Undergraduate Journal of Experimental Microbiology and Immunology (UJEMI) Vol. 29:1-8

A 6xHistidine tag insertion at the N-terminal signal peptide A^{42} and G^{43} may interfere with BrkA secretion and expression in *Escherichia coli* DH5α

Shini Chen, Haohua Li, Zhuohui Lin

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY BrkA is a type Vα autotransporter on the gram-negative human pathogen *Bordetella pertussis.* BrkA mediates serum resistance and cellular adherence; however, the secretion mechanism of BrkA has yet to be fully elucidated. We aimed to investigate the secretion of BrkA into the periplasm and extracellular space. We inserted a 6xHis-tag at the N-terminal signal peptide A^{42} and G^{43} on *brkA*-containing pDO6935. We then conducted an immunoblot assay using our test plasmid but did not observe BrkA secretion in *Escherichia coli* (*E. coli*) DH5α. Follow-up DNA sequencing of the newly constructed 6xHis-tagged BrkA coding plasmid showed that a frameshift mutation had been introduced in the *brkA* sequence, which resulted in a truncated and possibly unstable protein product. Since we were unable to isolate clones containing the desired 6xHis tag insertion in BrkA, we speculate that the 6xHis tag insertion near the signal sequence cleavage site may have resulted in the production of a toxic BrkA variant, which may have stalled in the Sec translocon resulting in cell death.

INTRODUCTION

Bordetella resistance to killing (BrkA), is a Type Vα autotransporter produced by the gram-negative human pathogen *Bordetella pertussis*. *B. pertussis* causes whooping cough, and it has been estimated worldwide that whooping cough causes nearly 295,000 deaths out of 48.5 million cases in total each year (1, 2). In addition, there was a 4% mortality rate among infants in low-income countries per year (2). Among the multiple virulence factors, BrkA has been known to mediate serum resistance and facilitate adherence of bacterium to host cells (3). In the BrkA secretion system, a 42 amino acid-long N-terminal signal peptide of the 103kDa BrkA precursor is cleaved by signal peptidase I (SPase I) (3). The passenger domain transits across the cytoplasmic membrane through the Sec translocation pathway (3, 4) . Periplasmic chaperones such as SurA are required for the correct folding of BrkA (5). Once the protein is in the periplasm, its N-terminal passenger domain (Q^{43} - N^{731}) is cleaved from the C-terminal translocation unit (A^{732} - F^{1010}) between residues N^{731} and A^{732} (3). The 30kDa translocation unit then inserts itself into the outer membrane (e.g., Omp85) and forms a pore to direct the passenger domain across the outer membrane (6, 7). The passenger domain is tightly associated with the bacterial surface, and its N terminal is exposed to an extracellular environment (3). Exploring the translocation of the BrkA is essential to gain insights into the determinants of the protein's pathogenesis.

His-tags, containing six or more histidine residues, have been widely used for protein expression and purification (8). His tags can be added to the C- or N-terminus of a protein. The relatively small size of His tags minimizes interference with the function and structure of proteins, including BrkA (9, 10). For example, 6xHis-tagged *bkrA* N-terminal passenger domain sequence (between 181-198 nucleotides) can be used to screen for functional displays of exogenous proteins expressed on the surface of *E. coli* (9). In addition, a 6xHis-tag insertion at 37 amino acids upstream of the C-terminal processing site of pRSETb confirmed that the C-terminal BrkA domain has formed a pore in lipid bilayer membranes to export the N-terminal domain in the formation of a hairpin loop in *E. coli* BL21(10). Despite the His-tag insertions at different locations of the BrkA, it has yet to be inserted between the N-terminal signal peptide and passenger domain to study the BrkA secretion system. Such

Published Online: September 2024

Citation: Chen, Li, and Lin. 2024. A 6xHistidine tag insertion at the N-terminal signal peptide A^{42} and G^{43} may interfere with BrkA secretion and expression in *Escherichia coli* DH5α. UJEMI 29:1-8

Editor: Shruti Sandilya and Ronja Kothe, University of British Columbia

Address correspondence to: https://jemi.microbiology.ubc.ca/

insertion might provide a better understanding of BrkA passenger domain secretion in the extracellular environment.

