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Steps Towards Understanding the Role Of DegP in Secretion of the *Bordetella pertussis* Autotransporter Protein BrkA

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SUMMARY BrkA is a type V secretion system found in *Bordetella pertussis*, the causative agent of whooping cough. Research has shown that the β-barrel formed by autotransporters is too small to allow for the translocation of the passenger domain by itself. However, periplasmic chaperones such as DegP, SurA, and Skp, can interact with certain autotransporters to keep them in a translocation-competent unfolded state prior to translocation across the outer membrane. DegP, a temperature-dependent chaperone and protease, is found in the periplasm of Gram-negative bacteria. It is unclear how DegP interacts with BrkA in the process of its translocation across the outer membrane. In this study, we validated whether *degP* was knocked out in the JW0157 *Escherichia coli* strain, performed site-directed mutagenesis to insert 6 histidine residues into the *brkA* plasmid, and conducted Western blot to determine the effect of DegP in BrkA secretion. The *degP* knockout strain was successfully validated, but the site-directed mutagenesis process and Western blots were unsuccessful. Further Western blot attempts are necessary in order to address the aim presented.

INTRODUCTION

The type V secretion system (TVSS) is one of the seven secretion system classes which belongs to one of the two general secretion architectures seen in Gram-negative bacteria (1). One thing that distinguishes this secretion system from the other types is that the translocation domain used in the outer membrane (OM) belongs to the same polypeptide containing the passenger domain that is secreted (1). This seemingly simplistic self-sufficient export system gave rise to the term “autotransporter” (1). The type V secretion systems contain many subtypes ranging from Va to Ve; this variation arises due to the different domain organizations which lead to an alternative transport mechanism (1).

Bordetella pertussis is a Gram-negative bacteria known to cause whooping cough (pertussis), an acute respiratory infection with dangerous complications such as bronchopneumonia and acute encephalopathy (2). This bacterium contains the virulence factor Bordetella resistant to killing (Brk) A, which confers classical-complement pathway resistance and mediates adherence (3,4). This protein is self-assembling and belongs to the Va secretion system (1). It consists of 4 regions: an N-terminal 42 amino acid long signal peptide, a 73 kDa N-terminal passenger domain, and a C-terminal translocation domain consisting of an N-terminal ~ 35 amino acid alpha-helical linker region and a ~ 30 kDa C-terminal β-domain predicted to form a β-barrel pore in the outer membrane (5).

The translocation of the 73 kDa BrkA passenger domain across the translocation pore is directed via the linker region, and when it crosses over, it gets cleaved but remains attached to the cell surface through non covalent interactions (3,5). Contrarily, other Va autotransporters, like immunoglobulin (Ig) A1, get released into the extracellular medium upon cleavage in an autocatalytic process (1). Moreover, the size of the translocation pore is around 2 nm, which highlights the fact that it is unlikely for a fully folded passenger domain to be able to pass through this pore. Therefore, the conformational state of the passenger domain in the periplasm remains uncertain (6). It is thought that an autotransporter maintains a translocation-competent unfolded state for proper translocation to the extracellular space (6). It has also been found that periplasmic chaperones such as DegP keep the autotransporters in a translocation-competent unfolded state, allowing for cell-surface expression and preventing their aggregation in the periplasm (7).

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DegP is a periplasmic chaperone and protease protein found in Gram-negative bacteria (8). It performs these functions by utilizing different conformations at various temperatures (8). Below 28℃, it has been reported that DegP prevents misfolded proteins from forming aggregates (chaperone function), while above 30℃, it degrades misfolded proteins (protease function) (8). However, the binary nature of this temperature-dependent phenotype has not been well characterized as its protease function has been observed even at 25℃ (8). DegP is essential for growth at high temperatures since misfolded outer-membrane proteins (OMPs) can form aggregates that can have toxic and cell-damaging effects on the cell (9). Furthermore, literature has shown that strains lacking *degP* led to a defective secretion of IcsA and EspP, which belong to the Va secretion system subtype similar to BrkA (7,10). However, another autotransporter in the Va subtype by the name of Hbp did not show a defect in translocation in a *degP* deficient background (10).

