Volume 10:1-10

UJEMI+

The Undergraduate Journal of Experimental Microbiology & Immunology (+Peer Reviewed)

Escherichia coli Secretion of a Variant in the *Bordetella pertussis* Autotransporter Protein BrkA Containing a Dual Polyhistidine-Tagged 112 Amino Acid Insert Between Asp-57 And Ala-58 of its Passenger Domain

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SUMMARY *Bordetella pertussis* is a Gram-negative bacterium which expresses the Bordetella resistance to killing A (BrkA) protein. BrkA mediates autotransporter functionality and undergoes a processing event to produce a cleaved and uncleaved protein. Despite advancements in the functionality of different regions of this autotransporter, there is a lack of understanding of the mechanistic dynamics driving BrkA translocation. This study aimed to generate a sizable amino acid insert into BrkA passenger domain, analyze the structural layout of the protein, and to determine surface-expression status, regardless of the increase in molecular weight. We hypothesized that inserting a 112 amino acid spacer into the N-terminal domain will not affect BrkA surface expression, but may modulate processing events in an *Escherichia coli* model. We constructed pSERC, containing two 6x histidine tags within a 336 base pair insert in the *brkA* passenger region. The amino acid sequence corresponding to the insert revealed passenger residue repetition and potential for a protease cleavage site. Finally, we confirmed that the 112 amino acid insert-containing BrkA passenger retained surface expression in an *E. coli* model and a processing event. Our findings report that BrkA translocation is retained following a substantial amino acid insertion, and further suggests a potential avenue for studying BrkA surface expression and the complex translocation pathway that the passenger follows from cytoplasm to outer-membrane.

INTRODUCTION

A constant struggle persists between the evolutionary dynamics of bacteria and the adaptive resiliency of the host immune response. Pathogenic bacteria have been shown to develop virulence factors that contribute to enhancing disease progression (1). *Bordetella pertussis* is a Gram-negative pathogenic bacterium and an agent of infection behind the manifestation of pertussis (2). Pertussis, more commonly known as whooping cough, is currently categorized as a worldwide endemic for its capacity to cause severe respiratory infection that leads to prolonged coughing, vomiting, and in some cases brain damage due to a lack of oxygen (3,4). This severe disease manifestation, primarily concentrated in infants, is due to the actions of an outer-membrane (OM) expressed Bordetella resistance to killing A (BrkA) protein. BrkA is an autotransporter which inhibits the bactericidal ability of the innate immune system in humans through the development of complement resistance (5). Despite the establishment of a vaccine, and the associated reduction in widespread dissemination of this pathogen, the recent resurgence of the infection, and reduced efficacy of the vaccine is becoming a growing concern (3).

Published Online: September 2024

Citation: Emery, Cormack, Ruehlen, Simard. 2024. *Escherichia coli* secretion of a variant in the *Bordetella pertussis* autotransporter protein BrkA containing a dual polyhistidine-tagged 112 amino acid insert between Asp-57 and Ala-58 of its passenger domain. UJEMI+ 10:1-10.

Editor: Shruti Sandilya, University of British Columbia

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Autotransporters such as BrkA are produced by the Type V secretion system family, a diverse and widespread collection of cell-surface proteins (6). Gram-negative bacteria deliver cell-surface proteins using the autotransporter secretion system (7). BrkA is a 103 kDa precursor protein that consists of three distinct domains: 1) N-terminal signal peptide to initiate translocation across the inner membrane (IM); 2) a passenger domain which establishes extracellular effector function; 3) a conserved C-terminal β-domain which has a short alpha-helical linker region fused to an amphipathic β-barrel for membrane anchoring (8). It has previously been established that within the periplasm, the β-barrel Assembly Machinery (BAM) is used to assemble the C-terminal domain within the outer membrane (OM) to form the β-barrel essential for protein translocation (9). Oliver et al. (8) found that the 103 kDa BrkA protein is effectively cleaved to reveal a 73 kDa N-terminal protein and a 30 kDa C-terminal membrane-bound domain (8). These proteins have been hypothesized to remain associated together on the bacterial surface following cleavage. The complex dynamics underlying subunit association is significant as it may underlie BrkA functionality dynamics (8).

Dynamics driving protein translocation have been shown to involve intramolecular regions of BrkA which display chaperone functionality (8). These chaperones have been shown to be important for the effective folding of the passenger domain, either concurrent with or following OM translocation (8). Although the functions of distinct components in the BrkA-related secretion system have been analyzed, uncertainty remains regarding the mechanistic dynamics of protein secretion, and processing events. Specifically, we sought to investigate whether the addition of genetic material confers structural flexibility to retain cleavage and translocation ability across the C-terminal β-barrel.