Here, we investigated whether a 6xHis tag insertion to the +1 position of the N-terminal BrkA signal peptide impacts the cleavage of SPase I. Previous studies have shown that SPase I recognized the consensus alanine residues at the -3 and -1 positions in the C region of the Sec-dependent non-lipoprotein signal peptide in *E. coli* (4, 11). However, it has been reported that BrkA is processed between residues of the signal peptide A^{42} and the passenger domain Q^{43} in *B. pertussis* and *E. coli* (3). In this paper, we inserted a 6xHis-tag between A^{42} and Q^{43} within *brkA*-containing pDO6935 to study whether the insertion interferes with BrkA expression and extracellular secretion. We obtained an out-of-frame *brkA* sequence. Subsequently, immunoblot analysis suggested interference of the His-tag to BrkA expression. We were unable to detect BrkA expression of the His-tagged 103kDa protein precursor or the 73kDa passenger domain.

METHODS AND MATERIALS

Preparation of liquid LB media, agar plates, and ampicillin. Filter-sterilized ampicillin (1000x) was prepared at 100 mg/mL and stored at -20 °C. Gibco liquid Luria-Bertani (LB) broth and LB agar were prepared based on the manufacturer's instructions (Fisher Bioregents), autoclaved, and stored at room temperature and 4°C, respectively. Ampicillin (100 ug/mL) was added to liquid LB broth and agar.

Extraction of *brkA*-containing pDO6935 plasmid from *E. coli* HB101 cells. Cells were cultured overnight at 37°C at 200 rpm in 4 mL LB broth with 1:1000 ampicillin. Plasmids were isolated and purified using the EZ-10 Spin Column Plasmid DNA Minipreps Kit and according to the manufacturer's instruction (BioBasic). Purified plasmids were measured using a NanoDrop 2000/2000c spectrophotometer and stored at -20°C.

6xHis tag insertion into *brkA*-containing pDO6935 using polymerase chain reaction (PCR). Forward (1iota_Fw1, 5'-atggtgatgCGCCGCCGCGGGCGCCA) and reverse (1iota_Rv1.1, 5'-caccatcacCAGGCGCCGCAGCCGCC) His-tag containing primers were obtained from the manufacturer (IDT). PCR reactions were performed in 25µL reaction volumes comprising DNA template (15ng/µL), 0.25µM of each primer, 12.5µL Q5 Hot Start High-Fidelity 2X Master Mix, and 0.5µL nuclease-free water. The reactions were performed with one minute of initial denaturation at 98ºC, followed by 25 cycles of 20 seconds of denaturation at 98ºC, 30 seconds of annealing at 68ºC, and four and a half minutes of extension at 72ºC. In the final extension step, the reaction was held at 72ºC for 3 minutes. Reactions were mixed with 6x Purple Loading Dye (New England BioLabs) before being run on a 1.0% agarose gel. 1 Kb Plus DNA Ladder (Invitrogen™) was used as a reference ladder. Gels were imaged using BioRad ChemiDoc™ MP Imaging System.

Ligation of 6xHis tagged *brkA*-containing pDO6935. His-tagged plasmid was ligated using a Q5 site-directed mutagenesis kit from New England BioLabs. 1µL PCR product of His-tagged *brkA*-containing pDO6935 was mixed with 5µL 2X Q5 kit KLD reaction buffer, 1µL 10X KLD enzyme mix, and 3µL nuclease free water. The mixture was incubated for five minutes at room temperature before transformation.

Heat shock transformation of the 6xHis-tagged *brkA*-containing pDO6935 into *E. coli* DH5α cells. 5µL of KLD mix was added to 50µL of chemically competent *E. coli* DH5α cells and incubated on ice for 30 minutes (12). Heat shock was then performed at 42ºC for 30 seconds. 950µL super optimal broth with catabolite repression medium (SOC) was added to the cells and gently shaken at 37ºC for 1h. 40-100µL cells were then spread onto LB agar plates containing ampicillin and incubated overnight at 37ºC.

