12-2012

Characterization of FhuA 104/149C: a Double Cysteine FhuA Mutant with Normal Binding and Diminished Transport

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A thesis

presented to

the faculty of the Department of Biology

East Tennessee State University

In partial fulfillment

of the requirements for the degree

Masters of Science in Biology

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by

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December 2012

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Keywords: TonB dependent transport, Ferrichrome, FhuA

ABSTRACT

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Iron is an essential element for most bacteria and is commonly acquired by siderophores, molecules secreted under iron restricted environment to bind ferric iron. Gram negative cells actively uptake these complexes via outer membrane-transport proteins such as FhuA in *Escherichia coli*. Structural analysis of receptors revealed a conserved β-barrel occluded by an N-terminal plug domain. The cell membrane TonB/ExbB/ExbD complex presumably supplies energy via interaction between the FhuA N-terminal TonB box and the C-terminal domain of TonB. In order to better understand the mechanism of action the FhuA mutant 104/149C, tethering the central β-strands 4 and 6 of the plug domain, was studied and showed severely reduced transport of radio-labeled ferrichrome. In the course of this study, this protein was HPLC purified for structural studies by crystallization and X-ray diffraction. In addition, protein interaction studies were performed with purified TonB-C terminal revealing no impact of the mutation on FhuA-TonB interactions.

ACKNOWLEDGMENTS

First, I would like to thank my committee chair and mentor, Dr. Ranjan Chakraborty, for his constant support, encouragement, and time. I would also like to thank my committee members, Dr. Dhirendra Kumar and Dr. Christopher Pritchett, for their support and advice as well as Dr. Elias Fernandez at the University of Tennessee for his collaboration and Erin Story for the creation and initial studies of the mutant used in this research. I would like to extend my sincere thanks to the Department of Health Sciences faculty for their continued support, advice, and friendship. For their technical support and advice, I would like to thank Bill Wright, Robin Grindstaff, Tom Barber, Brian Hill, Sean Stacey, Ralitsa Borisova, and Megan Carr. Funding for this research was provided by the Department of Health Sciences, College of Public Health, NIH grant GM069367, RDC grant 03-008M, the ETSU Ronald McNair program as well as a student research grant and thesis scholarship, both awarded by the ETSU graduate school. Lastly, I would like to thank my husband and my family for their support of my education and career goals.

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CHAPTER 1

INTRODUCTION

Iron Availability

Iron is an important element for the growth and metabolism of both eukaryotic and prokaryotic organisms; however, at higher concentrations it can cause the formation of damaging hydroxyl radicals (15). Concentrations of iron within the human body are controlled by high-affinity iron-binding glycoproteins which bind free iron to store and transport it to cells for use. Two examples are lactoferrin in breast milk and transferrin which together serve to effectively reduce the concentration of free iron within the body to 10^{-18}M. As a result, host cells are protected against hydroxyl radicals as well as infection by pathogens (2, 55).

Bacteria maintain internal iron concentrations of 0.4-4.0μM for many different cellular functions and must overcome iron limitation to survive and multiply in the human body (54). As such, bacteria have evolved several different methods for the purpose of iron acquisition, one of which is the secretion of iron chelators known as siderophores. Siderophores bind ferric iron and transport it back to the cell. Iron acquisition via siderophore production is often important for the prolonged infection and survival of a pathogen (1,12,38).

Siderocalin is a molecule released by host neutrophils to bind ferric siderophores and prevent their return to the bacterial cell. In response, some pathogens produce siderophores that are able to avoid binding and hindrance by siderocalin such as *Bacillus anthracis*' petrobactin (1). *Mycobacterium tuberculosis* mutants deficient in siderophore production showed impaired ability to survive in low iron conditions and within infected macrophages (12). Further evidence for the importance of bacterial iron acquisition systems is the fact that many virulence factors are regulated by the concentration of iron in the bacterial cell and only activated under low iron

conditions. These virulence factors can be found in pathogens such as *Vibrio cholerae* and include shiga toxin produced by *Shigella dysenteriae,* and diphtheria toxin secreted by *Corynebacterium diphtheriae* (38). One exception to this is the porcine pathogen *Actinobacillus pleuropenumoniae* where the *fhu*CDBA operon is constitutively expressed (48).

Siderophores

In 1952, the fungus *Ustilago sphaerogena* was found to produce an organo-iron compound (41). The compound, ferrichrome, was later determined to belong to the iron-chelating family termed siderophores. Siderophores are low molecular-weight, iron-chelating compounds synthesized within the cell and secreted into the environment. Siderophores are classified into 5 different categories according to the functional groups involved in the chelation of iron including hydroxamate, catechol, α-hydroxy-carboxylate, phenolate, and mixed siderophores. Hydroxamate type siderophores have carboxyl groups involved in iron chelation such as with schizokinen (Figure 1A) and rhizobactin (5, 40). Catechol groups chelate iron with the use of catecholate groups composed of a benzene ring plus 2 hydroxyl groups. Enterobactin (Figure 1B) is a catechol type siderophore produced by many enteric bacteria including *Escherichia coli* and *Salmonella typhimurium* (38, 42, 50). The third functional group is carboxylic acid, indicating carboxylate-type siderophores such as staphyloferrin A (Figure 1C) isolated from *Staphyloccus hyicus* (29) and rhizoferrin isolated from *Rhizopus microsporus* (14). Phenolate siderophores have a thiazoline ring as a part of functional groups that chelate ferric iron and include one produced by both *E. coli* and *Yersinia enterocolitica*, yersiniabactin (Figure 1D) (20). Lastly, mixed type siderophores are those where 2 or more types of functional groups are involved in iron chelation such as the group of siderophores isolated from *Pseudomonas cepacia* known as ornibactins (Figure 1E). Ornibactins contain a carboxylate functional group

composed of β-hydroxyaspartic acid as well as hydroxamate groups located on the side chains of ornithine residues (36, 51).

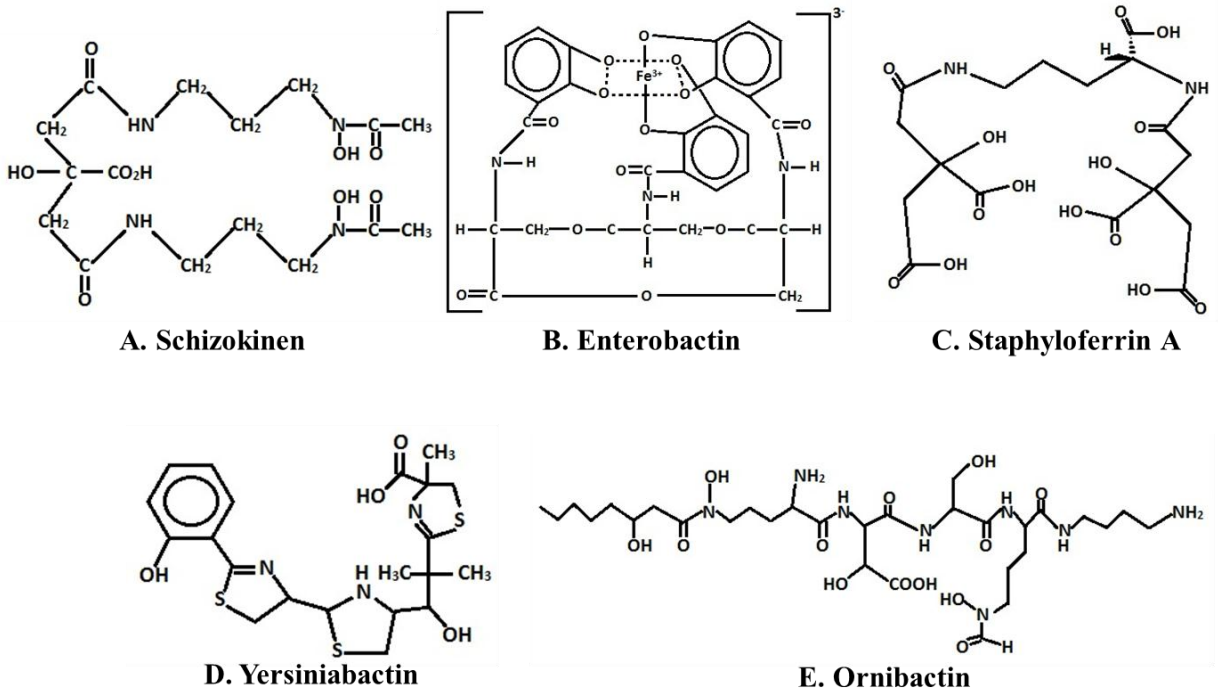

Figure 1. Examples of siderophore structures based on functional groups. (A) Schizokinen is an example of a hydroxamate type siderophore while enterobactin (B) is an example of catechol type. Staphyloferrin A (C) is carboxylate type siderophore, yersiniabactin (D) a phenolate and ornibactin (E) is a mixed type siderophore (20, 29, 36, 40, 50).

Uptake of Siderophore-Iron Complex

Upon excretion into the environment, siderophores chelate available ferric iron and return the iron to the bacterial cell. After chelation there are 2 possibilities for the uptake of the iron into the cell. The first is the release of the iron at the cell surface via reduction followed by transport into the cell cytoplasm; a method appearing to be employed by *Listeria monocytogenes* which produces a ferric reductase that acts at the cell surface and in the environment (3). Second is the transport of the entire siderophore-iron complex into the cell cytoplasm where iron is released from the complex via reduction by siderophore reductases or disassembly of the molecule such as in the cases of enterobactin and salmochelin (20). Transport across the cell

membrane of Gram-positive bacteria is most often accomplished by ABC transporters composed of 3 components: 1) a cell wall anchored extracellular binding protein, 2) a transmembrane permease complex (often composed of 1 or more homo or heterodimers), and 3) a cytoplasmic ATPase to provide energy for transport (3, 30). While ligand specific, these functions are often redundant such as with petrobactin uptake by *B. anthracis* where 2 different permases (FpuB, FatCD) and 3 distinct ATPases (FpuC, FpuD and FatE) have been identified (13). Gram-negative bacteria have 2 membranes, an inner and an outer on either side of a peptidoglycan cell wall. Ferric siderophore complexes are too large to be transported by diffusion through the porins present in Gram-negative bacteria outer membranes and thus require specialized outer membrane transporters (OM) for access into the periplasm where the siderophore is transported into the cell in a similar manner as that used in Gram-positive bacteria with the exception of a non-anchored periplasmic binding protein (3).

Ferric Siderophore Outer Membrane Transporters

Transporter Homology

Structural studies of TonB-dependent OM transporters have shown a considerable degree of sequential and structural homology. Four TonB dependent transporters BtuB, FhuA, IutA, and FepA were found to have sequential homology between 4 distinct regions. The first and second regions are the N and C-termini, respectively, and have high homology within short sequences. Regions 3 and 4 are less highly conserved but homology occurs over a longer sequence at residues 50 to 70 and 100 to 140, respectively (34). The comparison of *E. coli* transporters FhuA (18, 33), FecA (19, 57), and FepA (4) crystal structures to *P. aeruginosa* transporters FpvA (9) and FptA (10) illustrates the structural similarities (Figure 2).