Prior research conducted by Sun et al. (9) has demonstrated the functional importance of the passenger domain to retain translocation ability by generating a vector plasmid that replaced a region of the N-terminal passenger domain with different exogenous proteins (9). In this model, authors utilized a novel vector system and replaced the passenger domain of BrkA with alternate proteins, while retaining other functional components of BrkA (9). To note, the conserved N-terminal signal peptide, a 6X-His tag, the multiple-cloning-site (MCS), the sequence encoding the translocation determinants, and the region encoding the B-domain were all included (9). In this model, the MCS effectively replaced a part of the passenger domain, and the BrkA retained translocation capacity by exporting a range of exogenous proteins (9). This autotransporter system derived from BrkA displayed functional versatility expanding to GFP-expression, which offers initial evidence that the translocation unit (TU) is not limited to wild-type conditions to retain secretion. Building upon Sun et al.'s findings (9), we sought to investigate whether alterations to the wild-type BrkA passenger domain would impact the successful translocation of BrkA at the OM. While Sun et al.'s (9) model eliminated a significant portion of the passenger domain, our construct aimed to preserve all wild-type components and introduced a protein insert to Asp-57 to closely model Sun et al.'s site of exogenous protein insertion in the passenger domain (9). Spacer sequence of 112 amino acids was arbitrarily selected to analyze current restraints on autotransporter size selectivity. Sustaining BrkA surface expression following the introduction of an arbitrary spacer sequence could offer valuable insights into the dynamic processing events following translocation.

Considering passenger modifications did not prevent OM translocation of BrkA (9), we hypothesize that inserting a 112 amino acid spacer into the N-terminal domain will not interfere with surface protein expression compared to a single-histidine tagged BrkA control. This is based on previous analyses displaying N-terminal domain structure flexibility, without disruption to protein functionality. Understanding flexibility and size constraints of this secretion system is critical to further elucidate the mechanism by which the autotransporter system contributes to bacterial pathogenicity and toxicity in an *in vivo* model. To test this hypothesis, and evaluate modulations in protein expression, we designed a model for detecting BrkA. To enhance the expansion of the passenger domain, and improve antibody detection, we generated a randomized 112 amino-acid insert, composed of a polyhistidine tag, into the N-terminus of the passenger domain to analyze secretion specificity. This augmentation in protein size provided insight into the adaptability of this β-barrel secretion model. With the use of plasmid design tools such as SnapGene and Q5 ® Site Directed

Mutagenesis, we constructed an ampicillin-resistant BrkA-expressing pSERC. pSERC contains a 112 amino acid polyhistidine-tagged insert in the N-terminal region of the passenger domain. We transformed competent, non-pathogenic *Escherichia coli* DH5-α cells with pSERC. pSERC was sequenced to confirm insertion before employing a Western blot. Trypsin accessibility analysis confirmed BrkA surface expression following the proposed genetic engineering. Following *in vitro* analysis of BrkA autotransporter secretory mechanisms, we concluded that the BrkA model, including the 112 amino acid insert retained surface expression, offering preliminary insights into the size limits of autotransporter capacity, and the potential to retain pathogenicity despite significant genetic disruptions.

METHODS AND MATERIALS

Bacterial culture and isolation of pDO6935. *E. coli* DH5-α were cultured in Luria-Bertani (LB) broth or 1.5% agar plates containing 0.05 mg/mL ampicillin at 37°C. *E. coli* containing pDO6935 was obtained from stocks supplied by MICB 471 at the University of British Columbia and was grown in LB + 0.05 mg/mL ampicillin broth overnight before isolating plasmid using EZ-10 Spin Column DNA Cleanup Miniprep Kit (BioBasic) according to the manufacturer's instructions. Nanodrop2000 Spectrophotometer (Thermo Scientific) was used to measure DNA concentration and purity. Commercially available competent *E. coli* DH5-α cells were used for transformation.

Primer design. Primers were designed to include an insert region at the 5' end and a 3' overlap region flanking the Asp-57 site within the BrkA passenger domain using SnapGene (v7.1.0): 5'-caccaccacgaattcGCCGGGCAGGAAGGAG-3' (forward primer) and 5'-atgatgatgGTCCTGCGCATGCGG-3' (reverse primer). The BrkA overlap region was generated using the NEBase Changer. The insert region for each primer included 3xHis. The insert region for the forward primer also included an EcoRI site. The forward primer is 30 bp long and the reverse primer is 24 bp long. Length was chosen to keep the melting temperature of primers within 5°C of each other and the annealing temperature under 72°C. Melting temperatures of the primers were calculated by inputting annealing sequences of primers into the NEB Tm Calculator.

Construct development and validation. Primers were resuspended in nuclease-free water using the IDT calculation protocol to achieve a stock concentration of 100 µM. Primers were then diluted to 10 µM with nuclease-free water. Plasmid stock was diluted to 0.5 ng/µL in elution buffer from BioBasic EZ-10 Spin Column DNA Miniprep Kit. Purified pDO6935 was PCR amplified using our designed primers and the NEB Q5® Site-Directed Mutagenesis Kit, resulting in a linearized plasmid containing our construct. PCR was performed using 25 µL Q5® Hot Start High-Fidelity 2X Master Mix, 2.5 µL of each 10 µM primer, 1 ng template plasmid, 1.5 µL DMSO and nuclease-free water to achieve a reaction volume of 50 µL. PCR was performed with initial denaturation for 30 seconds at 98°C followed by 25 cycles of denaturation at 98°C for 10 seconds, annealing at 69°C for 10 seconds and extension at 72°C for 30 seconds, with a final extension at 72°C for 120 seconds. Linear PCR products were analyzed using agarose gel electrophoresis before performing ligation. Ligation was performed using 1 µL PCR product, 5 µL 2X KLD Reaction Buffer, 1 µL 10X KLD Enzyme Mix, and 3 µL nuclease-free water. Mixture incubated at room temperature for 5 minutes before being used to transform competent DH5-α *E. coli* cells and grown on ampicillin plates overnight at 37°C.