Based on the gap in the literature regarding the periplasmic folded state of autotransporters, and how DegP does not have a consistent effect on all autotransporters in the same subtype, we decided to investigate the effect knocking-out *degP* (**Δ***degP*) has on BrkA. We hypothesized that DegP is necessary for translocation of BrkA across the outer membrane.

METHODS AND MATERIALS

Bacterial Strains and Plasmids. Dr. Rachel Fernandez kindly provided the BrkA encoding plasmid pDO6935. Strains were ordered from the Horizon Discovery Keio Collection. JW0157 is the Δ*degP* and BW25113 is the strain used for the construction of JW0157. Bacterial cultures were grown in LB and plated in LB agar plates with the appropriate antibiotics. Kanamycin was supplemented at 50 µg/ml for plates and 25 µg/ml for culture. Ampicillin was supplemented at 100 µg/ml.

Q5 Site-Directed Mutagenesis Primer Design. Primers were designed for the insertion of the 6xHis tag between Q^{60} and E^{61} of the *brkA* reading frame in the pDO6935 plasmid (Table 1). Different codons for His were used to prevent complementary binding of the 5' end between the primers. The insertion site was chosen to be located in the N-terminus non-structural unfolded region after the signal peptide sequence and is based on a structural analysis of the BrkA protein using AlphaFold as well as previously shown in Sun *et al*. (11).

TABLE 1. Plasmids and Bacterial Strain

Plasmid/Strain	***Relevant Characteristics***
Plasmid	
pDO6935	Ampicillin resistance, *brkA*
pPALMC1	Ampicillin resistance, *brkA*, pDO6935 derivative with a 6x-Histidine insertion (D^{57}-A^{58})
Strain	
BW25113	F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*
JW0157	*F-, Δ(araD-araB)567, ΔdegP775::kan*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)*, *hsdR514*

Δ*degP* PCR primer design. Polymerase chain reaction (PCR) primers were designed for validation of *degP* knockout strain JW0157 (Table 2). Primers were designed to bind internally to the upstream gene *dgt* and the Kanamycin cassette, to amplify a 1.3 kbp region in the JW0157 strain and no amplification in the BW25113 strain.

TABLE 2. Sequence and Melting temperatures for primer

Primer	*Sequence (5'-> 3')*	*Tm (°C)*
SDM Forward	caccaccacGAAGGAGAGTTCGACCACCG	58
SDM Reverse	atgatgatgCTGCCCGGCGTCCTG	61
dgt-F	TGTGGCAGACCTTGAAGATG	57
kanR-R	CAGTCATAGCCGAATAGCCT	58

Q5 site-directed mutagenesis to insert a 6xHis tag into pDO6935. For the construction of a 6xHistidine (His) tagged pDO6935 plasmid, the NEB Q5 Site-Directed Mutagenesis kit and protocol were used. 12.5 µl Q5 Hot Start High-Fidelity 2X master mix, 10 µM of each primer, and 10 ng of pDO6935 in a 25 µl reaction were mixed for the initial amplification step. Thermocycler parameters for amplification were set to the following: initial denaturation at 98°C for 2 minutes; 25 cycles of denaturation at 98°C for 10 seconds, annealing at 69°C for 30 seconds and extension at 72°C for 3.5 minutes, and a final extension at 72°C for 2 minutes. The amplification product was treated with Kinase, Ligase, and DpnI (KLD). 1 µl of the amplification product was mixed with 5 µl of 2X KLD reaction buffer and 1 µl of 10X KLD enzyme mix in a 10 µl reaction. After 5 minutes of incubation, 5 µl of the KLD mixture was added to chemically competent cells, incubated for 30 minutes, heat shocked for 45 seconds, incubated on ice for 5 minutes, supplemented with 950 µl of LB and incubated for 60 minutes at 37°C and plated.

Genomic extraction PCR to validate Δ*degP* in JW0157 strain. BW25113 and JW0157 were grown overnight in LB with no antibiotics and Kanamycin respectively. and genomic DNA was extracted using the BioBasic genomic miniprep kit. Genomic DNA was quantified using the nanodrop PCR was done using 0.5 µl Invitrogen Platinum Taq DNA polymerase, 5 µl 10x taq buffer minus Mg with $(NH_4)_2SO_4$, 10 µM dNTP mixture, 1.5 mM $MgCl_2$, 0.2 µM each primer, 10 ng genomic DNA in a 50 µl reaction. Thermocycler parameters for PCR amplification: Initial Denaturation at 95°C for 2 minutes; 28 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 70 seconds, and a final extension at 72°C for 2 minutes. PCR amplification was run on a 1.5 % agarose gel with RedSafe and visualized on a BioRad ChemiDoc MP Imaging System.