Immunoblot assay. The protocol was adopted from Bio-Rad Laboratories Inc. Bulletin 6376. Briefly, 1mL of overnight *E. coli* LB culture was resuspended in 50µL of 2X Laemmli sample buffer (BioRad, Cat#: 1610737) and boiled for 3 minutes. The supernatant was collected.

12µL of lysate was run in each lane of 15-well 4–20% Mini-PROTEAN TGX Precast Protein Gel (BioRad, Cat#: 4561096). Separated protein was transferred using the Trans-blot Turbo transfer system (Bio-Rad Laboratories) to mini 0.2 µm PVDF transfer packs (BioRad, Cat#: 1704156). The membrane was stained with Ponceau S and blocked in 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) at room temperature for 45 minutes. Primary mouse anti-6xHis antibody (Invitrogen, Cat#: MA1-21315, 1:3000 dilution) was diluted in blocking buffer with 0.1% Tween20 and incubated with the membrane in the cold room overnight. Secondary antibody goat-anti-mouse-IgG HRP-conjugated secondary antibody (ThermoFisher, Cat#: 31430) diluted 1:10000 in blocking buffer with 0.05% Tween20 and was incubated with the membrane for 45 minutes. The membrane was washed with TBS, visualized with Clarity western ECL substrate (Bio-Rad Laboratories, Cat#: 170-5060), and imaged using the ChemiDoc MP Imaging System (Bio-Rad Laboratories).

Sanger and whole plasmid sequencing analysis of 6xHis-tagged *brkA*-containing pDO6935. Plasmid was prepared for Sanger sequencing in 0.2 mL PCR strip tubes in accordance with GENEWIZ's (Azenta Life Sciences) pre-defined guidelines. 500ng plasmid containing His-tag was mixed with 5µL of 5µM sequencing primer (5'-GCATGGCTCGTGCCTGAT-3') and nuclease-free water to a final volume of 15µL. Insertion of His-tag was confirmed using Snapgene software. In addition, pHAK whole plasmid was prepared for whole plasmid sequencing with a final concentration of 30ng/µL in sterile water and sequenced by Plasmidsaurus. Random sequence insertion was identified using Snapgene software.

RESULTS

A 6xHis-tag was inserted at A^{42} and Q^{43} on *brkA*-containing pDO6935 (pHAK). To study whether 6xHis-tag insertion interferes with BrkA secretion and expression, we used a site-directed mutagenesis kit to introduce this tag to *brkA*-containing pDO6935. We first designed a DNA construct using the *brkA* sequence available on NCBI (Ref: WP_015039078.1), to add an in-frame 18-bp 6xHis-tag sequence between the signal peptide passenger domain (Fig. 1A). The insertion is located directly downstream of the consensus