To validate the successful 112 amino acid insertion into BrkA's passenger domain, we performed EcoRI digestion on pSERC to confirm the generation of expected sizes of DNA fragments before performing whole plasmid nanopore sequencing. Isolated plasmids from selected colonies transformed with pSERC were digested with EcoRI-HF for 15 minutes at 37°C. Digested fragments were analyzed via agarose gel electrophoresis screening for fragments at 1487 bp, 1614 bp, and 3846 bp. Restriction sites within pDO6935 were identified using SnapGene. Original pDO6935 vector was also digested and used as a control. Samples that passed screening were diluted to 30ng/µL using elution buffer from EZ-10 Spin Column DNA Cleanup Miniprep Kit (BioBasic) in PCR tubes as per Plasmidsaurus sequencing

guidelines and sent for Plasmidsaurus sequencing. Sequencing results were analyzed using SnapGene and predicted protein structure was analyzed using ProtParam and PSIPRED.

Agarose gel electrophoresis. 1% agarose gels were prepared using 1g agarose powder in 100mL 1X TAE buffer with 5µL RedSafe Nucleic Acid Staining Solution. 6X Orange loading dye (Thermo Scientific) was added to DNA samples to a final concentration of 1X. O'GeneRuler™ DNA Ladder Mix (Thermo Scientific) was loaded alongside the samples. Gels were run at 100V for 90 minutes and visualized using Bio-Rad Laboratories ChemiDoc Imaging System.

Transformation of pSERC into *E. coli* DH5-α. NEB and Invitrogen DH5-α Competent Cells were transformed with pSERC using heat shock at 42°C for 30 seconds, followed by incubation on ice for 2 minutes and at 37℃ for 1 hour. Following incubation at 37℃, transformed cells were spread-plated onto LB agar plates containing 50 µg/mL ampicillin and grown overnight at 37℃. Selected colonies from the plates were grown in LB + 50 µg/mL Ampicillin broth overnight to be used for plasmid isolation and restriction digestion.

Plasmid isolation. Plasmid isolation was accomplished using the EZ-10 Spin Column DNA Miniprep Kit (BioBasic). Nanodrop2000 Spectrophotometer (Thermo Scientific) was used to measure DNA concentration and purity. Plasmids were extracted from NEB DH5-α cells to be used for restriction digestion, sequencing, and transformation of Invitrogen DH5-α competent cells for downstream analysis of protein expression.

Trypsin assay & lysis. pSERC transformed DH5-α and control pDO6935 transformed DH5-α cultures were pelleted, washed with PBS, resuspended in PBS + 200 µg/mL Trypsin or PBS controls and incubated at 37℃ for 10 minutes. Samples were washed again with PBS. Nanodrop2000 Spectrophotometer (Thermo Scientific) was used to measure protein concentration. Samples were resuspended in 2X Laemmli Sample Buffer (BioRad) with 5% BME for a final protein concentration of 15 µg/µL, denatured at 95℃ for 1.5 minutes, and centrifuged. Samples prepared likewise for pPALMC1 (6 amino acid insert, single 6xHistidine tag at D59 of BrkA) transformed *E. coli* were generously provided by A. Lalani, L. Bilal, M. MacAulay, P. Malhi (UJEMI manuscript in press, 2024).

SDS-PAGE & Western blotting. Prepared samples were loaded onto Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad) alongside Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder and run at 200V for 45 minutes using Bio-Rad Mini-PROTEAN Tetra Cell apparatus. Proteins separated on gel were transferred to a PVDF membrane using Trans- Blot Turbo Transfer Packs (Bio-Rad) and Trans-Blot Turbo Transfer System Machine (Bio-Rad). Following transfer, membranes were stained with Ponceau S Staining Solution and washed before blocking. Membranes were blocked with TBS-T + 5% skim milk for 1 hour while shaking at room temperature before incubating overnight at 4℃ in 10 mL primary 6x-His Tag Monoclonal Antibody (InvitrogenTM) diluted 1:1000 in blocking buffer. Membranes were washed 3 times for 5 minutes each with TBS-T while shaking and then incubated for 1 hour while shaking at room temperature in 15 mL Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HR (Invitrogen ™) diluted 1:100000 in blocking buffer. Following incubation, membranes were washed with TBS-T as previously described. Membranes were then incubated for 5 minutes in 7 mL Clarity Western ECL substrate reagents (Bio-Rad) before visualizing using Bio-Rad Laboratories ChemiDoc Imaging System.