Colony PCR to validate Δ*degP* in JW0157 strain. Colony PCR was performed on both BW25113 and JW0157. With a micropipette tip, touched a colony and transferred to 50 µl of nuclease-free water. The sample was then transferred into a thermocycler for 5 minutes at 94°C, then quantified using nanodrop. PCR was done using 0.5 µl Invitrogen Platinum Taq DNA polymerase, 5 µl 10x taq buffer minus Mg with $(NH_4)_2SO_4$, 10 µM dNTP mixture, 1.5 mM $MgCl_2$, 0.2 µM each primer, 10 ng genomic DNA in a 50 µl reaction. Thermocycler parameters for PCR amplification: Initial Denaturation at 95°C for 2 minutes; 28 cycles of denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 70 seconds, and a final extension at 72°C for 2 minutes. PCR amplification was run on a 1.5 % agarose gel with RedSafe and visualized on a BioRad ChemiDoc MP Imaging System.

Western blot. L. Bilal, A. Lalani, M. MacAulay, and P. Malhi (UJEMI in press, 2024) kindly provided the pPALMC1 plasmid (Table 1). JW0157 transformed with pDO6935 or pPALMC1 was grown overnight in LB supplemented with Kanamycin and Ampicillin. BW25113 transformed with pDO6935 or pPALMC1 was grown overnight in LB supplemented with Ampicillin only. Optical Density (OD) was measured to normalize. All samples were spun down and resuspended in an SDS-PAGE disruption buffer and boiled for 10 minutes at 95°C, then spun down and loaded. The Western blot was run using Mini-PROTEAN® TGX™ Precast Protein Gels and visualized on a BioRad ChemiDoc MP Imaging System. The primary antibody used was mouse anti-histidine diluted in 5% blocking solution at 1:1000. The secondary antibody used was goat anti-mouse HRP diluted in 5% blocking solution at 1:5000.

RESULTS

Sequencing of pDO6935-6xHis shows insertion and deletion mutations in BrkA. Initial cloning attempts of the proposed workflow with site-directed mutagenesis were unsuccessful. Two clones were sent for nanopore sequencing and another 5 were sent for anger sequencing. Sequencing results showed all clones contained either insertions or deletions resulting in frameshift mutations. One clone showed 2 separate single nucleotide insertions, while the rest had insertions or deletions from 50 to 300 nucleotides in length. However, they did show to have the 6x His tags incorporated in the correct insertion site.

JW0157 strain validated for deletion of *degP* gene and insertion of the Kanamycin cassette. Primers were designed to bind internally to the upstream *dgt* gene and the Kanamycin cassette (Table 2). We ran a genomic extraction PCR and colony PCR on the parent strain BW25113 (negative control) and the mutant strain JW0157 (experimental condition). Step 2 of the proposed workflow revealed a successful insertion of the Kanamycin cassette in place of the *degP* gene in JW0157 as compared to BW25113 (Figure 1). The bands obtained appeared at ~1300 bp in length, approximately the size of the expected product (Figure 1).