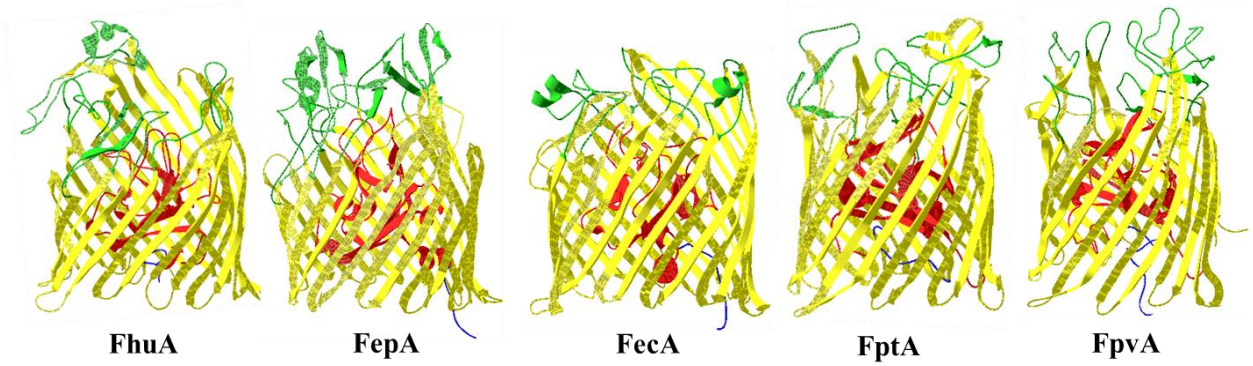

Figure 2. Comparison of the structural similarities of TonB dependent transporters. The 22 strand β-barrel (yellow) is occluded by a globular N-terminal domain (red). Contained in the N-terminus (blue) is the conserved TonB box, the extracellular loops (green) act as receptors for various types of siderophores (pdb files: FhuA, 1BY3; FepA, 1FEP; FecA, 1KMO; FptA, 1XKH; FptA, 1XKW).

The barrel domains vary in height and width between transporters. They are composed of a 22 strand β-barrel occluded by a globular N-terminal domain. Varying in length, size and number, extracellular loops extend above the lipid bilayer forming a binding pocket for specific ferric-siderophores and act as receptors with a high degree of binding specificity (44, 46). The plug domain of the transporter is composed of approximately 150 residues arranged in 4 α-helices and 6 β-sheets stabilized within the barrel by over 50 hydrogen bonds and multiple salt bridges. Residues in the extracellular loops of the plug domain also participate in ligand binding along with the barrel extracellular loops (33). Almost 27% of the plug domain is composed of highly conserved residues accounting for 32% of conserved residues in the protein. Conservation has not been found in the extracellular loops leaving the β-barrel to account for the remaining 68% of conserved residues (44).

Effects of Ligand Binding

A FhuA protein is able to bind and transport the siderophore ferrichrome after 3 hydroxamate groups bind ferric iron to result in a neutrally charged molecule. Aromatic residues line the FhuA binding site allowing it the ability to bind a variety of hydroxamate type

siderophores including the fungal siderophore ferrichrome. In contrast, the FepA protein binds and transports ferric enterobactin. Enterobactin is an aromatic molecule that binds ferric iron with 3 catecholate groups resulting in a net charge of negative 3. The FepA binding site is thus composed of positively charged residues and can bind other negatively charged ferric siderophores but restricts possible ligands based on size (26, 43).

Crystal structures comparing ligand-free and ligand-bound states of FhuA show that when ferrichrome enters the binding site it forms a total of 5 different hydrogen bonds: 3 with the plug residues Arg81, Tyr116, Gly99 and 2 with barrel residues Tyr244 and Tyr315. In addition, ferrichrome binding institutes a series of changes within the plug domain beginning with a shift of residues Thr80, Arg81, Gly99, and Gln100 towards the ligand. The alterations at residue 81 continue throughout the domain causing helix-1 to unfold towards the opposite side of the barrel and as a result, residue Trp22 is displaced by as much as 17Å. A similar motion has been observed in FecA as well and is believed to promote interaction of the N-terminal with the C-terminal of TonB (31, 33, 57).

TonB Complex

TonB is hypothesized to supply the energy required for transport of the ligand through the plug domain via a complex composed of TonB, ExbB, and ExbD found in the ratio 1:7:2 (22). TonB is anchored in the cytoplasmic membrane next to ExbB and ExbD and its C-terminal (residues 155 to 239) extends across the periplasm to interact with the N-terminus of the TonB dependent transport protein at a 5 amino acid long conserved sequence (Figure 3) known as the TonB-box (24, 34, 44, 47).

Transporter	Consensus Sequence					
FhuA	7	Asp	Thr	Ile	Thr	Val
BtuB	6	Asp	Thr	Leu	Val	Val
IutA	6	Glu	Thr	Phe	Val	Val
FepA	12	Asp	Thr	Ile	Val	Val

Figure 3. Consensus sequence of TonB dependent transporters. Numbers refer to the position of the first amino acid and the invariant residues are underlined (47).

This hypothesis further states that the energy "transduced" to transporters is provided to the TonB N-terminal via the proton motive force harnessed by ExbB and ExbD (22, 32, 38). X-ray crystal structures of the TonB C-terminal in complex with FhuA showed that the interaction of C-terminal TonB with the TonB box results in the formation of a 3 stranded β-sheet between the 2 proteins. In addition to the TonB box, the interaction also involves residues from the FhuA plug (Ala26, Glu56) and barrel, (Asn594, Ala591). This interaction causes the C-terminal of TonB to occlude about half of the periplasm exposed surface of the OM protein and places FhuA in such a manner that TonB could create a force perpendicular to the β-strands of the plug domain to cause their movement (17, 44, 45).

Mechanisms of Transport

As mentioned earlier, for the passage of the ligand through the barrel to occur, the plug domain must be either removed from the barrel or altered in such a way that it would no longer block the ligand's passage. Two possibilities for the movement of the plug domain to allow ligand passage is that it either drops out of the bottom of the barrel on a "hinge" (ball and chain), or it undergoes conformational change within the barrel. Several studies have been conducted on various proteins in the family such as FhuA and FepA in order to determine the mechanism of action. In the past, evidence against the first theory was the amount of energy required to break the large number of hydrogen bonds holding the plug in the barrel. Because the barrel is water-filled, however, the energy required would actually be much less than previously thought and is

estimated to be about 10 ATP (26).

Two of the salt bridges holding the plug domain within the barrel originate from 4 highly conserved residues termed the "lock region" (Figure 4). Two arginine residues within the plug domain (Arg93/133 in FhuA; Arg75/126 in FepA; Arg150/196 in FecA) and 2 glutamate residues within the barrel domain (Glu511/567 in FepA; Glu522/571 in FhuA; Glu541/587 in FecA) make up this region. Site-directed mutagenesis studies of FepA lock region residues showed that when altered to neutral residues, preventing salt bridges and hydrogen bonding, the transport capabilities of the protein were severely reduced. One possibility is that the results are due to the inability of the salt bridges to be reformed and help regain the proper conformation of the plug domain. The same results were observed when conserved glycine residues at positions 127 and 134 were altered to alanine. Glycine allows for a greater degree of rotation within proteins and these particular glycine residues have angles far greater than those allowed for other residues. From these data, the conclusion was made that these residues may allow for rotation of β-strands 5 and 6 to help create the transient channel for the transport of ferric enterobactin (7). An explanation for these data that fits the ball and chain hypothesis is that perhaps the proton motive force associated with the TonB complex causes protons to enter the barrel and lower the pH thus neutralizing the salt bridges and resulting in charge repulsions that might expel the plug (26).

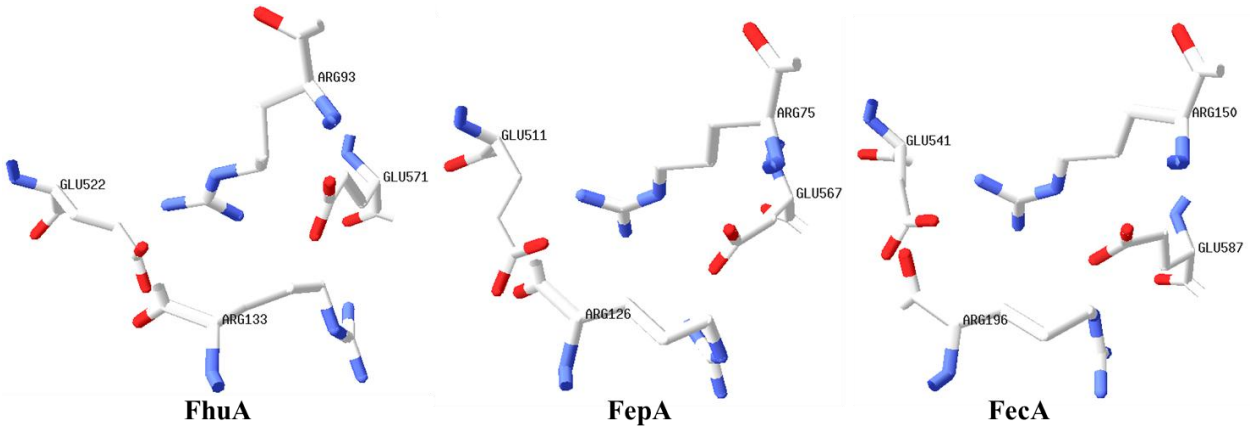

Figure 4. Comparison of lock region residues. Lock region residues are both spatially and structurally conserved across TonB dependent transporters such as FhuA (1BY3), FepA (1FEP), and FecA (1KMO).

The Coulton research group out of McGill University in Quebec, Canada is also responsible for several experiments performed on FhuA resulting in evidence for the conformational change hypothesis. The double cysteine mutants L109C/S356C and Q112C/M383C tether the plug domain to the barrel wall by the formation of disulfide bonds. Kinetics studies indicated that the ability of the protein to transport ferrichrome was not affected, thus the mechanism must not require complete removal of the plug domain from the barrel (16). A 1µM concentration of ferrichrome was used during these studies, however, making a concentration of iron available greater than that typically found in the bacterial cell. As a result, passive diffusion of the molecule into the cell may have occurred and resulted in inaccurate data. In addition, the disulfide tethers were created on a single side of the barrel, failing to take into account movement that may occur on the opposite side of the protein.

A 2007 study by Chakraborty et al. attempted to replicate Coulton's results by creating the FhuA mutants Y72C/S615C and P74C/S587C, where the plug domain was tethered to the barrel via a disulfide bond (8). FhuA mutant N104C/L149C was also created to tether plug β-strands 4 and 6 together (8). Kinetic studies showed that the N104C/L149C mutant had severely diminished transport with normal binding whereas the other 2 mutants had normal binding and

only slightly impaired transport. FhuA mutant protein G141A, where Gly141 is located between β-strands 5 and 6, also had severely diminished transport indicating a critical role in supporting the conformational change of these strands (8).

In contrast, similar studies on FepA by Klebba's group seem to provide evidence for the ball and chain hypothesis. They studied several cysteine mutants by fluorescent labeling techniques including the double cysteine mutant I14C/G300C. In their study, the formation of a disulfide bond between the plug domain and the barrel eliminated transport of enterobactin through FepA thus implying that the plug had to be dislodged from the barrel during transport (35). Residue 14, however, is a part of the FepA TonB box so the observed lack of transport might be attributed to the inability of FepA to interact with TonB.

Project Goals

Mutant FhuA 104/149C was created to tether β-strands 4 and 6 of the plug domain preventing their movement. If conformational change of the plug domain is required for transport to occur, then ferrichrome transport by this mutant protein should be diminished or severely reduced. Kinetics studies performed by a previous graduate student indicated that this mutant is capable of binding ferrichrome normally but is severely retarded in terms of its ability to transport the ligand when compared to wild type FhuA (8). The goals of this project were to carry out further structural and biochemical studies on the FhuA mutant 104/149C to determine 1) whether the diminished transport is due to unintended structural changes created by the mutation and 2) whether the mutation had a disruptive effect on the TonB binding interaction resulting in diminished transport. To achieve this, the FhuA mutant 104/149C protein was purified for structural studies by crystallization and X-ray diffraction and TonB binding interactions were studied using TonB-C terminal protein purified in the course of the study.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Escherichia coli strain BL21(DE3) was used to maintain and express all proteins in pET17b vector for protein expression. The strains were grown at 37°C and maintained on modified Luria-Bertani (Appendix) plates with 50-100 µg/mL carbenicillin at 4°C. Glycerol stocks of each strain were prepared for long-term storage by growth in 50mL of LB broth$_{Carb50}$ to an optical density (OD_{600nm}) of 0.6 to 0.9 then 1mL of the culture was added to 200µL of sterile 75% glycerol and stored at -80˚C. All materials and reagents, unless otherwise stated, were purchased from Fisher Scientific.