RESULTS

Constructed pSERC contains a double 6xHistidine insertion within *brkA*'s passenger domain. In order to investigate the impact of an insert region within BrkA's passenger domain on outer-membrane translocation in *E. coli*, we designed a plasmid which included a 336 base pair (112 amino acid) insert containing two 6xHistidine tags into *brkA*'s passenger domain. Sequencing results revealed that the 336 base pair insertion at Asp-57 within *brkA*'s passenger domain was successful with the remainder of downstream *brkA*

remaining in-frame (Figure 1A). As illustrated in Figure 1C, the 112 amino acid insert consists of two 6xHistidine tags, two EcoRI sites, and a 96 amino acid spacer sequence. These sequencing results suggest that pSERC contains the desired insert within the *brkA* gene and may be used further to investigate BrkA translocation.

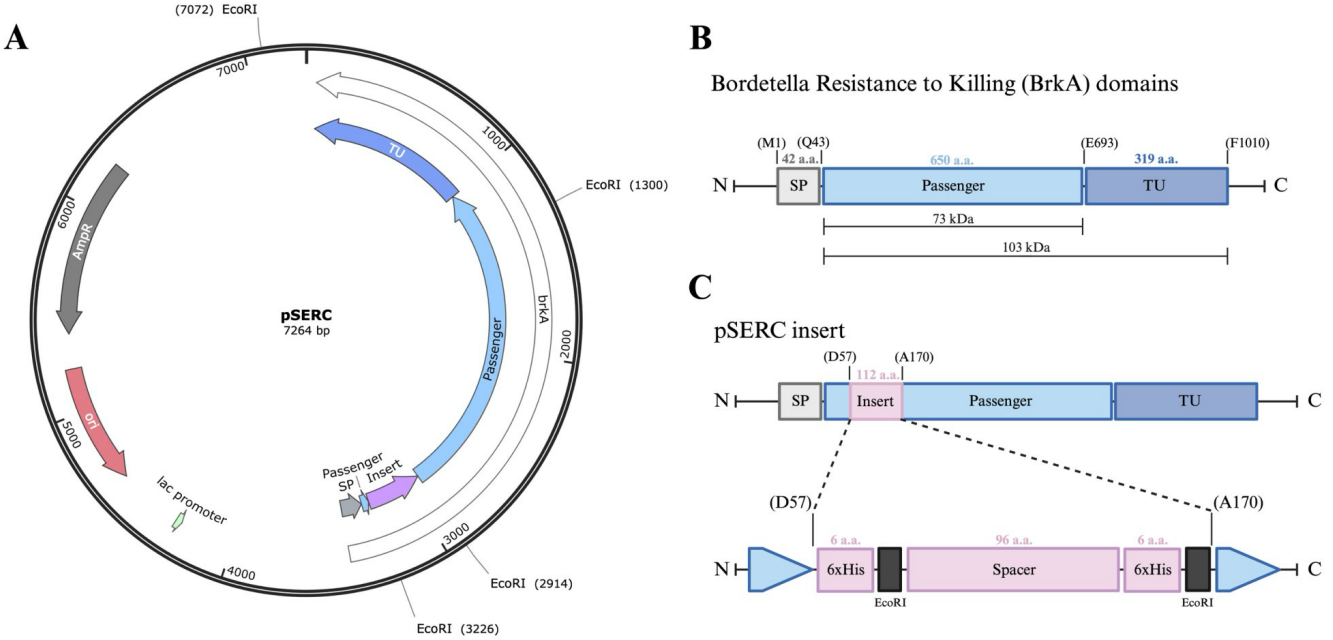


FIG. 1 Designing pSERC: 112 amino acid insert within BrkA's passenger domain contains two 6xHistidine tags. (A) 336 base pair insert (2914bp-3249bp) added to passenger domain of *brkA* in pSERC. (B) Domains in wild-type BrkA: SP (signal peptide), a.a. 1-42; Passenger domain, a.a. 43-692; TU (translocation unit), a.a. 693-1010. Amino acid length is "a.a.." Molecular weights computed using ProtParam. (C) 112 amino acid insert in pSERC BrkA: 6xHis (histidine tags), a.a. 58-63 and 162-167, respectively; Spacer sequence, a.a. 66-161.

To further validate the successful 112 amino acid insertion into BrkA's passenger domain, we performed EcoRI digestion on pSERC to confirm the generation of expected sizes of DNA fragments. pDO6935 was utilized as a negative control to aid in the identification of any size alterations in DNA fragments. pDO6935 contains two EcoRI sites at 1300 and 6735 bp, respectively, and we therefore expected to see two distinct DNA fragments following digestion: a 1491 bp band and a larger 5436 bp band (Figure 2A). Figure 2C (lane 1) shows two distinct bands which correspond to the aforementioned EcoRI generated pDO6935 fragments. Comparatively, pSERC contains four EcoRI sites: two identical sites to pDO6935, and two additional sites within the *brkA* passenger insert (Figure 2B). As predicted, EcoRI digestion of pSERC yielded four distinct DNA bands at approximately 1492, 1614, 3846, and 312 bp (Figure 2C). It should be noted that the bands ran at a slightly larger size than anticipated, likely due to an inconsistency between ladder separation and DNA fragment migration during SDS-PAGE. For whole-gel image, see Supplemental Figure S1. The generation of distinct DNA bands at the predicted sizes compared to the negative control further validates that pSERC contains the desired 112 amino acid insert at Asp-57 within *brkA*'s passenger domain.