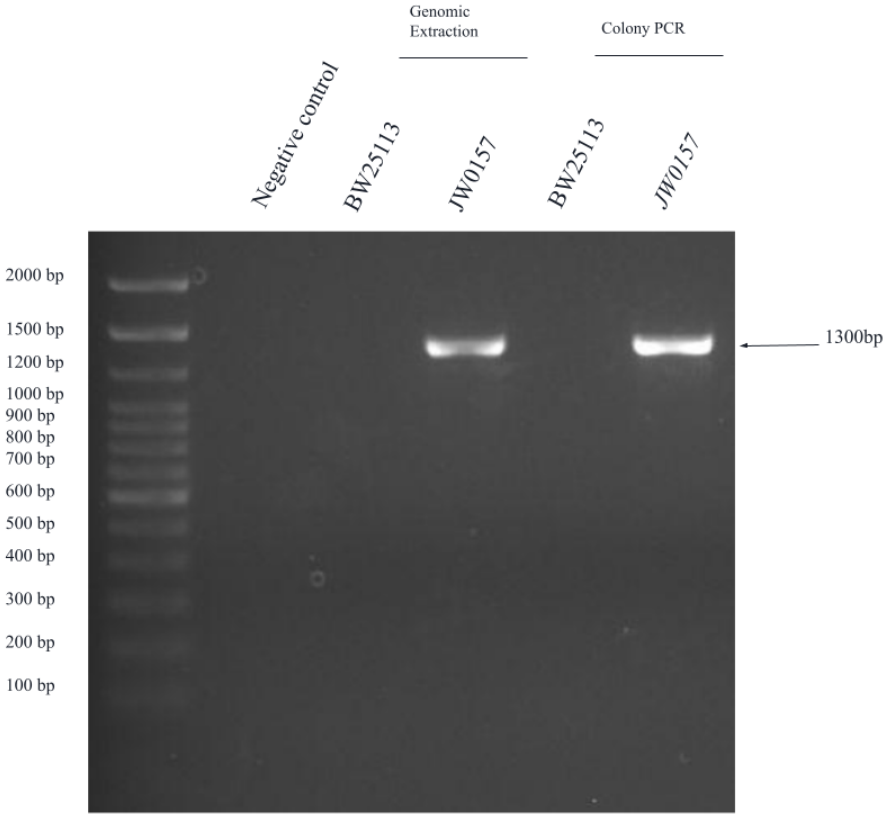

FIG. 1 PCR validation shows insertion of the Kanamycin cassette and knockout of *degP* in JW0157.

Western blot only shows a 103 kDa band for JW0157 transformed with pPALMC1. In the Western blot conducted, BW25113 with pPALMC1 acted as positive control while JW0157 with pPALMC1 was the experimental condition. JW0157 and BW25113 transformed with pDO6935 acted as the negative controls. Protein concentrations were loaded at 1X and a ½ dilution for each condition in the SDS-PAGE. The Western blot results showed only a 103 kDa band for the JW0157 with pPALMC1 plasmid for both the 1X and ½ dilution concentrations loaded, but no banding was seen for the BW25113 transformed with pPALMC1 in either concentration (Supplementary Figure S1). Furthermore, background bands can be visualized at 35 and 30 kDa in all the lanes (Supplementary Figure S1).

DISCUSSION

Our study aimed to determine what role the periplasmic chaperone protein known as DegP plays in BrkA secretion. Previous research suggested a temperature-dependent mechanism associated with DegP which could either prevent the misfolding of proteins as a chaperone or degrade proteins as a protease (9). Utilizing a PCR approach, we were able to validate that *degP* was knocked-out in JW0157 as compared to its wild-type parent strain BW25113.

BrkA is a virulence factor that allows *Bordetella pertussis* to evade the innate immune response by conferring resistance to the classical complement pathway and mediating adherence (3,4). Further, there is a gap in the literature regarding the translocation mechanism of autotransporters (6). However, it is known that periplasmic chaperones such as DegP can

interact with some of the TVSS such as IcsA, where it keeps the protein in an unfolded translocation-competent state (7,10). Hence, this work will provide more insight into the BrkA mechanism and the role of DegP as a potential therapeutic target to combat *B. pertussis,* the causative of whooping cough (2).

Inconclusive results cannot suggest whether DegP plays a role in BrkA maturation. Due to the suggested interaction of DegP with some TVSS autotransporters (10), we expect it to play a role in BrkA maturation (Figure 2). We predicted that pPALMC1 in BW25113 to yield a 103 kDa and 73 kDa band representing the unprocessed periplasmic BrkA and the translocated cleaved passenger domain respectively (Figure 2A). In the JW0157 strain, we expected to only see a 103 kDa band illustrating that BrkA is unable to be secreted (Figure 2B). However, in our Western blot, only the experimental condition, JW0157 strain transformed with pPALMC1, showed a banding at 103 kDa. The positive condition, BW25113 strain transformed with pPALMC1, showed no banding besides the background bands that were positioned at 35 and 30 kDa in all the lanes. Due to the lack of banding in the BW25113 positive condition, the findings are inconclusive.