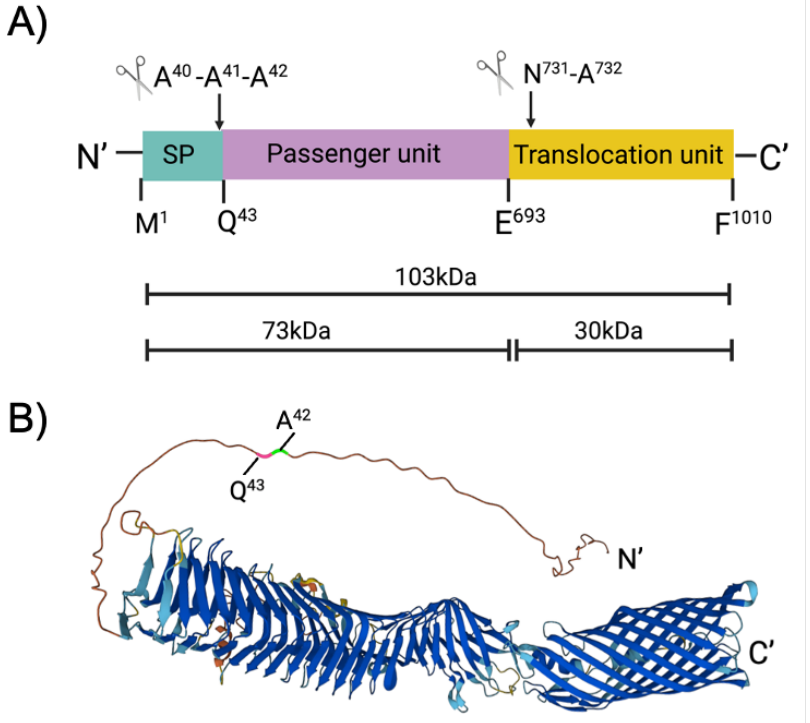
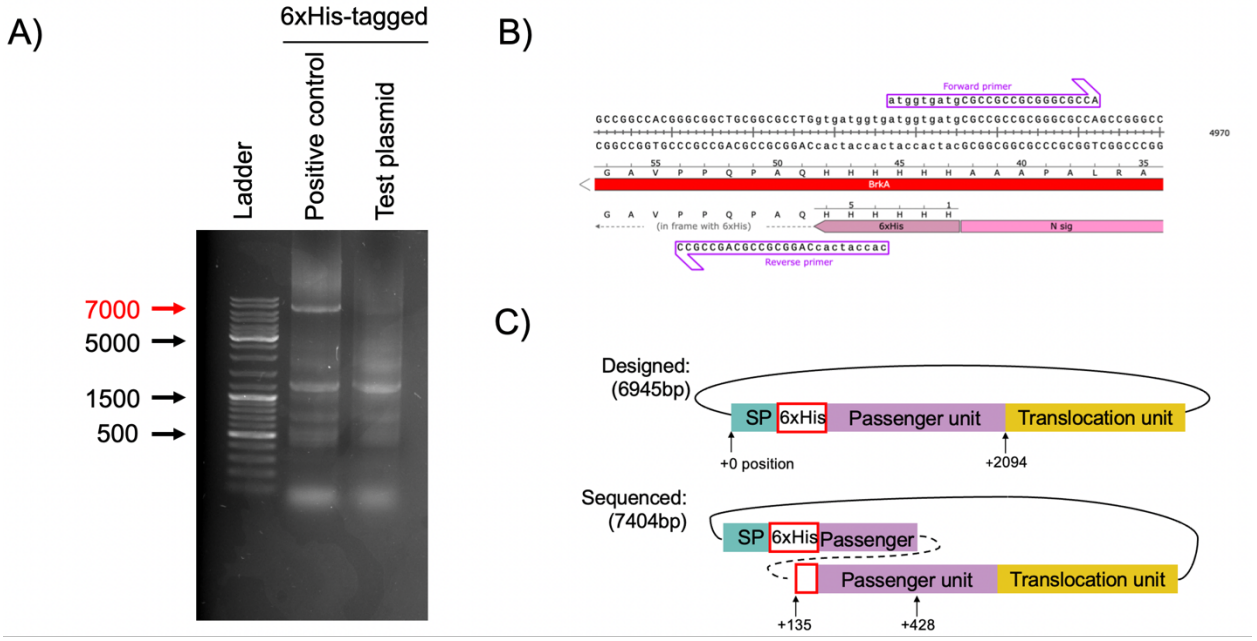


FIG. 1 Domain structure of BrkA. (A) Protein domain map of BrkA from *Bordetella pertussis*. The positions and lengths of amino acid sequences for signaling peptide (SP), passenger domain, and translocation unit were indicated. The specific cleavage sites were illustrated by arrows. The molecular weight was annotated. (B) The three-dimensional, tertiary structure prediction of *Bordetella pertussis* BrkA was derived from AlphaFold. The base pairs upstream and downstream of 6xHis-tag insertion were coloured. Green, A^{42}; Purple, Q^{43}. Created with Biorender.com.

AXA SPase I recognition site (11). Due to the small size of the insert, we attempted overlap-based ligase-free ligation of the gradient PCR product with *E. coli* DH5α, which produced only wild-type transformants, confirmed by Sanger sequencing. We then attempted to optimize the primer annealing temperatures with no success (Fig. S1). We then optimized the

conditions of the Q5 polymerase reaction (Fig. S2-3). We performed two PCR reactions with DNA templates from different sources and Q5 polymerase, one of which amplified the desired 7kb full plasmid with a 9bp half-His tag overhang on each end (Fig. 2A, B). The kinase, ligase, and DpnI (KLD) reaction of the positive control product was transformed into *E. coli* DH5α and the resulting colonies were inoculated and sequenced with nanopore sequencing. The KLD reaction resulted in a few insertions and deletions and one major 293 bp duplication downstream of the His tag (Fig. 2C). The sequence between nucleotide 135 and 428 was duplicated, resulting in an out-of-frame BrkA, consisting of a part of the passenger domain and the entire translocation unit (Fig. 2C).