Protein Detection

Protein expression was verified via SDS-PAGE analysis and Western Blotting.

SDS-PAGE Analysis

A 10% separating gel (Table 1) was used to separate proteins by size to verify the presence of the target protein. The gel was made by mixing the first 5 ingredients of the separating and stacking gels according to Table 1. TEMED and 10% ammonium sulfate were added to begin polymerization after each solution was deaerated by vacuum pump for 5-20 minutes. The separating gel was pipetted into to the gel caster and allowed to polymerize for 20-30 minutes prior to deaeration and polymerization of the stacking gel. Samples were prepared by centrifugation and resuspension of cells in an equal volume of sample buffer prior to being boiled for 5 to 10 minutes. Fifteen to 40 µL of each sample was loaded into lanes, with the first lane containing 10µL of Bio-Rad Precision Plus® Dual Color protein standard. Each gel was run for 60 minutes at 200 volts or 60 mAmps (Bio-Rad). Gel visualization was accomplished by

placement in Coomassie blue stain for 30 minutes followed by destaining for 30 minutes to an hour in destaining solution. Gels were stored in 3% acetic acid until preserved by drying. The composition of SDS-PAGE buffers and solutions can be found in the Appendix.

Table 1. SDS-PAGE gel mixtures

Solution	Volume	
	Stacking Gel	**Separating Gel**
30% Bis-Acrylamide	1.33 mL	6.66mL
Stacking Gel Buffer	2.50 mL	Ø
Running Gel Buffer	Ø	5.0 mL
10% SDS Solution (pH 7.2)	0.1 mL	0.2 mL
Distilled Water	6.1 mL	8.0 mL
TEMED	5µL	10µL
10% Ammonium Persulfate	50µL	100µL

Western Blot

Polyclonal rabbit antibodies against FhuA (ProSci) were used with the western blot technique to directly determine the presence of the transport protein in a sample (11). Primary antibody was preabsorbed overnight at 4˚C with empty BL21(DE3) cells resuspended in 1mL phosphate buffered saline and boiled for 10 minutes. In preparation, SDS-PAGE gels were acclimated in transfer buffer with fiber pads, and Whatman #2 filter paper for 5 to 20 minutes. A Millipore® polyvinylidene floride (PVDF) membrane was rinsed first in methanol, followed by distilled water prior to acclimation in transfer buffer. An SDS-PAGE gel was placed against the membrane, avoiding bubbles, and then placed between 2 pieces of filter paper and fiber pads. The resulting "sandwich" was placed in the transfer apparatus with ice-cold transfer buffer and run at 25-30 volts overnight at 4˚C (Bio-Rad). Protein blotted membranes were equilibrated in blocking buffer for 1 hour to bind excess protein then left in a 1:100 primary (anti-FhuA) antibody/blocking buffer solution overnight at 4˚C. Membranes were subjected tosix 5 minute rinses in 50mL wash buffer to remove excess or loosely bound antibody then left in 1:5000

solution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) antibody/blocking buffer overnight at 4˚C. Six 5 minute wash buffer rinses were performed prior to development in HRP substrate in the dark for 30 minutes to 2 hours. The composition of all solutions and buffers can be found in the Appendix.

Estimation of Protein Concentration by Bicinchoninic Acid Assay

Protein concentrations were estimated using the Pierce® Bicinchoninic Acid (BCA) Protein Assay kit. The working reagent was made by mixing BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1M sodium hydroxide) with BCA reagent B (4% cupric sulfate) in a 50:1 ratio to obtain a green solution. When mixed with protein, the alkaline environment allows reduction of Cu^{2+} to Cu^{+} by cysteine, tyrosine, and tryptophan residues. The cuprous ion forms a complex with the bicinchoninic acid, resulting in the formation of a purple color, the intensity correlating to the concentration of protein present (50, 53, 56).

An aliquot of the sample ranging from 1µL to 100µL was mixed with a specified diluent for a final volume of 100µL. To this, 2mL of the kit's working reagent was added and the solution incubated at 37°C for 30 minutes. The optical density was then measured at a wavelength of 562nm using 1mL of the diluent as a blank. The concentration of the sample was calculated using the equation of the line of best fit from the standard curve derived for the specific diluent.

Standard curves were determined by mixing a stock solution of BSA at 2mg/mL in 0.9% saline with the diluent according to Table 2. A 2mL aliquot of working reagent was added to 100µL of each standard solution in triplicate and incubated at 37°C for 30 minutes. Using Microsoft® Excel, resultant OD_{562} measurements were plotted against the concentration of BSA

in µg/mL to obtain the equation of the line of best fit.

Table 2. BCA assay standard curve dilution table. For each diluent, the following table was used to create a 5-2000ug/mL standard curve (53).

Standard Curve for Bicinchoninic Acid Protein Assay				
Vial	**Diluent (uL)**	**Volume (uL)**	**Source**	**Final BSA Conc. (ug/mL)**
A	0	300	Stock	2000
B	125	375	Stock	1500
C	325	325	Stock	1000
D	175	175	Vial B	750
E	325	325	Vial C	500
F	325	325	Vial E	250
G	325	325	Vial F	125
H	400	100	Vial G	25
I	400	100	Vial H	5
J	400	0		0

Extraction and Purification of FhuA 104/149C

Growth and Expression

FhuA mutant 104/149C pET17b in BL21 (DE3) was grown in 1 liter LB_{Carb50} broth cultures to an OD_{600} of 0.4 to 0.6 (about 3 hours) then induced with 1mM IPTG, supplemented with 50µM of $CuSO_4$ to prevent reduction of the engineered disulfide bonds, and allowed to incubate for 3 hours. Cells were harvested by centrifugation at 8,000 rpm for 5 minutes and stored at -80˚C until use.

Extraction

To isolate the outer membrane protein, cells were resuspended in 20mL of ice-cold Buffer A (Appendix) per liter of culture harvested and homogenized overnight at 4˚C. Resuspended cells were aliquoted into 3 50mL beakers, 30 mL apiece, placed in a sodium chloride ice-bath and sonicated for 1 minute at a relative output of 0.8. The process was repeated

for a total sonication time of 5 minutes. This process was repeated until all of the resuspended cells had been sonicated, replacing the melted ice as needed.

After sonication, the homogenate was centrifuged at 8,000 rpm for 10 minutes to remove cell debris and the resultant supernatant centrifuged at 30,000 rpm for 90 minutes. Upon completion, the supernatant was saved as fraction A and the pellet homogenized in Buffer B for 30 minutes at room temperature prior a second round of centrifugation at 30,000 rpm for 90 minutes. Resultant supernatant was saved as fraction B1 and the process was repeated to obtain fraction B2. Buffer D containing 2% β-D-octylglucoside was placed on the resultant pellet overnight at 4˚C followed by homogenization at room temperature for 30 minutes. Buffer D homogenate was then centrifuged at 30,000 rpm for 90 minutes. Solubilized FhuA mutant protein was found in the resultant supernatant and saved as fraction D for further purification. All fractions and the end pellet were analyzed via SDS-PAGE to verify the presence or absence of mutant FhuA protein in each. Composition of buffers can be found in the attached appendix.

Purification

FhuA 104/149C was purified from fraction D via high performance liquid chromatography (HPLC, Bio-Rad Biologic DuoFlow). The anion exchange column (All Tech Hema IEC DEAE) was equilibrated with Mobile Phase (MP) A (Appendix) prior to injection of 10mL of fraction D. A gradient from 100% MP A to MP B was used to elute the protein, resultant fractions were analyzed via SDS-PAGE for the presence of the FhuA protein. Fractions containing the protein were pooled and dialyzed overnight at 4˚C in MP A to reduce the concentration of salt prior to exchange of the Triton-X100 detergent with lauryldimethylamine-oxide (LDAO) detergent via a DEAE Sepharose CL-6B anion exchange column. LDAO detergent forms smaller micelles and serves to facilitate solubilization of the protein as well as

the crystallization process. Dialyzed samples were loaded into the column and rinsed with 20mL of MP A with LDAO detergent. Elution of the protein was performed with 20mL of MP B with LDAO detergent where 2mL fractions were collected and analyzed via SDS-PAGE to identify protein containing fractions. These fractions were pooled and concentrated to 5mg/mL in a Corning® Spin-X® UF 6 spin filter. All buffers were supplemented with 50µM $CuSO_4$ to prevent reduction of the engineered disulfide bonds during extraction and purification.

Extraction and Purification of TonB C-Terminal

Growth and Expression

A 135 residue (109-244) fragment of TonB's C-terminal was histidine tagged at the N-terminal and amplified from TonB C-terminal/pET32 plasmid DNA via polymerase chain reaction (PCR) with the following primers at an annealing temperature of 48˚C: forward 5'-CATATGCATCATCACCATCACCAC**AAA**AAGGTACAGGAGCAG-3', (NdeI restriction site is underlined and TonB residue 109 is in bold) and reverse 5'-AAGCTT**TTA**CTGAATTTCGGTGGT-3' (HindIII restriction site underlined and TonB stop codon in bold). Enzymes were removed from the resultant PCR product using the GE® IllustraGFX® kit then digested for 3 hours at 37˚C with NdeI and HindIII restriction enzymes prior to overnight ligation at 4˚C with restriction digested, gel-purified pET17b (see appendix for reaction mixtures). Subsequent heat-shock transformation of 1µL of the ligation into Novablue yielded plasmid DNA for transformation into the BL21 (DE3) host for expression. The culture was grown in 1 liter LB_{Carb50} broth cultures to an OD_{600} of 0.4 to 0.6, induced with 1mM IPTG and allowed to incubate for 3 hours. Cells were harvested by centrifugation at 8,000 rpm for 5 minutes and stored at -80˚C until use.

Extraction

To isolate the protein, harvested cells were resuspended in 20mL of ice-cold Buffer T per liter and homogenized overnight at 4˚C. Resuspended cells were aliquoted into 30mL fractions in 3 50mL beakers, and placed in a sodium chloride ice-bath. The cells were sonicated for 1 minute at a relative output of 0.8. The process was repeated 4 times for a total sonication time of 5 minutes. After sonication, the homogenate was centrifuged at 8,000 rpm for 10 minutes to remove cell debris and the resultant supernatant diluted 1:1 with Ni-NTA Equilibration Buffer (23, 39).

Purification

The histidine tagged TonB C-Terminal was purified from the extract/Equilibration Buffer via gravity filtration through 3mL HisPur™ Ni-NTA columns. Ni-NTA columns were first equilibrated to room temperature with 6mL of Ni-NTA Equilibration Buffer then loaded with the protein extract mixture and the flow through collected. The Ni-NTA column was next washed with 18mL of Ni-NTA Wash Buffer collected in three, 6mL fractions and the protein eluted with 9mL of Ni-NTA Elution Buffer collected in three, 3mL fractions. Fractions containing TonB C-terminal were pooled and dialyzed overnight at 4˚C against Ni-NTA Equilibration Buffer. The column was regenerated by the passage of 30 mL MES Buffer and 30mL deionized water prior to re-equilibration with Ni-NTA Equilibration Buffer. Columns were stored as 50% slurry in 20% ethanol at 4˚C (52).

The affinity chromatography purified protein was further purified by high performance liquid chromatography (HPLC, Bio-Rad Biologic DuoFlow). The “All Tech Hema IEC DEAE” anion exchange column was used for further purification. Equilibration of the column with Buffer T (Appendix) occurred prior to injection of 2mL of concentrated TonB C-terminal and

eluted with Buffer U. The eluted fractions were analyzed via SDS-PAGE for the presence of pure protein. Fractions containing pure protein were pooled and concentrated using an Amicon® Centricon Plus-20 spin filter.