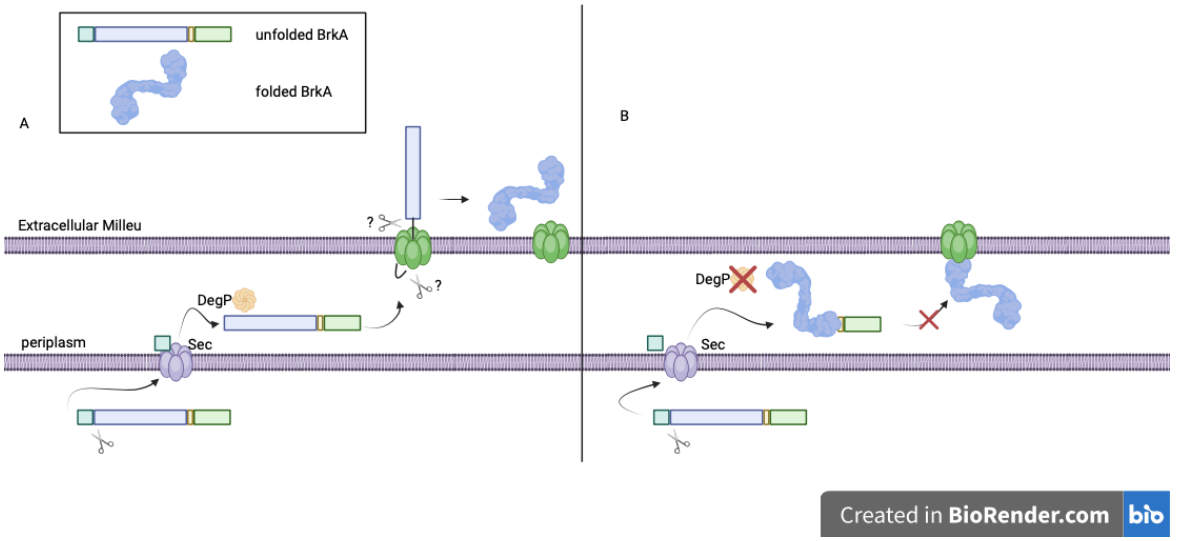

FIG. 2 Pathway for BrkA translocation and proposed DegP periplasmic chaperone involvement. Signal peptide with Sec machinery allows passage of BrkA through the inner membrane. (A) DegP is proposed to prevent premature folding until BrkA reaches the surface of the outer membrane through its β-barrel domain. (B) Without DegP chaperone function, BrkA may prematurely fold, preventing its translocation through the outer membrane.

Workflow. Due to the inconclusive nature of our results, we believe that the first step in the continued study of the effect of DegP on BrkA secretion is the completion of the original study design. Following the validation of *degP* knockout in JW0157 via PCR, the next step is to repeat site-directed mutagenesis to insert a 6xHis tag into the pDO6935 *brkA* plasmid or use a plasmid that has been validated for containing 6xHis tag in the *brkA* reading frame (Figure 3.1). If the site-directed mutagenesis is repeated, sequence the modified plasmid to ensure that the proper insertion of the 18 bp construct has occurred, before transformation into JW0157 (*ΔdegP*) and BW25113 (*degP*+) strains (Figure 3.3). Finally, by conducting a trypsin accessibility assay (TAA) before running SDS-PAGE and Western blot will help suggest the role of DegP in the maturation process of BrkA (Figure 3.4). This would differentiate between whether DegP is playing a role in BrkA translocation or autoprotease function.