Nanopore sequencing and structural analysis reveals the location and conformation of the double 6xHistidine tag within pSERC BrkA. To investigate the location and conformation of the 112 amino acid insert within the BrkA protein, we compiled nanopore sequencing data and conducted various structural analyses. SnapGene and PsiPred analyses revealed a general lack of organized secondary structure within the insert region, with the majority of the insert existing in a coiled conformation (Figure 3A). Finally, the general location of the 112 amino acid insert was considered by examining a three-dimensional model of BrkA using AlphaFold. As illustrated in Figure 3C, the insert was predicted to be located at the N-terminal passenger region following the signal peptide cleavage site, and was inserted outside the folded region of the passenger domain. Taken together, these results suggest that

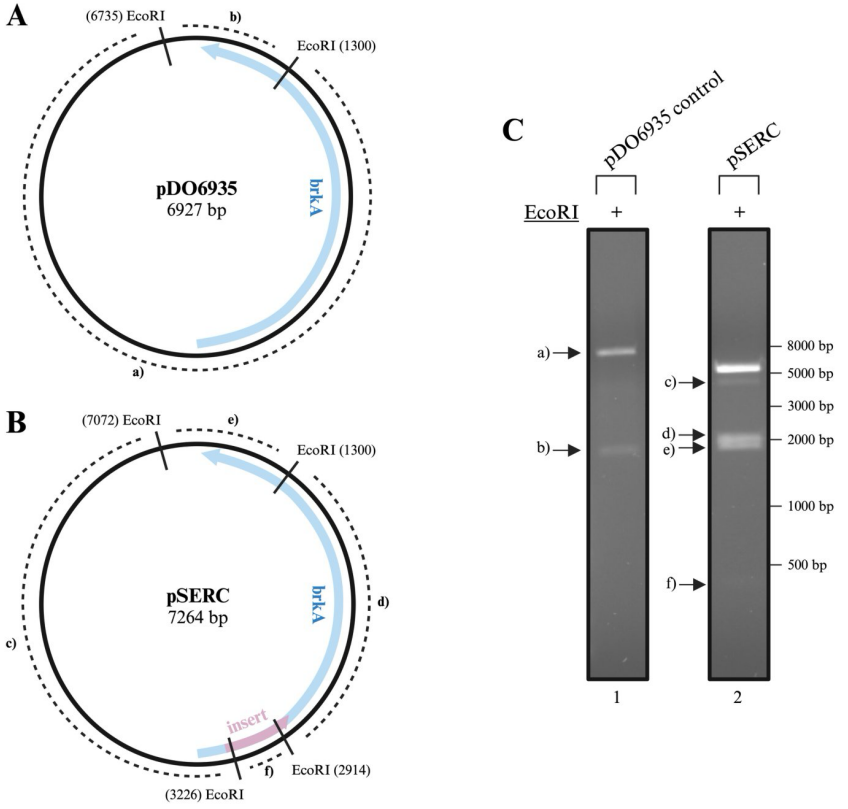
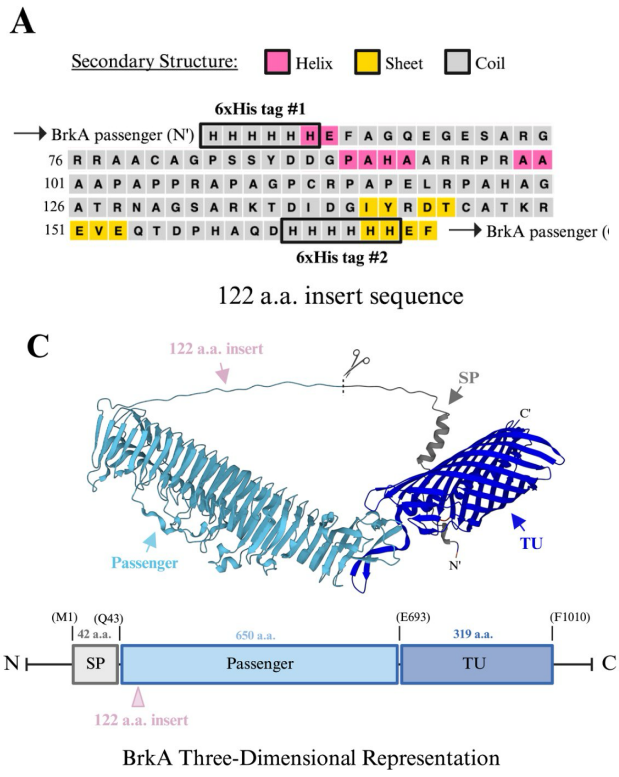


FIG. 2 112 amino acid insertion into *brkA*'s passenger domain validated using EcoRI digestion sites. (A) Fragments produced from EcoRI digestion of pDO6935 control plasmid. (B) Fragments produced from EcoRI digestion of pSERC. Insert region contains two additional EcoRI sites. (C) EcoRI digestion of purified pDO6935 (control) and PSERC (112 a.a. insert) plasmid. Arrows a) and b) correspond to DNA fragments produced from pDO6935 (Figure 2A), and arrows c) - f) correspond to DNA fragments produced from pSERC (Figure 2B).