TonB C-Terminal Binding Assays

In order to determine whether the generated mutations have had an effect on the ability of the protein to interact with the TonB C-terminal, TonB binding assays were conducted. FhuA proteins in the pET17b vector and BL21 (DE3) host were grown in 1 liter LB_{Carb50} broth cultures to an OD_{600} of 0.4 to 0.6, induced with 1mM IPTG, supplemented with 50µM of $CuSO_4$ as necessary, and allowed to incubate for 3 hours. Cells were harvested by centrifugation at 8,000 rpm for 5 minutes. Harvested cells were resuspended in 20mL of ice-cold Buffer A and stirred overnight at 4˚C. Resuspended cells were sonicated for 1 minute at a relative output of 0.8. The process was repeated for a total sonication time of 7 minutes. After sonication, the homogenate was centrifuged twice at 8,000 rpm for 10 minutes to remove cell debris and the resultant supernatant diluted 1:1 with Ni-NTA Equilibration Buffer.

The cell extract was then passed through a 3mL HisPur™ Ni-NTA column as described in TonB C-terminal purification. The concentration of FhuA protein in the flow through was estimated and incubated with 10-fold molar excess of ferric ferrichrome, deferri-ferrichrome, or no ferrichrome for 30 minutes at room temperature. Purified C-terminal TonB was added in a 1:1 molar ratio and the mixture incubated for 1 hour at room temperature. After incubation, the mixtures were passed through a 3mL HisPur™ Ni-NTA column washed and eluted as previously described; a different column was used for each FhuA protein. Flow through and all other fractions were analyzed by SDS-PAGE. If the ability of the protein to interact with TonB has not been affected then the transport protein and TonB C-terminal should be eluted together after

incubation with ferric-ferrichrome, whereas if it has been negatively affected, or if ferric-ferrichrome is not present for binding, then only a single band matching the TonB C-terminal should be observed in elution fractions (23, 39).

Protein Crystallization

The conditions described by Locher et al. for the hanging drop vapor-diffusion method of McPherson were used for crystallization of FhuA 104/149C and FhuA 104/149C in complex with ferrichrome (33, 37). Twenty-four well plates were used to grow crystals from 1µL of the concentrated protein at 10µg/µl, and 2µL of reservoir solution composed of: 33% polyethylene glycol (PEG) 2000, 0.45M NaCl, 0.15M sodium phosphate buffer pH 6.2, 0.5% n-octyl-2-hydroxyethylsulfoxide (OES) detergent (BACHEM) and 50µM $CuSO_4$ to maintain disulfide tethers. The drop was mixed by gentle pipetting on a glass coverslip, inverted and equilibrated against a 1 mL reservoir at room temperature (33). The coverslips were sealed with Corning ® silicon grease and the process was repeated for each well. Crystals were preserved by flash cooling in liquid nitrogen cooled liquid propane prior to using X-ray diffraction to determine the structure of the mutant FhuA protein at the University of Tennessee, Knoxville with the help of Dr. Fernandez (this work is still pending). All crystallization supplies were purchased through Hampton Research.

CHAPTER 3

RESULTS

Bicinchoninic Acid Protein Estimation Standards

BCA protein assay standard curves were prepared for 2 different diluents, Buffer A with LDAO, and Ni-NTA Equilibration Buffer. Each standard was prepared in triplicate according to the standard curve table above (Table 2). The standard curve using Buffer A with LDAO as a diluent was determined to have a best-fit line with the following equation: Y=0.0008X + 0.0633 where Y is the optical density measured at 562nm and X the protein concentration in µg/mL (Figure 5). Similarly, the standard curve with Ni-NTA Equilibration Buffer as the diluent was found to have a best-fit line with the equation: Y=0.0008X+0.0311 (Figure 6).

BCA Protein Assay Standard Curve for Buffer A w. LDAO & 50uM $CuSO_4$

BSA Concentration		2000	1500	1000	750	500	250	125	25	5	0
O.D. 562nm	1	1.754	1.379	1.080	0.855	0.623	0.448	0.321	0.219	0.139	0.137
	2	1.799	1.466	1.058	0.813	0.637	0.407	0.364	0.214	0.165	0.140
	3	1.718	1.411	1.035	0.770	0.676	0.428	0.295	0.194	0.162	0.127
Average		1.757	1.419	1.058	0.813	0.645	0.428	0.327	0.209	0.155	0.135
Corrected		1.622	1.284	0.923	0.678	0.511	0.293	0.192	0.074	0.021	0.000

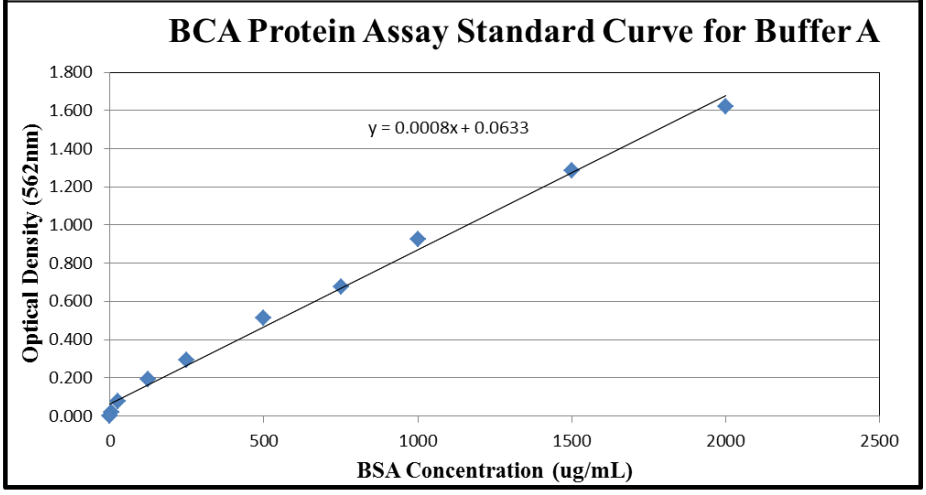

Figure 5. Results for BCA standard curve with Mobile Phase Buffer A with LDAO.

BCA Protein Assay Standard Curve for Ni-NTA Equilibration Buffer

BSA Concentration		2000	1500	1000	750	500	250	125	25	5	0
O.D. 562nm	1	1.752	1.379	0.924	0.806	0.603	0.335	0.301	0.166	0.121	0.205
	2	1.667	1.320	0.932	0.839	0.623	0.385	0.260	0.140	0.124	0.130
	3	1.783	1.226	1.015	0.819	0.681	0.375	0.246	0.137	0.133	0.242
Average		1.734	1.308	0.957	0.821	0.636	0.365	0.269	0.148	0.126	0.192
Corrected		1.604	1.178	0.827	0.691	0.506	0.235	0.139	0.018	-0.004	0.000

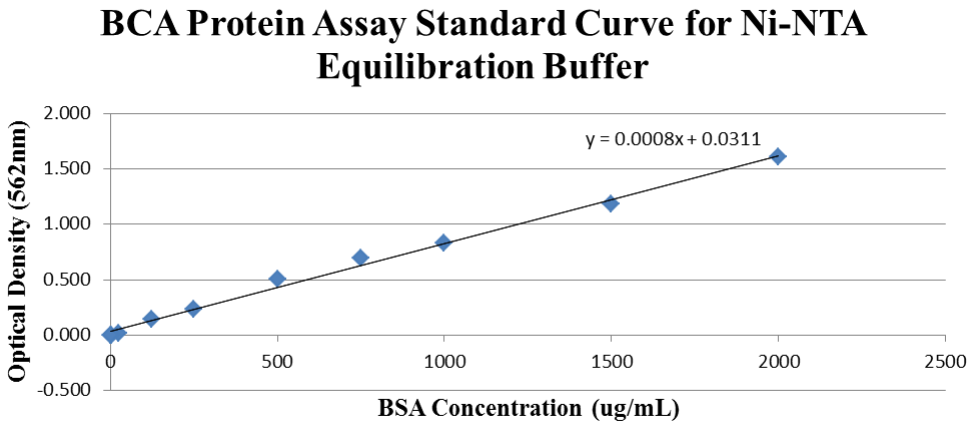

Figure 6. Results for BCA standard curve with Ni-NTA Equilibration Buffer

Protein Extraction and Purification

FhuA 104/149C

FhuA mutant protein 104/149C was created by genetically altering the non-conserved residues Asn104 and Leu149 to cysteine residues in order to engineer a disulfide bond tethering β strands 4 and 6, thus restricting their movement during transport. The Swiss pdb viewer theoretically confirmed the formation of a disulfide bond due to energetically favorable conditions (21). While these changes are not predicted to alter protein structure in any other form, it is still possible that unintended structural changes have occurred. Obtaining the crystal structure of the mutant would help determine what, if any, changes have been introduced.

Expression of FhuA 104/149C pET17b in BL21(DE3) was induced with 1mM IPTG and supplemented with 50μM $CuSO_4$ at an O.D. $_{600}$ of 0.4 to 0.6 and allowed to continue for 3 hours

prior to harvesting by centrifugation and storage at -80˚C. A total of 30 liters of culture suspensions were used; cells were collected and sonicated, 10 liters at a time, in Buffer A for extraction of the mutant protein. Supernatant was treated as described to obtain Fractions A, B1, B2 and D. All fractions and the final pellet were analyzed by SDS-PAGE to verify the presence of FhuA 104/149C (78kDa) in the original sonicated supernatant and Fraction D (Figure 7, black arrow).

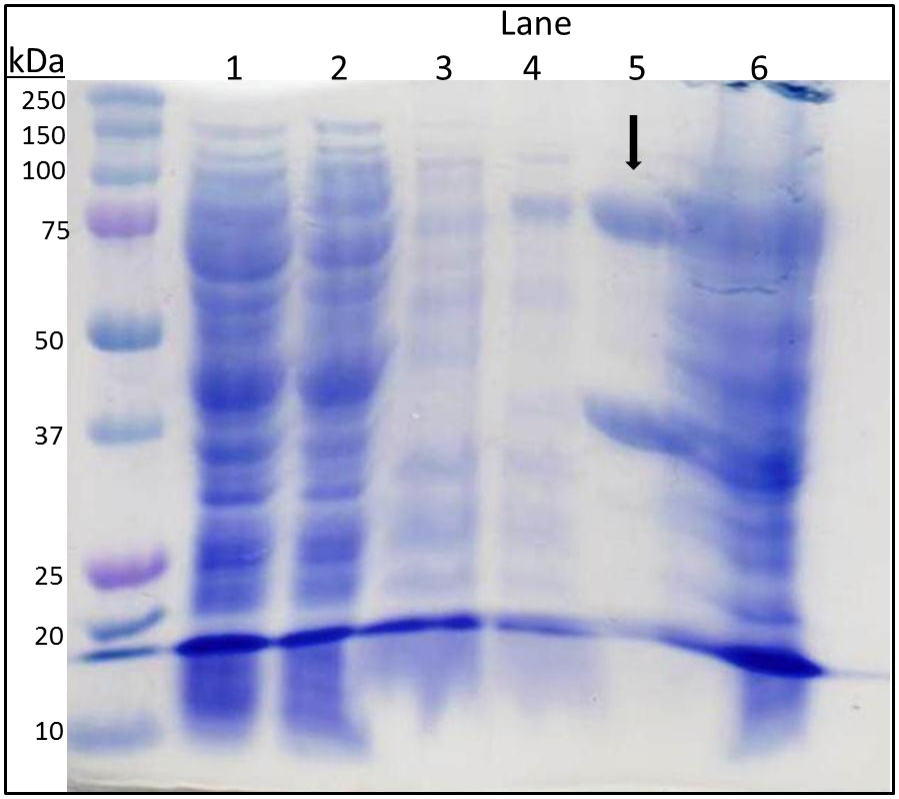

Figure 7. SDS-PAGE analysis of FhuA 104/149C extraction. The lane to the far left is the Bio-Rad® Dual Color protein standard with molecular sizes in kiloDaltons. Lanes 1 through 6 contain sonicated supernatant, Fraction A, Fraction B1, Fraction B2, Fraction D and the final pellet, respectively. The arrow points to the 78kDa band corresponding to solubilized FhuA 104/149C in lane 5.

