrkA before and after cleavage. Rather, we observed the average molecular weight of the two. Although ladder spillover at 90 kDa occurred, it is unlikely to account for our observed bands. This is due to the decreasing loading concentration in our pENS lanes, corresponding to proportionally decreasing band intensity in the blot. Potential reasons for the singular band include experimental errors, such as insufficient concentrations of β-mercaptoethanol (BME) during sample preparation. BME is important in Western blot sample preparation to reduce

disulphide bonds in proteins and contributes to overall denaturation. Insufficient concentrations of BME during preparation may compromise the complete unfolding of proteins and can potentially result in a singular band that fails to separate during SDS-PAGE. The same experimental lanes of pENS and pDO695 were repeated in the second Western blot (Figure 3) but transformed into *E. coli* UT2300. The use of different bacterial strains may introduce variations in the processing of BrkA, potentially contributing to the observed differences in band patterns. This may warrant the need for a generalized exploration of how different strains influence overall BrkA expression.

Our second Western blot showed a 61 kDa band in the cell lysate of pOCS UT2300 (figure 3). We anticipated the engineered OmpT cleavage site to result in a 61 kDa protein which is then released into the supernatant, given its positioning before the BrkA CAR region of the passenger hypothesized to be non-covalently associated with the OM. However, the presence of the 61 kDa band was only observed in the lysate. This indicates that our engineered cut site was functional. Furthermore, its presence in the pOCS OmpT+ UT2300 lane but not the OmpT- UT5300 lanes suggests that this cleavage was mediated by OmpT.

The absence of the 61 kDa band in the pOCS UT2300 supernatant, while likely suggesting that the passenger domain remains bound to the cell surface after cleavage, may have alternative explanations to consider until the observed results can be replicated. Although we do observe a 61 kDa band in the pOCS UT2300 lysate there is an absence of the expected 105 and 75 kDa bands in the lane which creates the possibility of other factors producing the observed result. First, an insufficient supernatant protein concentration in these lanes may have contributed to the lack of detection. A280 protein concentrations for both Western blots were estimated using a NanoDrop spectrophotometer and then used to normalize the sample concentrations which can lead to the overdilution of samples. Additionally, the samples were grown in LB media which contains peptides that likely persisted throughout the experiment, potentially influencing readings and subsequent dilutions. These factors may warrant repeating the Western blot procedure to confirm our current conclusion. The unexpected 105 and 75 kDa bands in the supernatant of the pENS(+) UT2300 and pOCS(+) UT5600 lanes also raised concerns about potential contamination. A possible explanation could be the use of a 0.45 μm filter to process the supernatant which might have sheared any remaining bacteria and released its contents into the supernatant leading to downstream observations. Although we do observe a 61 kDa band in the pOCS UT2300 lysate there is an absence of the expected 105 and 75 kDa bands in the lane which can be inferred as unidentified factors producing this result. Given the current data, the most plausible inference is that BrkA is cleaved but remains associated with the cell surface. However, a repetition of the Western blot procedure, while considering the potential experimental errors described above, may be needed to fully validate this conclusion.

Several alternative hypotheses may also explain these results. After OmpT cleaves at the engineered cleavage site, it is plausible that the passenger becomes unstable and is no longer protected from proteolysis as previously hypothesized. The CAR region may be involved in keeping the passenger folded and would potentially require constant association with the passenger domain to maintain structural integrity. Additionally, there is potential for the involvement of other proteases aside from OmpT, which raises questions about additional factors that are involved in autochaperone cleavage and secretion. Exploring these hypotheses with the newly constructed pENS and POCS plasmid could provide valuable insights into the complex interplay of factors governing BrkA autotransporter folding and processing.

Conclusions In summary, we made two plasmid constructs, pENS and pOCS, to investigate the region of the BrkA autotransporter passenger domain interacting non-covalently with the OM β-barrel post-cleavage. The functionality of both constructs was verified by the detection of uncleaved and cleaved BrkA at both the wild type and engineered cut sites using anti-His tag immunoblotting. Cleaved BrkA at the engineered OmpT site was detected in the protein lysate instead of the supernatant, suggesting potential cell-bound retention of the passenger rather than secretion into the extracellular space. These findings provide crucial insights into the intricacies of BrkA autotransporter processing, prompting further inquiry.

Future Directions A key focus for future investigations lies in optimizing the detection of proteins in culture supernatants. Despite the use of 0.45 µm syringe filters in the current study, Western blot analysis revealed band sizes indicative of proteins expected to be exclusively present in cell lysates, in several supernatant lanes. Therefore, exploring alternative filter sizes to eliminate contaminating bacteria before centrifugal protein concentration may prove beneficial. Additionally, the utilization of 3 kDa protein concentrator columns for culture supernatant concentration posed challenges, particularly as the anticipated extracellularly soluble fragment had a predicted size of 61 kDa. This led to filter clogging and likely retention of LB broth media proteins. To overcome these limitations, future investigations could explore the efficacy of centrifugal filters of larger sizes to enhance the efficiency of media component removal and expedite concentration, enabling higher loads of the protein of interest onto SDS-PAGE gels.

In the immediate future, replication of the second Western blot experiment with the proposed changes in supernatant protein isolation is crucial to validate observed results against our hypothesized model for non-covalent association. Exploring and comparing alternative OmpT consensus sequences could offer valuable insights into potential variations in cleavage efficiency. Additionally, investigating cloned OmpT cleavage sites in a different protein or altering its location within the BrkA autotransporter could provide further understanding of its viability and impact on processing. Alternative cleavage sites can be systematically probed and validated using immunoblot to determine the relationship between OmpT processing of BrkA and its localization after cleavage. Different affinity tags besides 6XHis can also be explored concurrently to understand potential impacts in processing. Finally, a quantitative analysis of cleavage efficiency may offer a comprehensive view of the dynamics of BrkA autotransporter processing once the mechanisms of cleavage and translocation have been fully elucidated.

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CONTRIBUTIONS

Laboratory Work. JG took the lead in developing the project idea and workflow, as well as cloning, transformation, and sequencing. CY, AZ, and DT took the lead in optimizing and executing the protocols for protein extraction, SDS-PAGE, and western blot. All members participated in all tasks.
Manuscript. AZ took the lead in writing the figure 2 and 3 results, discussion, conclusion, and acknowledgements. AZ also contributed to the introduction and future directions. CY took the lead in writing the methods for protein extraction, SDS-PAGE, and western blot, as well as annotating the western blots. CY also contributed to figure 1, and the future directions section. DT took the lead in writing the abstract, introduction, references, and helped with the discussion. JG took the lead in writing the methods for all cloning and transformation steps, creating figure 1 and supplemental figure S1, and analyzing the results. JG also contributed to the formulation of ideas for the introduction, discussion, and future directions. All authors proofread and contributed ideas for every section of the manuscript.

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