BrkA expression was undetectable using the 6xHis-tagged BrkA encoded by pHAK.

FIG. 2 Designed 6xHis-tag insert in *brkA*-containing pDO6935. (A) PCR amplified 6xHis-tagged positive control and test plasmids were confirmed using 1% agarose gel. The expected band size was indicated by a red arrow (~7000bp). (B) In-frame 6xHis-tag insertion was illustrated. The location of the 6xHis tag, and forward and reverse primers were annotated in SnapGene software. (C) Alignment of nanopore-sequenced plasmid with our design, highlighting the major differences. Arrows denote locations (in base pairs) relative to the start codon in the designed plasmid.

To detect BrkA expression, we extracted proteins from overnight cultures of *E. coli* DH5α strains expressing two distinct 6xHis-tagged BrkA. Immunoblot analysis using anti-His antibody was performed to detect the expression of 6xHis-tagged BrkA on pDO6935. pHAK encodes an insertion at the +1 site relative to A^{42} while the positive control pDO6935-H encodes the same His tag at +18 (after G^{59}) relative to A^{42}, where the BrkA expression has been confirmed (Fig. 3). We were able to detect 6xHis-tagged BrkA expressed by positive control at roughly 103kDa and 73kDa. We failed to detect pHAK-encoded truncated 6xHis-tagged BrkA by the 293 bp duplication (Fig. 2C, 3).

DISCUSSION

BrkA, as a virulence factor of *Bordetella pertussis,* mediates the resistance to complement killing via the classical pathway by an unknown mechanism (1). BrkA secretion has been studied in a non-pathogenic *E. coli* using pDO6935 (3). In the proposed model of BrkA secretion, the N terminal signal peptide directs the export of BrkA precursor from the cytoplasm to the periplasm via the Sec system (Fig. 4). Then the signal peptide is cleaved, and the protein is released in the periplasm (7). Subsequently, the passenger domain is transported across the outer membrane (3). However, the exact mechanism behind signal peptidase SPase I cleavage of N-terminal BrkA signal peptide is unknown. It has been reported that SPase I recognizes consensus sequence AXA at position -3 to -1 to the cleavage site (13). Meanwhile, previous studies showed that BrkA is processed between residue A^{42}

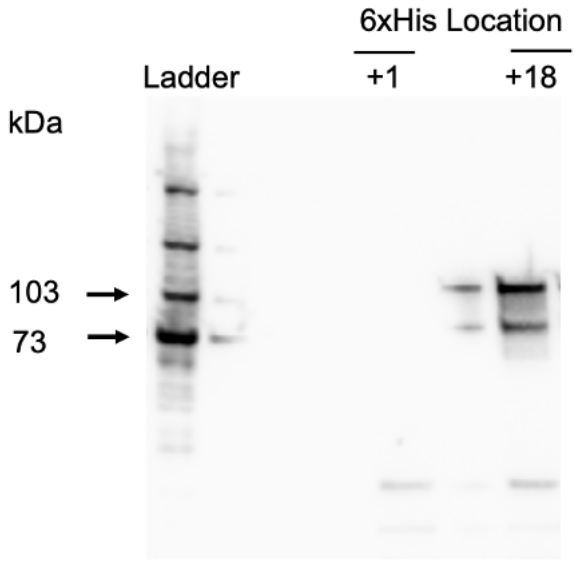


FIG. 3 Immunoblot detection of 6xHis-tagged BrkA. Protein lysates of two clones of 6xHis-tagged BrkA-expressing *E.coli* DH5α were separated on SDS-PAGE and blotted for 6xHis tag. 6xHis-tag insertion locations are shown in relation to A^{42}. +1 by test plasmid pHAK, +18 by positive control.