FhuA 104/149C was initially purified using the HPLC program "Fraction D Initial Run" where 20mL of Mobile Phase A (MP A; Appendix) was followed by a gradient of MP A to 15% MP B over 8mL, then 15 to 30% MP B over 30mL run volume. A gradient to 100% MP B was performed over a 14mL run volume, followed by 10mL of 100% MP B. The column was

equilibrated between runs with 25mL of MP A. SDS-PAGE analysis revealed that FhuA 104/149C was eluted between 16 and 19% MP B along with a 37 kDa porin (Figure 8).

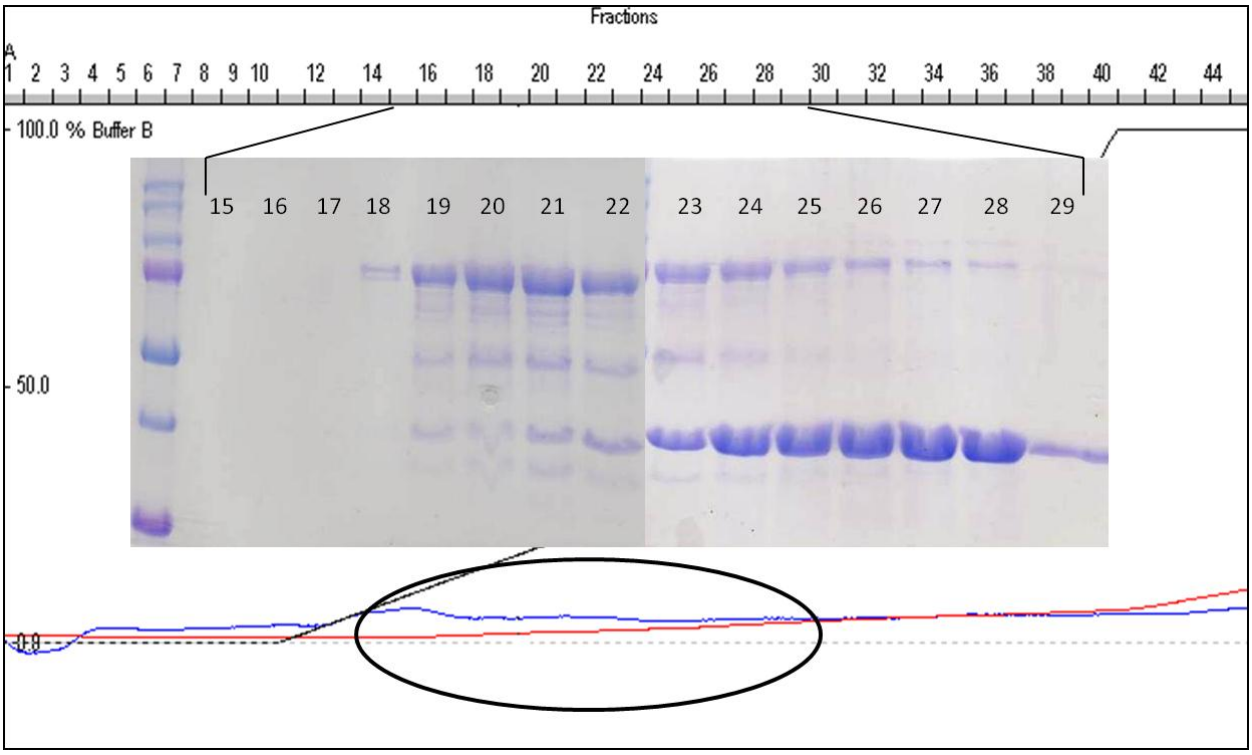

Figure 8. Chromatograph and SDS-PAGE analysis of "Fraction D Initial Run". HPLC fractions 15 through 29 corresponded to the circled peak and were analyzed by SDS-PAGE revealing FhuA 104/149C elution between 16-19% Mobile Phase Buffer B.

FhuA 104/149C was found to be best separated from contaminating proteins via the "Revision T2" program with a gradient of MP A to 16% MP B over 8mL, then 16 to 19% MP B over 30mL run volume collected in 1 mL fractions (Figure 9). FhuA 104/149C containing fractions were separated based on the amount of contaminating porin: "pure", presence of no contaminating porin; "FhuA with less porin", presence of some contaminating porin; and "FhuA with more porin", where there was the presence of a lot of contaminating porin. In order to reduce the concentration of sodium chloride in the sample in order to purify further via HPLC, similar fractions were pooled and dialyzed overnight at 4°C in 1 liter MP A with continuous stirring. Porin containing samples were loaded onto the HPLC and FhuA 104/149C eluted via the

"Revision T2" program. The process was repeated until SDS-PAGE analysis showed that all of the contaminating porin had been eliminated.

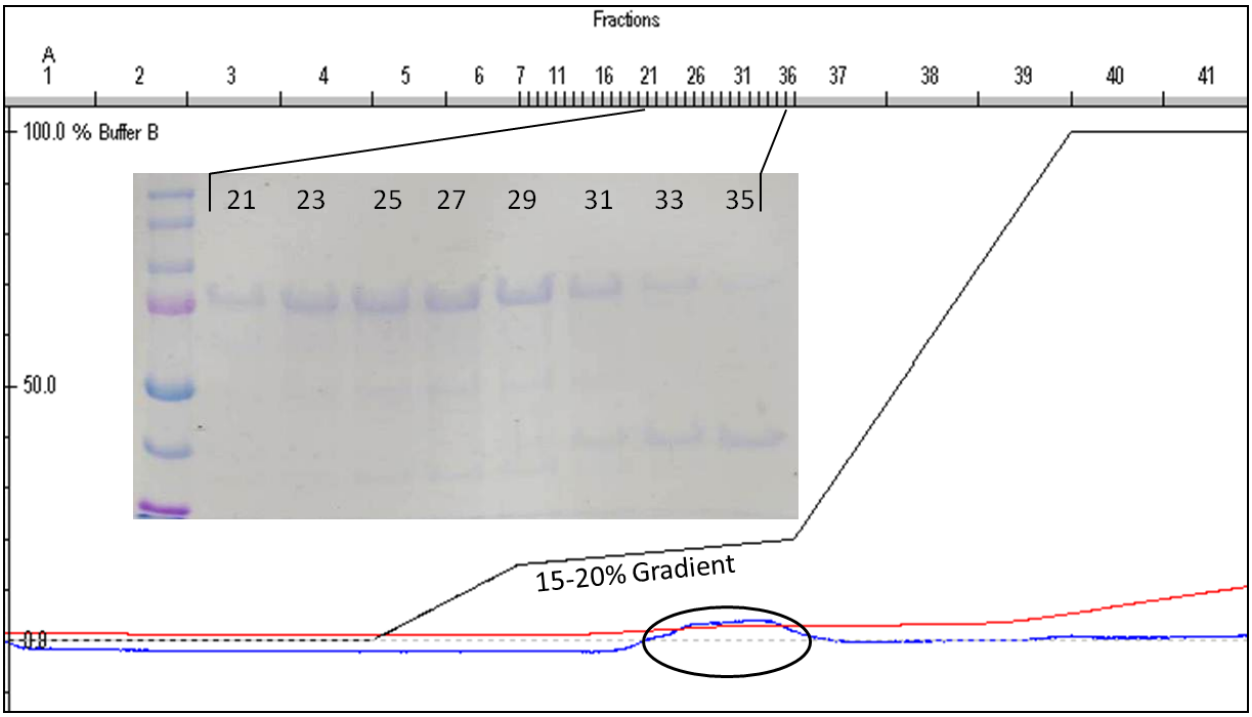

Figure 9. Chromatograph and SDS-PAGE analysis of "Program T2". HPLC fractions 20 through 36 correspond to the circled peak, every odd numbered fraction was analyzed by SDS-PAGE revealing FhuA 104/149C eluted along with a 37kDa porin. Fractions 20-29 were considered "pure" and 30-37 as "FhuA with less porin".

HPLC fractions containing pure FhuA 104/149C were concentrated to a final volume of 10mL per 10L of harvested cell culture via an Amicon® Centricon Plus-20 spin filter with a 30 kDa cut off. The filter was rinsed with 5mL of distilled, deionized water for 30 minutes at 3,000xg then 5mL of MP A for 50 minutes at 3,000xg. FhuA 104/149C fractions were loaded on to the filter to a volume of 10mL then centrifuged for 60 minutes at 2,500xg until all fractions were processed.

Concentrated fractions of FhuA 104/149C were loaded 10mL at a time on to a DEAE Sepharose CL-6B anion exchange column and rinsed with 20mL of MP A with LDAO detergent. Elution of FhuA 104/149C was accomplished with 20mL of MP B with LDAO detergent and

collected in 2 mL fractions. Analysis of LDAO detergent exchange elution fractions by SDS-PAGE showed FhuA 104/149C primarily in fraction 2 (Figure 10), though fractions 1 through 5 were pooled. Corning® Spin-X® UF 6 spin filters with a 30 kDa cutoff were used to concentrate the pooled fractions to approximately 5mg/mL at 7,000xg using protein concentrations estimated via BCA protein assays.

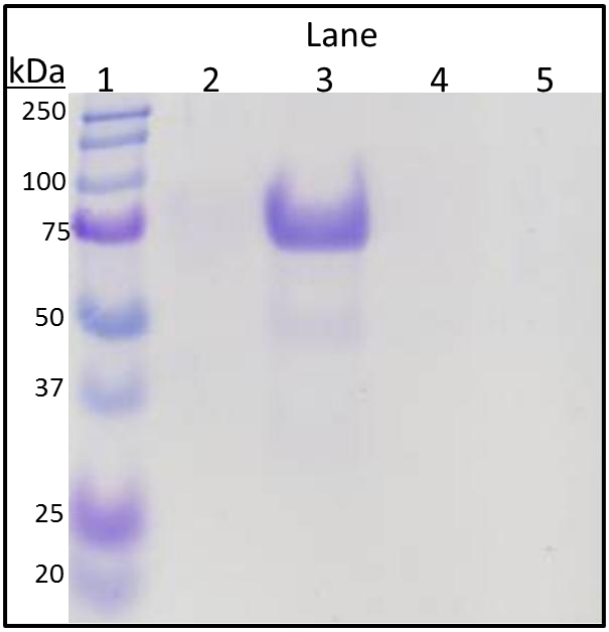

Figure 10. SDS-PAGE analysis of LDAO detergent exchange fractions. SDS-PAGE analysis of LDAO detergent exchange flow through (Lane 1), and elution fractions 2, 4, and 5 (Lanes 3, 4, and 5, respectively). FhuA 104/149C was observed primarily in fraction 2 (Lane 3).

TonB C-Terminal

Five, 1 liter cultures of hisidine tagged TonB C-Terminal in the pET17b vector and BL21 (DE3) host were grown to an OD_{600} of 0.4 to 0.6, induced with 1mM IPTG and incubated for another 3 hours. Cells were harvested by centrifugation at 8,000 rpm for 5 minutes and stored at -80˚C until use. To isolate the protein, harvested cells were resuspended in 100mL of ice-cold Buffer T and homogenized overnight at 4˚C. After sonication as described, the homogenate was centrifuged at 8,000 rpm for 10 minutes to remove cell debris and the resultant supernatant

diluted 1:1 with Ni-NTA Equilibration Buffer for a final volume of 200mL (Figure 11A).

Histidine tagged TonB C-terminal was purified from the extract/Equilibration Buffer via gravity filtration through 3mL HisPur™ Ni-NTA columns. Purification was performed with 50mL aliquots of protein extract mixture at a time until all protein extract had been processed. SDS-PAGE analysis revealed the presence of TonB C-terminal (15kDa) in elution fractions 2 and 3, with fraction 3 appearing to contain pure protein (Figure 11B). Elution fractions number 2 contained most of the desired protein along with a contaminating protein at 75 kDa. These fractions were pooled and dialyzed overnight at 4°C against 1 liter of Ni-NTA Equilibration Buffer. The protein mixture was then loaded on to the Ni-NTA column a second time and eluted in 11, 1mL fractions in an attempt to separate the TonB C-terminal from the contaminating protein. SDS-PAGE analysis revealed that the attempt was unsuccessful and both proteins were found in all elution fractions (Figure 11C).