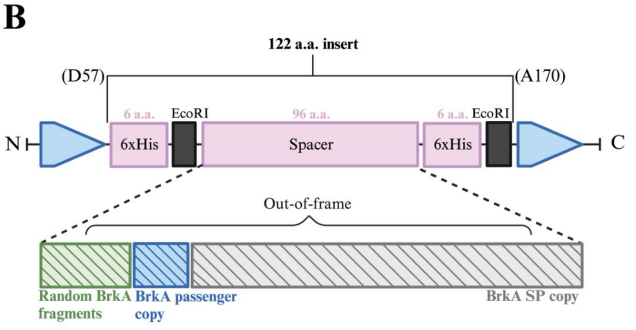


FIG. 3 Structural analysis of the 112 amino acid insert of the pSERC BrkA protein. (A) Secondary structures within the 112 a.a. insert of pSERC BrkA. Structure predictions computed with PsiPred. (B) Composition of the 96 a.a. spacer sequence. (C) Three-dimensional protein diagram illustrating BrkA's protein domains. TU (translocation unit) in dark blue; Passenger (light blue); SP (signal peptide) ingrey; 112 a.a. insert (pink). Scissors denote the location of the SP-passenger domain cleavage site.

the double 6xHistidine insert may be expressed at the N-terminal of the BrkA passenger, without introducing any significant structural changes to BrkA.

BrkA with a 112 amino acid insert is trypsin accessible in *E. coli*, suggesting surface expression. Finally, we aimed to determine whether BrkA containing the 112 amino acid insert would be translocated across the OM and surface-expressed. For pSERC-derived BrkA,

which contains 112 amino acid insert and two 6xHistidine tags, the predicted protein sizes were 110.95 kDa for uncleaved BrkA (passenger and translocation unit) and 76.63 kDa for cleaved BrkA (passenger alone) (Figure 4A). For pPAMLC1-derived BrkA, we predicted an uncleaved protein size of 99.68 kDa, and 65.37 kDa for the cleaved conformation (Figure 4B). Despite these predictions, both pSERC and pPALMC1-derived BrkA exhibited identical band sizes in the absence of trypsin (Figure 4C), with uncleaved BrkA running at approximately 120 kDa, and cleaved BrkA running at approximately 85 kDa across both conditions. However, in the absence of trypsin, pSERC-derived BrkA exists in the 85 kDa cleaved form at a greater proportion than the uncleaved form, and the opposite is observed for pPALMC1-derived BrkA (Figure 4C). In the presence of trypsin, the 85 kDa cleaved form of BrkA (Figure 4C) is absent in both the pSERC and pPAMLC1-derived conditions. The absence of cleaved BrkA when incubated with trypsin suggests cell-surface accessibility of the protein. For whole-gel image, see Supplemental Figure S2.