Trypsin accessibility assay is a process where the enzyme trypsin degrades proteins on the surface of cells. Without this assay, only the effect of DegP on the autoprotease function of BrkA can be inferred (Figure 4A). This means that the β-barrel is inserted into the OM and the passenger domain is translocated across, however, the loss of DegP has somehow inhibited the cleavage of BrkA that happens after its translocation which normally would

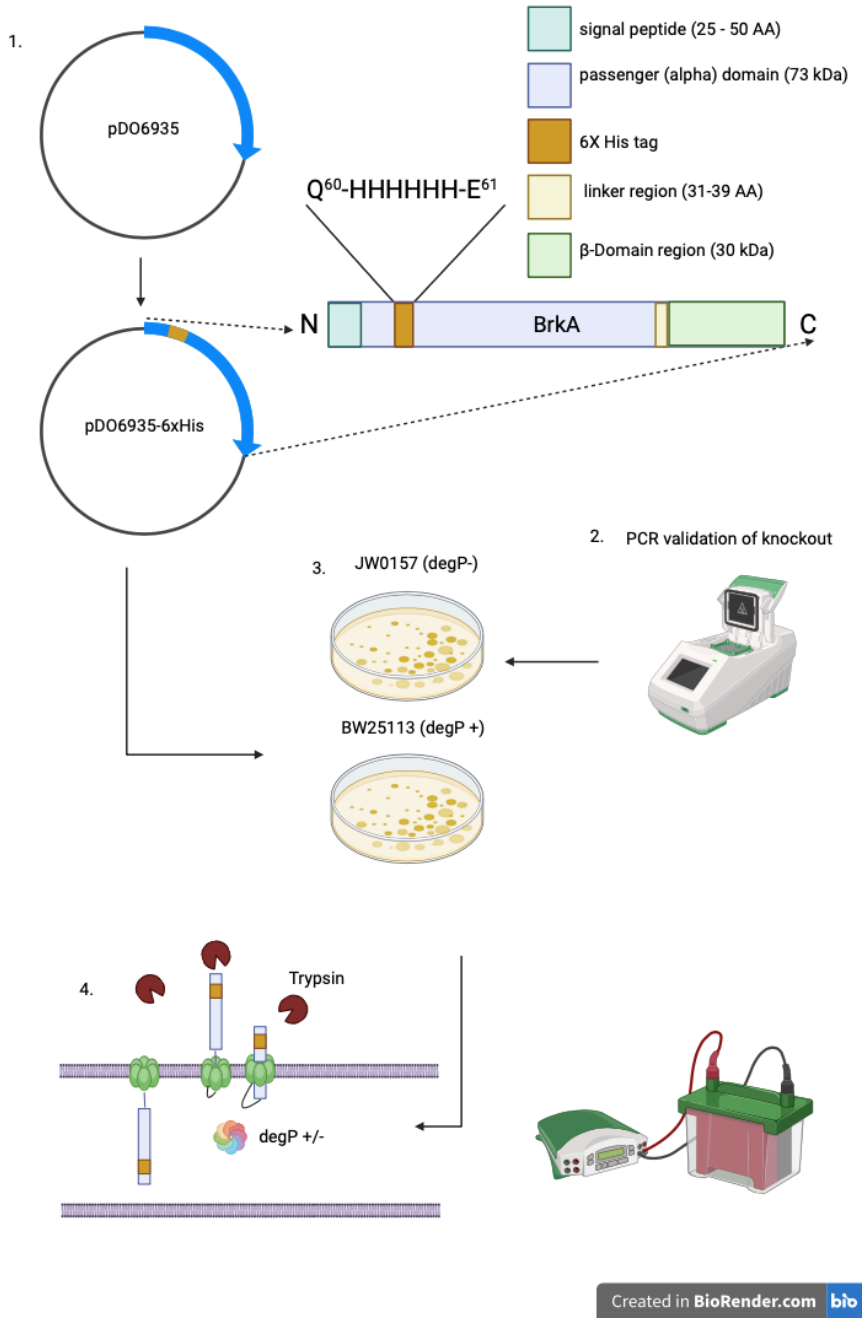

FIG. 3 Proposed workflow for determining *ΔdegP* on BrkA translocation. 1. Construction of detection system through insertion of 6xHis tag into pDO6935 BrkA plasmid. 2. Validation of *ΔdegP* through PCR. 3. Transformation of pDO6935-6xHis into JW0157 (*ΔdegP*) and BW25113 (*degP*+). 4. Determining function of DegP on BrkA translation with Western blot and Trypsin accessibility assay.