Because the desired protein is only about 15 kDa in size and the contaminating protein was 75 kDa, the Ni-NTA elution fractions were passed through an Amicon® Centricon Plus-20 spin filter with a 30 kDa cut off in an attempt to separate the 2 proteins. After being rinsed with 5mL of distilled, deionized water and 5mL of Ni-NTA Equilibration Buffer, the elution fractions were centrifuged at 2,500xg. SDS-PAGE analysis revealed that this was also ineffective, the smaller protein (TonB C-terminal) had been retained and concentrated along with the contaminating proteins, possibly due to aggregation as a result of the large amount of protein present.

A 1 to 2 mL volume of concentrated TonB C-terminal was loaded onto the "All Tech Hema IEC DEAE" anion exchange column and run with the HPLC program "TonB Initial Run" where 20mL of Buffer T was followed by a gradient of Buffer T to 100% Buffer U over 50 mL,

then followed by 10mL of 100% Buffer U. The column was equilibrated between runs with 25mL of Buffer T. SDS-PAGE analysis verified pure TonB C-terminal in fractions 3 through 24 (Figure 11D). These fractions were pooled and concentrated to 95mg/mL, as determined by BCA analysis, with an Amicon® Centricon Plus-20 spin filter at 2,000xg.

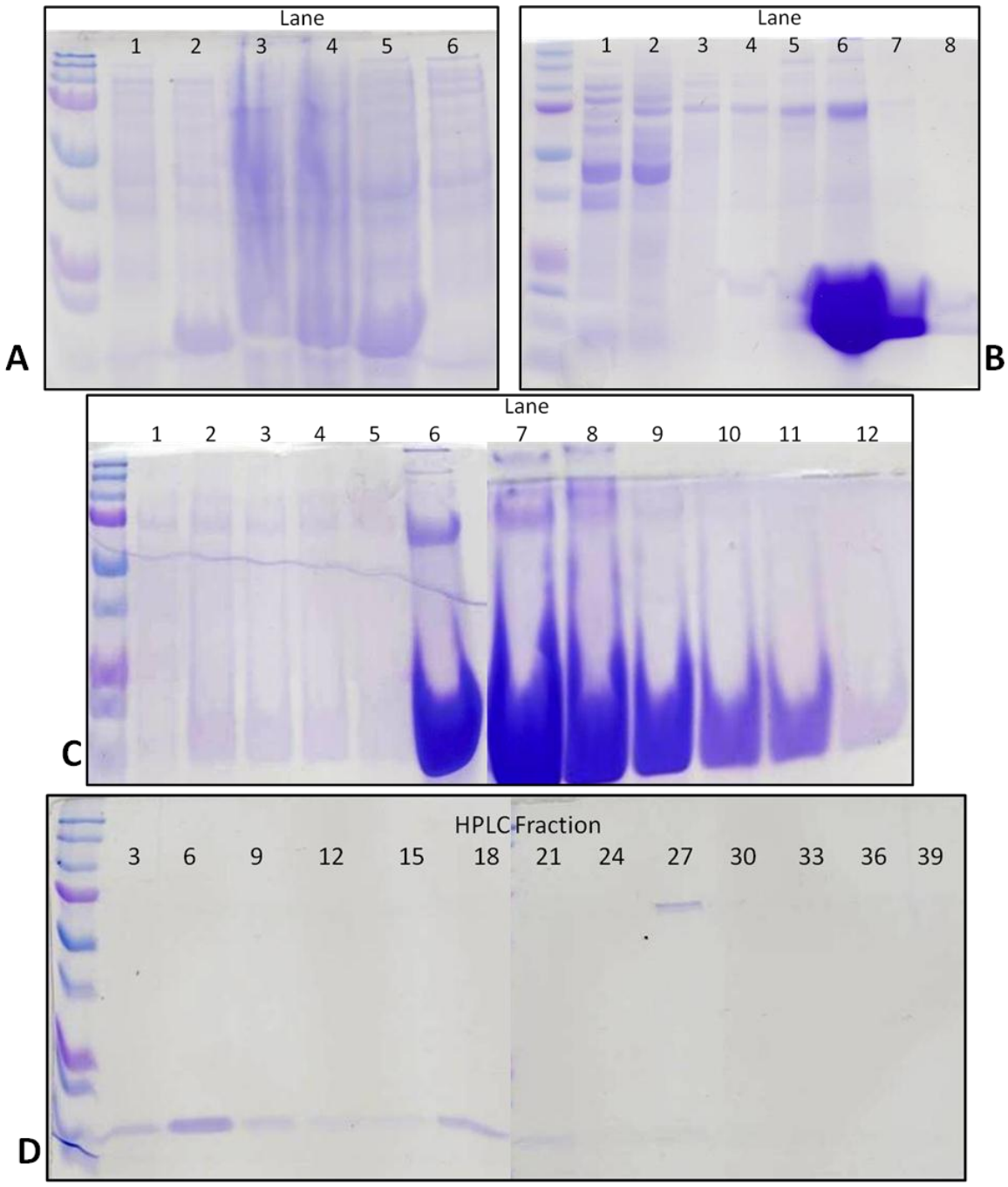

Figure 11. SDS-PAGE analysis of TonB C-terminal extraction and purification. (A) Lanes 1 through 6: TonB C-terminal pET17b BL21(DE3) not induced, TonB C-terminal pET17b BL21(DE3) 3 hour induced sample, cell suspension in Buffer T, sonicated cell suspension, sonicated supernatant, and Ni-NTA flow-through. (B) Lanes 1 through 6: Ni-NTA flow-through, Wash fraction 1, Wash fraction 2, Wash fraction 3, Elution fraction 1, Elution fraction 2, Elution fraction 3, and MES regeneration flow-through. (C) Ni-NTA elution fractions 1 through 11 from pooled and dialyzed TonB C-terminal containing fractions. (D) HPLC fractions obtained from "TonB Initial Run", fractions 3 through 24 were saved as "pure".

TonB-C Terminal Binding Assays

Wild Type FhuA

A 1L broth culture of FhuA pET17b in BL21 (DE3) was grown to an OD_{600} of 0.4 to 0.6 induced with 1mM IPTG and allowed to incubate for 3 hours. Harvested cells were resuspended in 20mL of ice-cold Buffer A and stirred overnight at 4˚C prior to sonication. Afterwards, the homogenate was centrifuged twice to remove all cell debris and the resultant supernatant was diluted 1:1 with Ni-NTA Equilibration Buffer.

Analysis by SDS-PAGE of the cell extract after passage through a Ni-NTA column showed that FhuA was present in the column flow through indicating that it passed through unhindered (Figure 12A). The concentration of protein in the flow through was determined by BCA to be 61 µg/µL and it was further estimated that the FhuA protein comprised 10% of the total protein giving it a concentration of about 78.2µM. A 10mL aliquot was incubated with 123µL of 95 mg/mL purified C-terminal TonB for 1 hour at room temperature then passed through a Ni-NTA column. All flow through, wash and elution fractions were analyzed by SDS-PAGE (Figure 12B). FhuA was not observed to be present in the elution fractions containing TonB C-terminal indicating that there was little to no binding interaction.

Next, 10-fold molar excess of deferri-ferrichrome (78.2µL of 100mM stock) and ferric ferrichrome (97.75µL of 80mM stock) were added to 10mL aliquots of FhuA Ni-NTA flow-through and incubated for 30 minutes at room temperature then for another hour after the addition of 123µL of purified TonB C-terminal. The resulting mixtures were also passed through Ni-NTA columns immediately after incubation. Flow-through and all other fractions were again analyzed by SDS-PAGE (Figures 12C and 12D). In both cases, protein bands corresponding to FhuA were found in elution fractions containing TonB C-terminal (Figure 12, black arrows).

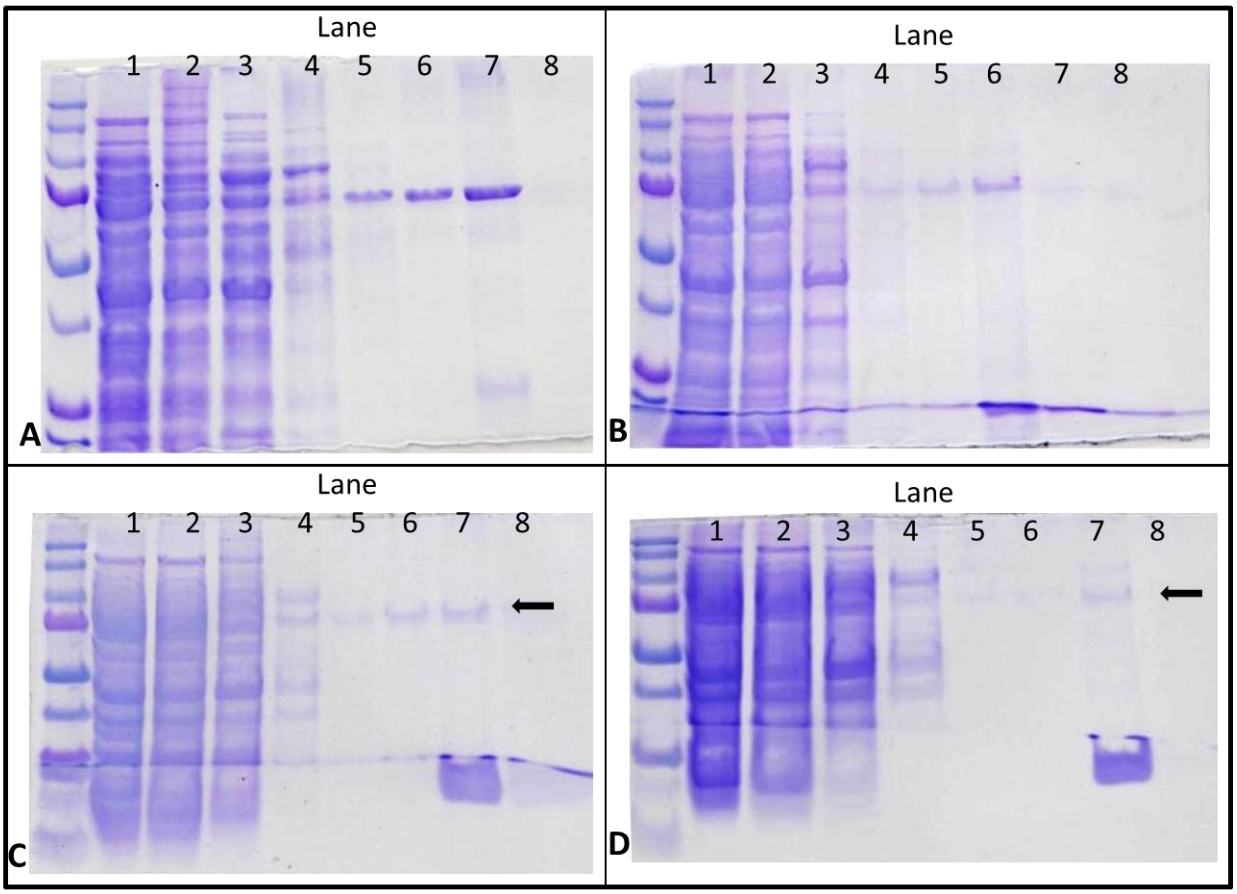

Figure 12. SDS-PAGE analysis of wild type FhuA/TonB C-terminal binding assays. (A) FhuA cell extract. (B) FhuA and TonB C-terminal. (C) FhuA, deferri-ferrichrome and TonB C-terminal. (D) FhuA, ferrichrome, and TonB C-terminal. For all figures: Lane 1 is the mixture prior to passage through Ni-NTA column, lane 2 is Ni-NTA flow through, lanes 3 through 5 are Wash fractions 1, 2 and 3(respectively), and lanes 6 through 8 are Elution fractions 1, 2 and 3 (respectively). Black arrows point to wild type FhuA in elution fraction 2.