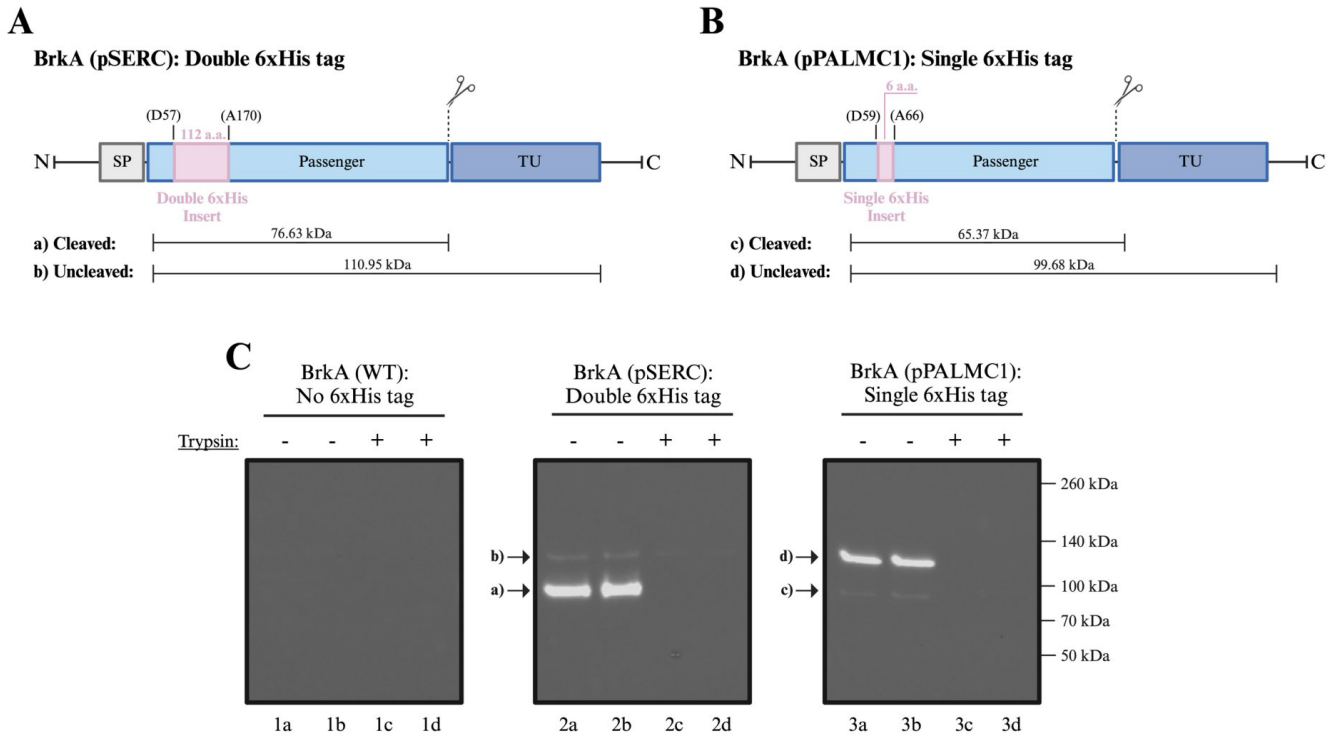


FIG. 4 Trypsin analysis identifies surface expression in both single and double 6xHistidine-tagged BrkA. (A) Fragments produced from double 6xHis-tagged BrkA with 112 a.a. insert. (B) Fragments produced from single 6xHis-tagged BrkA. Molecular weights predicted using ProtParam. (C) Detection of cell surface 6xHis for DH5-alpha *E. coli* cells transformed with pDO6935 (WT BrkA), pSERC (double 6xHis-tagged BrkA), and pPALMC1 (single 6xHis-tagged BrkA) using trypsin-accessibility analysis.

DISCUSSION

Traversing both the cytoplasmic and OM within a Gram-negative bacterium follows a complex pathway that has not been fully characterized. In the case of *B. pertussis*, a sec-dependent translocation mechanism has been implicated in the secretion of its notable BrkA protein (8). Next, the C-terminal domain of BrkA inserts itself into the OM, behaving as a β-barrel channel and allowing for passenger domain insertion through the OM into its integral location (8).

Building upon this idea, we sought to discover how the insertion of a 336 base pair insert -112 amino acids - containing a double histidine tag, into the N-terminus of the passenger domain would interfere with the surface expression of BrkA in an *E. coli* model (Figure 5). In our model, cleaved surface expressed BrkA was absent in the presence of protease activity, indicating the absence of detectable surface-expressed BrkA passenger following digestion.

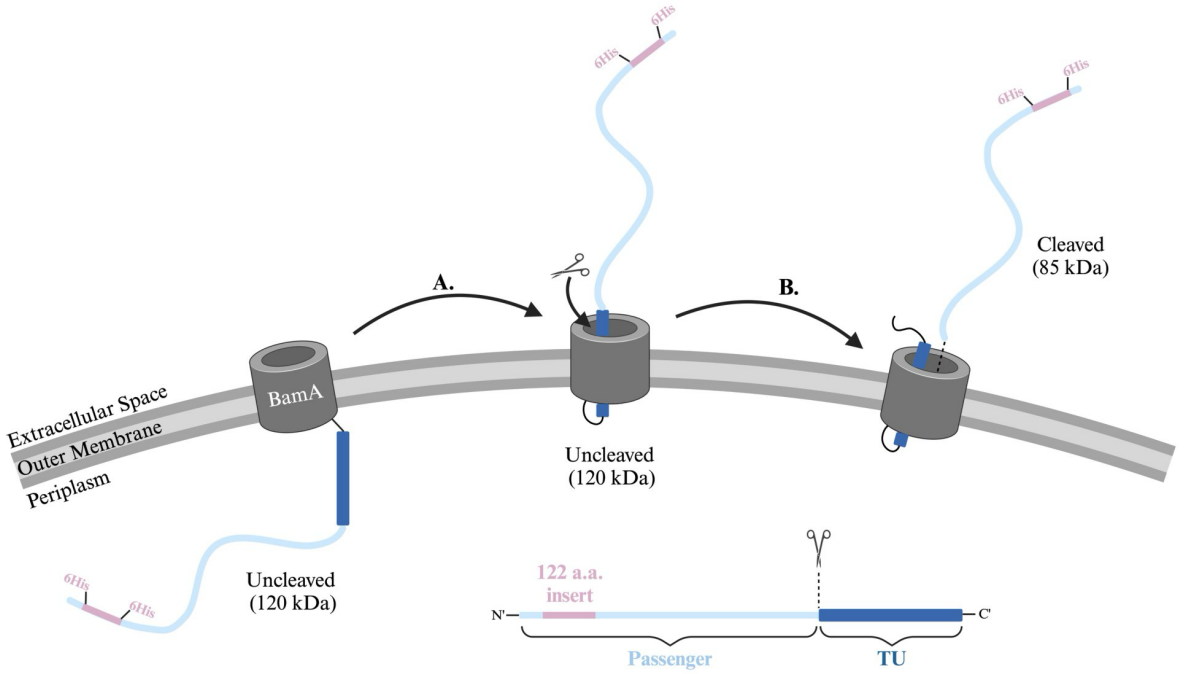


FIG. 5 Working model of pSERC-derived BrkA surface expression across *E. coli* outer membrane. BrkA is initially translocated into the periplasm where the N-terminal signal peptide (not shown) is cleaved. (A) The TU forms an amphipathic beta-barrel and folds into the OM. Passenger domain is translocated through the OM, remaining bound to the TU. Passenger folds in the extracellular space (not shown). (B) Following translocation, beta-barrel autoproteolytic activity cleaves the passenger which remains non-covalently bound to the cell surface. Figures created with Biorender.com

This suggests that the translocation of BrkA was not inhibited following a 112 amino acid insert despite the addition of a 336 base pair insert in the passenger domain.