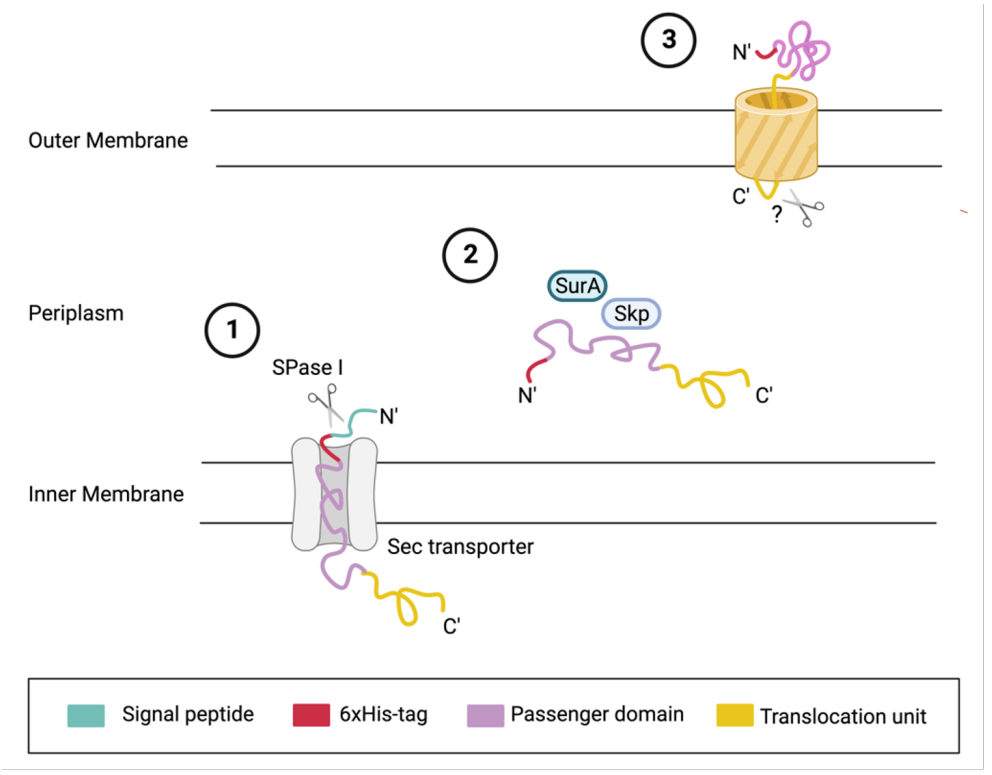


FIG. 4 Hypothetical secretion working model of 6xHis-tagged *brkA*-containing pDO6935. 1) BrkA precursor sequence translocates across the inner membrane via the Sec transporter. SPase I recognizes and cleaves at +1 of the AAA (A^{40}-A^{41}-A^{42}), releasing the sequence to the periplasm. 2) Periplasmic chaperones such as SurA and Skp will ensure the correct conformation of the protein sequence. 3) The translocation unit directs the 6xHis-tagged passenger domain across the outer membrane to the extracellular domain. The unknown protease cleaves at Q^{731}- A^{732} of the translocation unit in an Omp-independent manner (3). Created with Biorender.com.

and Q^{43}. Therefore, a deeper understanding of BrkA biogenesis and secretion is required to elucidate the mechanism behind BrkA signal peptide cleavage. This would allow a better understanding of *B. pertussis* bacterial pathogenesis.

In this study, we have investigated BrkA biogenesis and secretion in the *E. coli* DH5α model system. We inserted a 6xHis-tag into *brkA*-containing pDO6935 using a site-directed mutagenesis kit. The SPase I cleave after A^{42}, leaving the 6xHis tag retained on the disordered N-terminus of the passenger domain (Fig. 1B). The His-tag-containing pDO6923 was named pHAK, and it allows for the examination of BrkA expression using an anti-His antibody. The insertion locus permits the optimal detection of the outer membrane and secreted BrkA and ideally minimizes the interference with passenger domain secretion and interactions with chaperones (Fig. 1B).