Mutant FhuA 104/149C

A 1L broth culture of FhuA 104/149C pET17b in BL21 (DE3) was grown to an OD_{600} of 0.4 to 0.6 induced with 1mM IPTG, supplemented with 50µM $CuSO_4$, and allowed to incubate for another 3 hours. Cells were harvested by centrifugation at 8,000 rpm for 5 minutes then resuspended in 20mL of ice-cold Buffer A and stirred overnight at 4˚C. Resuspended cells were sonicated for 7 minutes and the homogenate centrifuged twice to remove all cell debris. The resultant supernatant was diluted 1:1 with Ni-NTA Equilibration Buffer.

After cell extract was passed through a Ni-NTA column as previously described SDS-PAGE analysis showed that FhuA 104/149C was present in the column flow through but not the elution fractions (Figure 13A). The concentration of protein in the flow through was determined by BCA to be 54 µg/µL and it was estimated that FhuA 104/149C protein comprised 10% of the total protein giving it a concentration of about 68.4µM. A 10mL aliquot was incubated with 108µL of 95 mg/mL purified C-terminal TonB for 1 hour at room temperature then passed through a 3mL Ni-NTA column. All flow-through, wash, and elution fractions were analyzed by SDS-PAGE. FhuA 104/149C was not observed to be present in the elution fractions containing TonB C-terminal, indicating little to no binding interaction (Figure 13B).

Next, 10-fold molar excess of deferri-ferrichrome (68.4µL of 100mM stock) and ferric ferrichrome (85.5µL of 80mM stock) were added to 10mL aliquots of FhuA 104/149C Ni-NTA flow through and incubated for 30 minutes at room temperature and for an hour after the addition of 108µL of purified TonB C-terminal. Immediately after incubation, the resulting mixtures were passed through Ni-NTA columns. Flow-through and all other fractions were analyzed by SDS-PAGE revealing in both cases protein bands corresponding to FhuA104/149C in elution fractions containing TonB C-terminal (Figures 13C and 13D, black arrows).

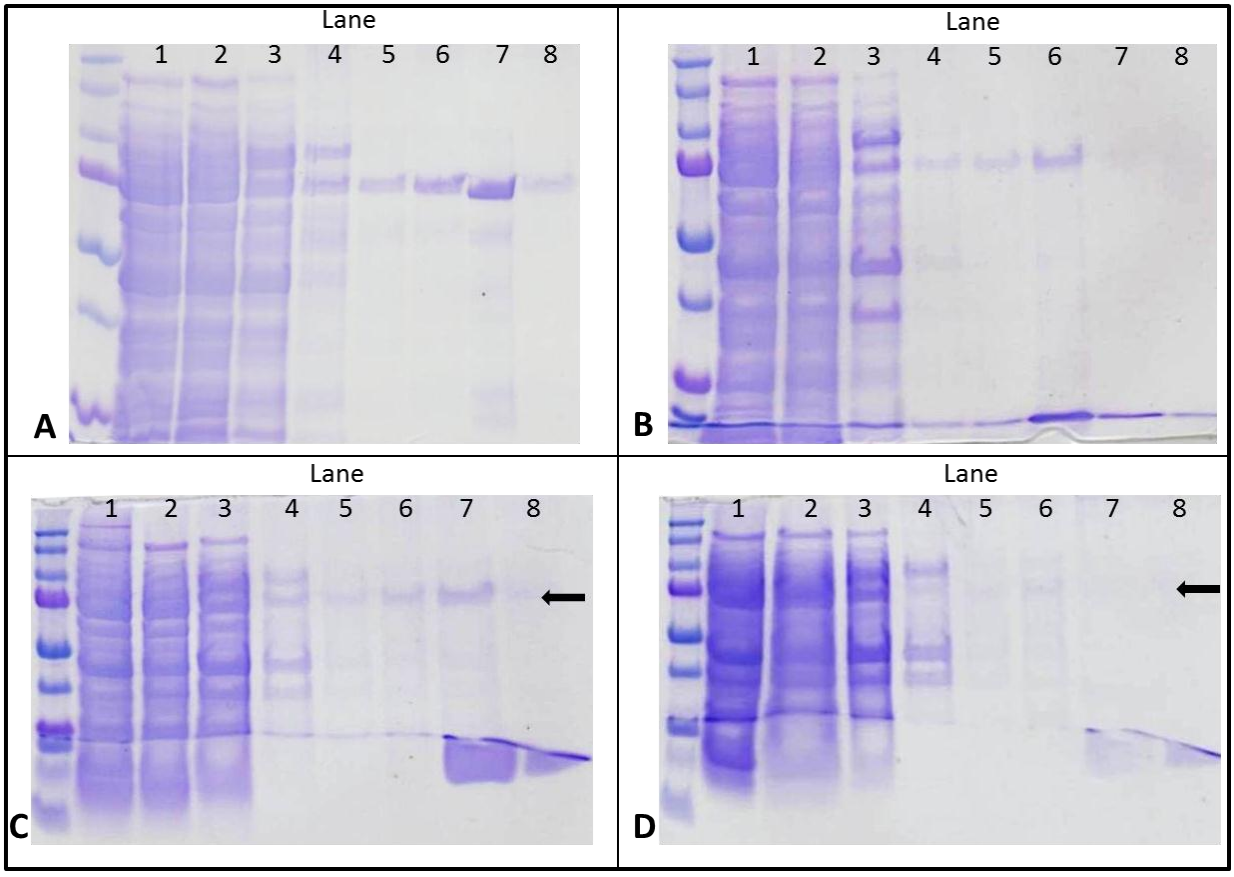

Figure 13. SDS-PAGE analysis of FhuA 104/149C/TonB C-terminal binding assays. (A) FhuA 104/149C cell extract. (B) FhuA 104/149C and TonB C-terminal. (C) FhuA 104/149C, deferri-ferrichrome and TonB C-terminal. (D) FhuA 104/149C, ferrichrome, and TonB C-terminal. For all figures: Lane 1 is the mixture prior to passage through Ni-NTA column, lane 2 is Ni-NTA flow through, lanes 3 through 5 are Wash fractions 1, 2 and 3(respectively), and lanes 6 through 8 are Elution fractions 1, 2 and 3 (respectively). Black arrows point to FhuA 104/149C in elution fraction 2.

Comparing Wild Type and Mutant FhuA

In order to confirm the presence and/or absence of FhuA proteins in elution fractions containing the TonB C-terminal, elution fraction number 2 from each experiment were analyzed side-by-side by SDS-PAGE and western blotting. Results showed that neither FhuA protein was present in the elution fractions from the initial cell extract. Neither were they present in elution fractions when incubated with TonB C-terminal alone. Conversely, both FhuA proteins were confirmed as being present in elution fractions after incubation with deferri-ferrichrome or ferric ferrichrome plus TonB C-terminal (Figure 14A and 14B).

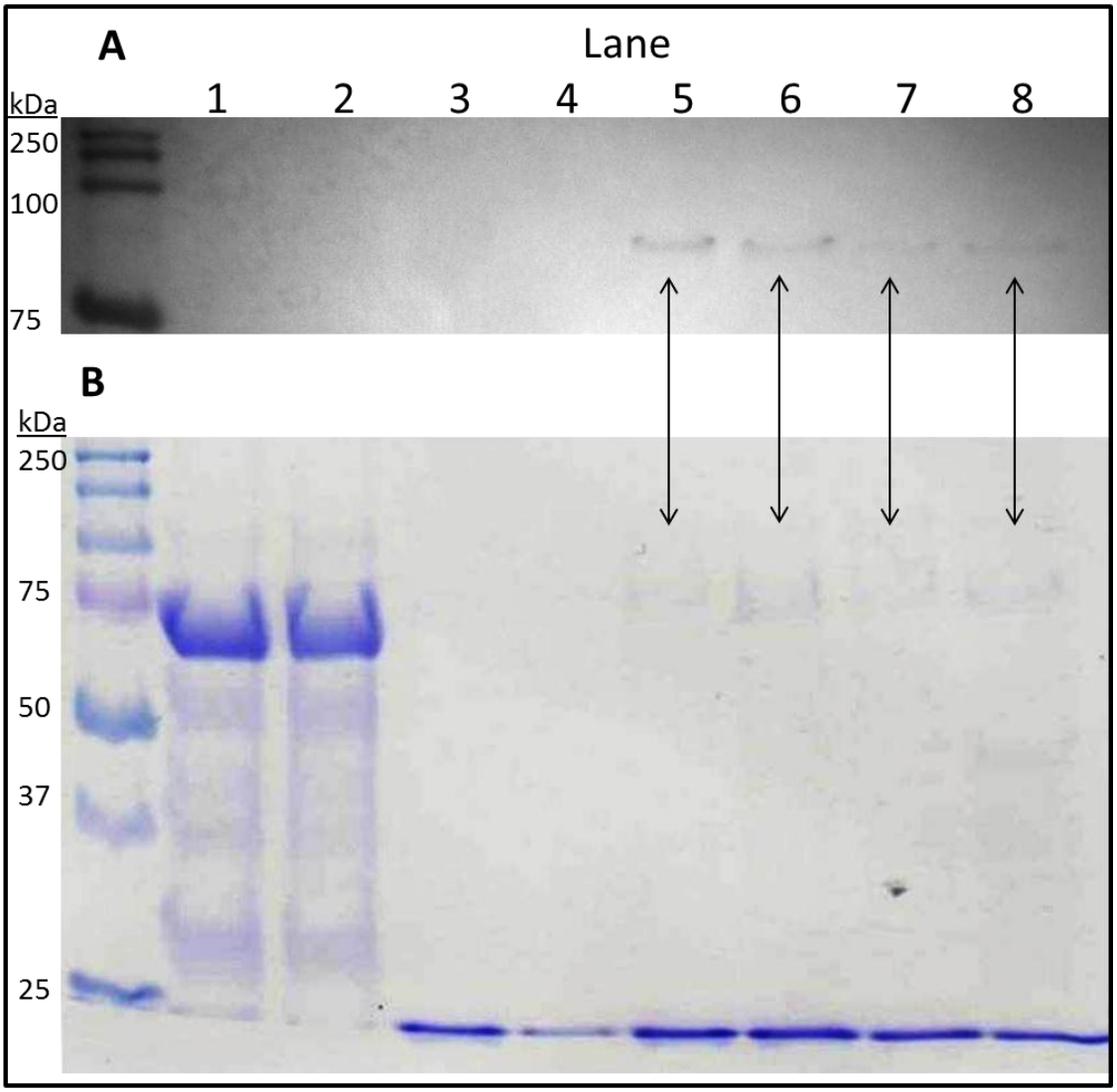

Figure 14. Side-by-side comparison of TonB C-terminal binding assays. Lanes 1 through 8 are the second elution fractions from FhuA cell extract, FhuA 104/149C cell extract, FhuA and TonB C-terminal, FhuA 104/149C and TonB C-terminal, FhuA, deferri-ferrichrome, and TonB C-terminal, FhuA 104/149C, deferri-ferrichrome and TonB C-terminal, FhuA, ferrichrome and TonB C-terminal and FhuA 104/149C, ferrichrome, and TonB C-terminal, respectively. (A) Western blot confirming presence of FhuA proteins in TonB C-terminal binding assays. (B) SDS-PAGE side-by-side comparison of TonB C-terminal binding assays. Black arrows point to where FhuA wild type and mutant proteins were detected.

Crystallization of FhuA 104/149C

Two 24 well crystallization plates were set up for crystallization of FhuA 104/149C and 1 for crystallization of FhuA 104/149C with ferric ferrichrome in a 1:1 molar ratio. Crystals were

observed within 3 days and monitored every 7 days. Crystals 100µm or larger were selected for flash cooling and x-ray diffraction (Figure 15).