result in a 73 kDa band. Hence, why we expect to only see a 103 kDa banding for JW0157 transformed with pPALMC1, but a 103 and 73 kDa banding for BW25113 transformed with pPALMC1. By including a TAA, it allows us to state whether DegP plays a role in the BrkA translocation process. If DegP only affects the autoprotease function of BrkA, then Western blot should show a fainter band at 103 kDa for the experimental condition, due to trypsin degrading uncleaved BrkA expressed on the surface (Figure 4B). But, if the same intensity banding is seen as in the Western blot without TAA, this indicates that nothing has been translocated across the OM to be exposed to trypsin (Figure 4C). In order to know that TAA has worked as intended, BW25113 containing pPALMC1 is believed to show only a 103 and 73 kDa band in the no TAA condition, while in the TAA condition, it is believed that it will only show a 103 kDa band. This is because the 73 kDa band indicates the cleaved passenger domain on the surface of the cell, where it will be exposed to trypsin. To summarize, based on the outcome of the Western blot with a TAA, we can state whether DegP impacts the autoprotease function of BrkA or its translocation process.

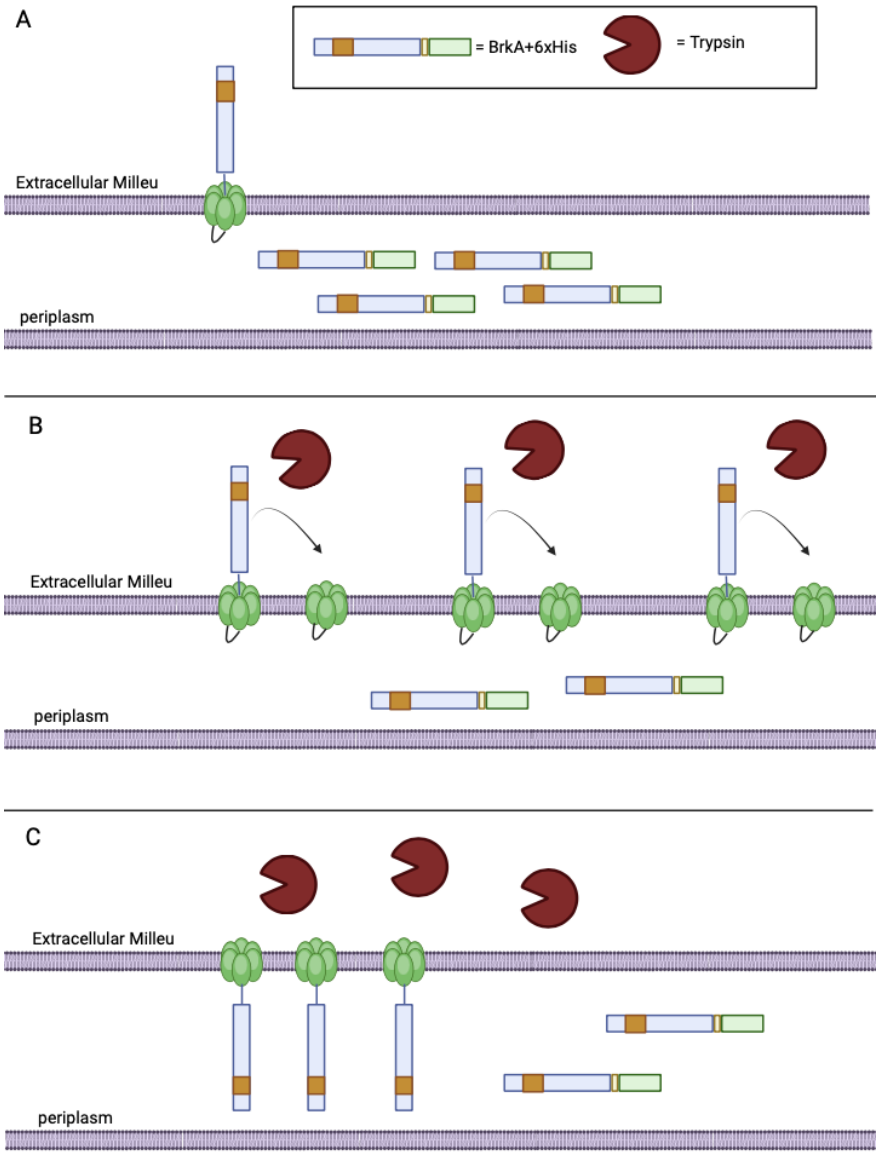

FIG. 4 Illustration of how JW0157 cells containing pPALMC1 treated with and without TAA would look like if DegP played a role on BrkA autoprotease function versus translocation process. (A) This figure illustrates why it is expected that a Western blot without a TAA can only infer that DegP impacts the autoprotease function of BrkA if no 73 kDa band is seen for the JW0157+pPALMC1 strain. (B) This figure illustrates why a fainter band at 103 kDa is expected in a Western blot with TAA for the JW0157+pPALMC1 cell relative to its counterpart in the Western blot without a TAA, if the assumption that DegP only impacts BrkA autoprotease function is true. (C) This figure illustrates why no change in the band intensity at 103 kDa is expected in a Western blot with TAA for the JW0157+pPALMC1 cell relative to its counterpart in the Western blot without a TAA, if the assumption that DegP impacts BrkA translocation process is true. This figure was created on Biorender.

Limitations This study is limited due to its inconclusive nature, further experimentation needs to be conducted to test our hypothesis. This includes the construction of the 6xHis tagged plasmid and Western blot with and without TAA to determine the effect of DegP on the translocation of BrkA or its autoprotease function.

The site of insertion of the 6xHis tag is also a limitation. It is unknown what the potential effects of inserting a 6 amino acid sequence at the site chosen for detection will have on the maturation or folding process of BrkA, potentially altering the results (12). Also, DegP contains both a chaperone and protease function (8) and by knocking out the gene for this protein may result in an induction of compensatory expression of other periplasmic chaperones or proteases such as SurA or OmpT respectively. This effect could alter potential results associated with DegP. For example, a study done on DegP, SurA, and Skp has shown all three chaperones affect the secretion of TVSS proteins known as IcsA and EspP, illustrating a redundancy in their function (10). Hence, in order to determine the necessity of DegP while limiting the potential effects of compensatory expression, we can utilize double knock-out strains as opposed to just a single knockout.