Using nanopore sequencing, we confirmed the insertion of 6xHis-tag directly downstream of the AXA SPase I recognition site and in between A^{42} and Q^{43} through Sanger sequencing. However, we observed repeated sequences downstream of the 6xHis tag resulting in a truncated BrkA (Fig. 2C). We performed immunoblotting using an anti-His tag antibody to detect BrkA expression. We observed BrkA expression at 73kDa and 103kDa in the positive control only representing the 6xHis-tagged passenger domain with and without the transmembrane domain attached, respectively (Fig. 3). In the positive control, His tag is inserted at +18 (after G^{59}) relative to A^{42}, suggesting that the signal peptide was cleaved and BrkA was secreted into extracellular space. However, BrkA expression was not detected in our test plasmid pHAK, suggesting that there was no stable expression of BrkA due to repeated sequences resulting in truncation (Fig. 2C). In other words, the duplication and resulting in-frame signal peptide, 6xHis tag, part of passenger domain, likely produces a truncated 20kDa product, incapable of secretion and is likely prone to degradation (Fig. 2C). This then blocks subsequent BrkA extracellular secretion, so no band was observed at 73kDa (Fig. 3). We hypothesize that the absence of these bands might be due to the reduced stability of the truncated BrkA encoded by our test plasmid. Due to duplication in sequence between nucleotides 135 and 428, BrkA translation is terminated earlier, resulting in truncated products. This unstable BrkA protein may then be degraded instead of secreted. Another possibility for no detection in BrkA secretion could be inserting the His tag too close to the SPase I recognition site. This scenario may hinder the SPase I signal peptide recognition and cleavage process, such that SPase I cannot recognize and cleave the signal peptide properly. The three-dimensional structure of SPase I-preprotein interaction in *E. coli* was determined (14). Despite the consensus that the SPase I recognition site is often designated as A-X-A at the -3 and -1 positions relative to the passenger domain, the modeling of a signal peptide into the *E. coli* SPase I active site has suggested that -8 to +1 residues are also critical for protein-protein interaction (11). The 6xHis-tag insertion changed the amino acid at the +1 position from polar glutamine to positively charged histidine. As a result, the change in amino acid might impact the SPase I affinity to signal peptide and can be recognized as unstable. The unstable preprotein-SPase I structure may also affect the catalytic activity of SPase I, interfering with its ability to bind to the cleavage site. Furthermore, the His tag might jam the Sec translocon. Moreover, the BrkA variant might quickly get degraded once it is translated, which can be the reason why no BrkA secretion but non-specific small peptides were observed in the immunoblot. However, further studies are required to understand how this interference of signal peptidase cleavage due to His-tag insertion in the cleavage site took place.

In addition, passenger domain A52 to P600 is critical for protein stability. Previous studies (3) have reported A^{52} to P^{600} BrkA mutant that an intact 103kDa BrkA was detected after being treated with trypsin in *E. coli* UT5600, suggesting that amino acid 52 to 600 are important maturation of BrkA protein in periplasm. According to Fig. 2C, the duplication site was observed in the passenger domain, possibly interfering with interaction with periplasmic chaperones to prevent aggregation. SurA and Skp have been reported to interact with the passenger domain at amino acids residue 361 of Type Vα secretion system EspP in the periplasm in *E. coli* O157:H7 (15).

Conclusions Overall, we inserted a 6xHis tag into the *brkA*-containing pDO6935. Sequencing data indicated a duplication in the *brkA* sequence downstream of the 6xHis-tag insertion site.

BrkA secretion and the expression of neither the His-tagged 103kDa BrkA precursor nor the 73kDa passenger domain could be detected by immunoblot assay. Furthermore, the 6xHis tag was inserted close to the SPase I cleavage site, which may have interfered with SPase I cleavage and subsequent secretion of BrkA.

Future Directions In the future, it is possible to investigate the chemical bonding between SPase I and the catalytic site of the signal peptide. We can also try to determine how many amino acid residues are needed for the cleavage of the signal peptide with the proper construct of pHAK. Moreover, we can insert the His-tag to a few amino acid residues downstream of Q^{43} to see if BrkA expression can be detected and if the signal peptide is truly cleaved between A^{42} and Q^{43}. In addition, we can also assess the recognition site of SurA and Skp to the passenger domain of BrkA.