It is important to note that our findings were produced somewhat serendipitously, and there is limited available research to serve as the foundational context for our results. It is therefore challenging to directly apply the generation of a double polyhistidine insert with a large spacer region within BrkA to existing research.

As discussed, Sun et al. (2015) replaced the BrkA functional passenger domain with foreign protein by inserting genetic material between two restriction digest sites (9). Additionally, a 6xHis tag was inserted within the N terminal of the passenger domain, downstream of the signal peptide. The researchers noted that they purposefully constructed their vector with N-terminal BrkA residues between the signal peptide and the polyhistidine tag to ensure cleavage efficiency was not altered positively or negatively (9). This suggests that alterations to the N-terminus of the BrkA protein could potentially modulate cleavage efficiency. Sun et. al (2015) previously indicated the effector function of the passenger domain, which called into question the capacity to modify the domain while retaining functionality. Introducing a 112 amino-acid insert and retaining surface expression broadens understanding on the flexibility of protein translocation, in regards to size, for this Type V system. Although Sun et. al (2015) previously displayed maintenance of surface expression despite GFP addition to the passenger domain, our introduction of novel secondary structures while retaining wild-type passenger domain indicates a broader capacity to modulate *brkA* to further analyze the development of pathogenicity for *B. pertussis.*

Barnes et al. (2001) previously showed that secretory functionality is critical for bacterial pathogenesis. Specifically, secretion of the 73 kDa protein effectively aids in the inhibition of the classical pathway of complement activation, to prevent C4 accumulation (5). As protein translocation was retained despite the addition of a sizable insertion into the effector domain, this model provides subsequent evidence that *B. pertussis* is capable of adapting to complex mutations constantly occurring over evolution, thus leading to sustained pathogenicity.

Limitations Normalization of the loaded samples was limited for SDS-PAGE by the pPALMC1 samples that were generously provided by A. Lalani, L. Bilal, M. MacAulay, P. Malhi (UJEMI manuscript in press, 2024). Since the samples came pre-prepared and pre-diluted in Laemmli buffer and BME, we were unable to measure the protein concentration in those samples. Therefore, we were not able to normalize the pPALMC1-derived BrkA to PDO6935 and pSERC-derived BrkA. For this reason, we did not quantitatively compare between these conditions for our Western blot results. Therefore, we cannot determine whether there is more expression of BrkA in transformants in the pPALMC1 condition compared to the Pserc condition featuring the 112 amino acid insert. We can only conclude whether the double 6xHistidine insert is still expressed.

Conclusions To conclude, we created a construct, pSERC plasmid containing two polyhistidine tags within a 336 base pair insert that was located within the N-terminal region of the *brkA* passenger region. The corresponding 112 amino acid insert revealed passenger residue repetition. Finally, the 112 amino acid insert-containing BrkA passenger was shown to be surface expressed in an *E. coli* model.

Future Directions To mitigate our limited ability to compare across conditions, we could perform the trypsin assay and lysis using transformants containing pSERC, pPALMC1 or pDO6935, and measure protein concentration to normalize across conditions. Our research found that the pSERC-derived BrkA condition produced bands of the same size as the pPALM-derived

BrkA condition despite having a larger insertion. We discussed how we predicted that there might be a cleavage site in the spacer region between the two 6xHistidine tags. Future studies could explore the functional potential of this spacer to confirm if a protease region exists. Another avenue for future research with pSERC is studying complement evasion. BrkA passenger domain plays an important role in the evasion of the innate immune system through binding to complement C4 (5). Future research could test if increasing the size of the BrkA passenger domain, which was done to create pSERC, is correlated with host immune response.

ACKNOWLEDGEMENTS

We would like to thank Dr. David Oliver, Herieth Ringo and Jade Muileboom for their continuous guidance and support for our project. We would also like to thank the other teams in MICB 471 this term for their support, especially A. Lalani, L. Bilal, M. MacAulay, P. Malhi who generously provided us with pPALMC1 transformed *E. coli* samples. Lastly, we would like to thank the Department of Microbiology and Immunology at the University of British Columbia for providing access to the laboratory resources used in our project. We would also like to thank the anonymous reviewer for constructive feedback on this manuscript.

CONTRIBUTIONS

All authors contributed to the lab work completed in MICB 471. N.C contributed to the abstract and references, and completed the introduction. H.E completed methods, limitations, and future directions. M.R contributed to the abstract and references, and completed the discussion and conclusion. A.S completed the results, designed and generated the figures, wrote the figure captions, and generated the supplemental materials.

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