Conclusions In this study, we hypothesized that knocking out *degP* would prevent the proper translocation of BrkA across the outer membrane of Gram-negative bacteria. To test our hypothesis, we aimed to construct a 6xHis tagged BrkA plasmid. Then, to test how the

secretion of BrkA was affected, we aimed to compare wildtype BW25113 to our *degP* knockout JW0157 strain by performing a Western blot with and without trypsin accessibility assay. Overall, we were able to confirm that *degP* was knocked out in JW0157, however, we were unable to create a 6xHis tagged *brkA* plasmid or conduct a conclusive Western blot.

Future Directions The first step for future directions would be to retry the Western blot with and without the trypsin accessibility assay to see if DegP plays a role in BrkA autoprotease function or translocation across the OM. As previously mentioned, double knockout studies could also be conducted to rule out compensatory expression due to knocking out *degP.*

Furthermore, studies to investigate the use of DegP as a potential therapeutic target to prevent the surface expression of BrkA in *Bordetella pertussis* can be performed. If DegP can be shown to play a role in BrkA autoprotease function or BrkA translocation, it can become a potential therapeutic target for future drug development to treat *Bordetella pertussis* infection. The additional experiments mentioned in the discussion can determine if DegP is necessary to maintain the unfolded state of the BrkA passenger domain for effective translocation through the β-barrel domain. Designing therapeutic agents that can chemically inhibit the function of periplasmic chaperone proteins like SurA has been studied (13). By designing therapeutic agents that can inhibit DegP, we can potentially alter the translocation of BrkA in *B. pertussis*, ultimately inhibiting its effector functions such as resistance to the classical complement pathway and host cell adherence (3,4). This mechanism could serve as a method of reducing the incidence of whooping cough from *Bordetella pertussis* infection.

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CONTRIBUTIONS

This study is the result of the collaborative effort of all authors. VH made the figures/table, worked on the methods, results, discussion, and limitations. NK worked on the title, abstract, results, discussion, workflow, limitations, conclusion, future directions, and acknowledgments. KS worked on discussion, workflow, study limitations, future directions, introduction, and figures. AT came up with the project idea, worked on the introduction, discussion, workflow, limitations, future direction, references, and created figures. Every author worked on revision as well as conducting the experiments and lab analyses. Co-authorship should be considered equal for all authors.

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