ACKNOWLEDGEMENTS

This project was funded by the Department of Microbiology and Immunology at UBC. We acknowledge Dr. David Oliver for providing insightful guidance to facilitate the success of the project and manuscript. We would also like to express gratitude to Herieth Ringo for her expertise and inspiring suggestions for troubleshooting the experiments and formatting the manuscript. In addition, we also like to thank Jade Muileboom for ordering reagents, assisting in submitting sequencing materials, and organizing the lab. In addition, we would like to thank Team 2-α for sharing their protocols and reagents. Lastly, we would like to thank the two anonymous reviewers for constructive feedback on this manuscript.

CONTRIBUTIONS

S.C.: wet lab experiments, methods and materials, figure development, discussion, future direction, conclusion, review and editing;
H.L: wet lab experiments, abstract, result, methods and materials, figure analysis, review and editing;
Z.L: lead on proposal conceptualization and development, wet lab protocol adaption and experiments, introduction, methods and materials, discussion and future direction, lead on illustration development, figure legends and analysis, reference formatting, review and editing.

REFERENCES

1. Locht C, Antoine R, Jacob-Dubuisson F. 2001. *Bordetella pertussis*, molecular pathogenesis under multiple aspects. Current Opinion in Microbiology 4:82–89.
2. Lutwick L, Preis J. 2014. Pertussis, p. 359–371. *In* Emerging Infectious Diseases. Elsevier.
3. Oliver DC, Huang G, Nodel E, Pleasance S, Fernandez RC. 2003. A conserved region within the *Bordetella pertussis* autotransporter BrkA is necessary for folding of its passenger domain. Molecular Microbiology 47:1367–1383.
4. Auclair SM, Bhanu MK, Kendall DA. 2012. Signal peptidase I: Cleaving the way to mature proteins. Protein Science 21:13–25.
5. Ruiz-Perez F, Henderson IR, Leyton DL, Rossiter AE, Zhang Y, Nataro JP. 2009. Roles of Periplasmic Chaperone Proteins in the Biogenesis of Serine Protease Autotransporters of *Enterobacteriaceae*. J Bacteriol 191:6571–6583.
6. Passerini de Rossi BN, Friedman LE, Gonzalez Flecha FL, Castello PR, Franco MA, Rossi JPFC. 1999. Erratum to: "Identification of *Bordetella pertussis* virulence-associated outer membrane proteins." FEMS Microbiology Letters 180:351–351.
7. Oliver DC. 2005. Structure/function studies of the *Bordetella pertussis* autotransporter protein BrkA: Secretion and folding.
8. Booth WT, Schlachter CR, Pote S, Ussin N, Mank NJ, Klapper V, Offermann LR, Tang C, Hurlburt BK, Chruszcz M. 2018. Impact of an N-terminal Polyhistidine Tag on Protein Thermal Stability. ACS Omega 3:760–768.
9. Sun F, Pang X, Xie T, Zhai Y, Wang G, Sun F. 2015. BrkAutoDisplay: functional display of multiple exogenous proteins on the surface of *Escherichia coli* by using BrkA autotransporter. Microb Cell Fact 14:129.
10. Shannon JL, Fernandez RC. 1999. The C-Terminal Domain of the *Bordetella pertussis* Autotransporter BrkA Forms a Pore in Lipid Bilayer Membranes. J Bacteriol 181:5838–5842.
11. Paetzel M, Karla A, Strynadka NCJ, Dalbey RE. 2002. Signal Peptidases. Chem Rev 102:4549–4580.
12. Chang AY, Chau VW, Landas JA, Pang Y. 2017. Preparation of calcium competent *Escherichia coli* and heat-shock transformation 1:22–25.

13. Szałaj N, Benediktsdottir A, Rusin D, Karlén A, Mowbray SL, Więckowska A. 2022. Bacterial type I signal peptidase inhibitors - Optimized hits from nature. European Journal of Medicinal Chemistry 238:114490.
14. Paetzel M, Dalbey RE, Strynadka NCJ. 2002. Crystal Structure of a Bacterial Signal Peptidase Apoenzyme. Journal of Biological Chemistry 277:9512–9519.
15. Leva R, Bernstein HD. 2009. Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. Proc Natl Acad Sci USA 106:19120–19125.