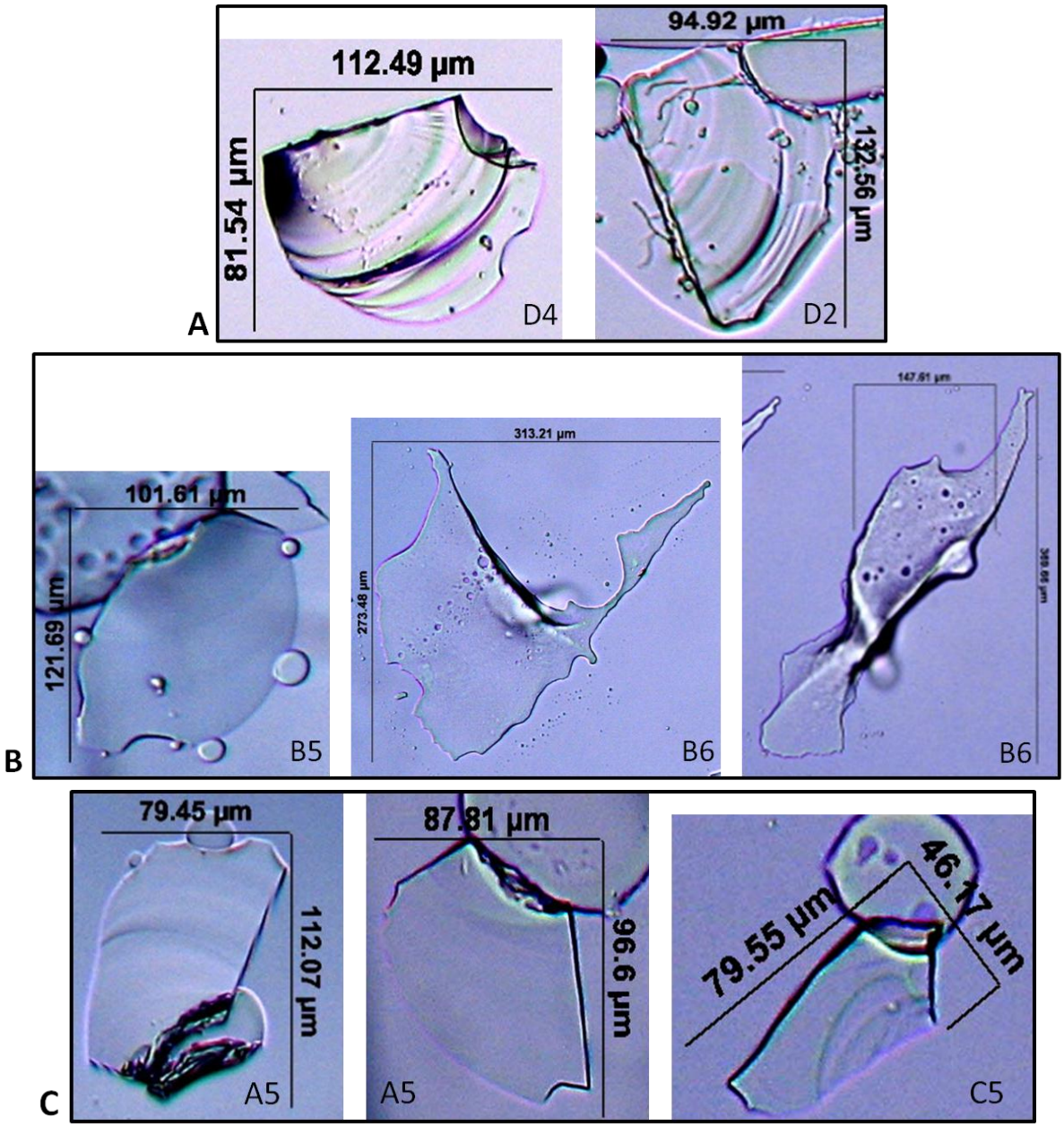

Figure 15. Images of FhuA 104/149C crystals. (A and B) Crystals of FhuA 104/149C, measurements for B6 crystals are 273.48µm x 313.21µm and 147.61µm x 369.66µm. (C) Crystals of FhuA 104/149C and ferrichrome complexes.

CHAPTER 4

DISCUSSION

The goal of this project was to further characterize the FhuA 104/149C mutant which is capable of binding ferric ferrichrome but is deficient in transport as compared to the wild type. This mutant contains 1 engineered disulfide bond tethering plug domain β-strands 4 and 6 together restricting movement. The observed decrease in this mutant's ability to transport ferrichrome supports the hypothesis that the plug domain undergoes conformational change during transport as opposed to dropping out of the barrel. It is possible, however, that the disparity in ferrichrome transport between the wild type and the mutant is an artifact of the mutagenesis. Perhaps the mutation of residues 104 and 149 to cysteine caused unintended changes in the structure which negatively affected the mechanism of transport or prevented interaction with the C-terminal of TonB inhibiting energy transduction.

HPLC purified FhuA 104/149C was crystallized both alone and with a 1:1 molar ratio of ferric ferrichrome. The crystal structure of FhuA 104/149C in free and ligand-bound states will be determined and compared to the structure of wild type FhuA. To determine if the interaction with TonB C-terminal has been compromised, TonB binding assays of cell extracts containing wild type FhuA and FhuA 104/149C were conducted using appropriate controls. Passage of the extracts through Ni-NTA columns confirmed that neither FhuA protein interacted with the column while passage of wild type FhuA after incubation with the 135 residue TonB C-terminal indicated that little to no binding occurred between FhuA protein and TonB C-terminal. When FhuA was incubated with either deferri-ferrichrome or ferric ferrichrome prior to exposure to TonB C-terminal, FhuA was found in elution fractions containing TonB C-terminal. This indicates that an interaction with enough affinity to pull FhuA from the cell extract occurred. In

all instances, the FhuA 104/149C mutant behaved similarly to the wild type protein. Incubation of both FhuA proteins with deferri-ferrichrome should have yielded results similar to that of FhuA and TonB C-terminal alone because deferri-ferrichrome does not bind to FhuA and thus would not signal the conformational changes required for FhuA to interact with TonB. The results obtained, however, can be explained by the fact that a complex cell extract was used in this experiment containing iron. The iron present was chelated by the supplied deferri-ferrichrome forming ferrichrome, which subsequently bound to FhuA/FhuA 104/149C protein and induced conformational changes resulting in an interaction with TonB. While these results were unexpected, they do not detract from the conclusion that both wild type FhuA and FhuA 104/149C were capable of, and underwent, ferrichrome-dependent interaction with pure TonB C-terminal.

Previous studies by Klebba's group have indicated that TonB C-terminal binds non-specifically to a variety of proteins including the TonB dependent transporter FepA, porin OmpA, and lysozyme (23). These experiments, however, were performed with a 69 residue fragment (170-239) which is significantly smaller than the 135 residue fragment used during this study. It is possible that the smaller TonB C-terminal fragment used by Klebba's group was unable to achieve its native folding allowing it to bind non-specifically. In addition, crystallographic data have shown that TonB C-terminal fragments less than 100 residues in length form dimers with a poor binding interaction between TonB C-terminal and FhuA. Larger C-terminal TonB fragments are observed as monomers that have an efficient interaction with the FhuA TonB box (27, 28). The results of this study are consistent with these observations and particularly show that the 135 residue TonB fragment binds to the ligand-bound receptor and requires the presence of ferrichrome for its interaction with FhuA.

Other studies have shown that the C-terminal of TonB interacts not only with the conserved TonB box but also with barrel residues exposed to the periplasm (6, 25, 39). That interaction alone, however, does not appear to occur with enough affinity for FhuA to be eluted along with the TonB C-terminal in this study and may also be dependent on the presence of iron-bound ferrichrome.

This study sought to further characterize the FhuA 104/149C mutant and its ability to interact with the TonB C-terminal and concluded that this interaction was not inhibited as compared to the wild type. This indicates that the disparity in active transport between the wild type and the mutant does not appear to be due to a lack of supplied energy from TonB. Further studies of this mutant would help confirm the results previously obtained. Specifically transport studies of this mutant in the presence of 0.5mM dithiothreitol would confirm the structural and functional integrity of the mutant by reducing the engineered disulfide bonds and restoring the protein to wild type functionality. Study of a FhuA mutant such as the tetra-cysteine FhuA 72/615/109/356C is also needed to observe the results of tethering the plug domain to the barrel at 2 different locations. In conclusion this study further characterized the FhuA 104/149C mutant and provided evidence of ferrichrome dependent binding between TonB dependent receptors and the TonB C-terminal.

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APPENDIX
Description of Media and Buffers

Modified Luria-Bertani Broth

	500mL	1L
Tryptone	5.0 g	10 g
Yeast Extract	2.5 g	5 g
NaCl	2.5 g	5 g
Agar	7.5 g for plates	

Dilute to the appropriate volume with ddH_2O and pH to 7.5, autoclave

SDS-PAGE Solutions and Buffers

30% Bis-Acrylamide:	Purchased from Bio-Rad		
Running Gel Buffer:	Tris	36.3 g	
	ddH_2O to 200mL, pH to 8.8 with HCl		
Stacking Gel Buffer:	Tris	3.0 g	
	ddH_2O to 50mL, pH to 6.8 with HCl		
2x Loading Buffer:	Stacking Gel Buffer	2.5 mL	
	10% SDS	4.0 mL	
	Glycerol	2.0 mL	
	2-mercaptoethanol	1.0 mL	
	Bromophenol blue	2.0 mg	
	ddH_2O	10.0 mL	
Tank Buffer (4X):	Tris	12 g	
	Glycine	57.6 g	
	SDS	4 g	
	ddH_2O to	1L	
Stain Stock:	Coomassie blue R-250		2.0 g
	ddH_2O		200 mL, stir and filter
Stain:	Coomassie blue R-250		12.5 mL
	Methanol		50 mL
	Glacial acetic acid		10 mL
	ddH_2O to		100 mL
Destaining Solution:	Methanol		500 mL
	Glacial acetic acid		100 mL
	ddH_2O		400 mL

Western Blot Transfer Solutions

Transfer Buffer:	20mM Tris	2.4 g, pH 8.3	
	150mM Glycine	11.26 g	
	ddH_2O to	1 L	
Tris-Buffered Saline (TBS, 2X):	20mM Tris	9.7 g, pH 7.5	
	500mM NaCl	117 g	
	ddH_2O to	2 L	
Wash Buffer:	Tween – 20	0.5 mL	
	TBS (1X)	1 L	

Blocking Buffer:	Bovine Serum Albumin (BSA)		6.0 g
	TBS (1X) to		200 mL
Substrate Buffer:	0.1M Tris	1.2g, pH 9.5	
	0.1M NaCl	0.58 g	
	5mM $MgCl_2$	0.2 g	
	ddH_2O to	100 mL	
Substrate:	a) 4-chloronapthol		60 mg
	Methanol, ice-cold		20 mL
	b) 30% H_2O_2		60 µL
	Substrate buffer		100 mL
	Mix immediately before immersion of membrane		

FhuA Extraction/Purification Buffers

Buffer A:	50mM Tris, pH 7.5	6.06 g	
	5mM Benzamidine	0.783 g	
	ddH_2O to	1 L	
Buffer B:	50mM Tris, pH 7.5	6.06 g	
	5mM Benzamidine	0.783 g	
	2% Triton-X100	20 mL	
	ddH_2O to	1 L	
Buffer D:	50mM Tris, pH 7.5	6.06 g	
	5mM Benzamidine	0.783 g	
	2% Triton-X100	20 mL	
	5mM EDTA	0.1901 g	
	2% β-D-octylglucoside		2.0 g
	ddH_2O to	1 L	
Mobile Phase A:	50mM Tris, pH 7.5		6.06 g
	3mM Sodium Azide		0.195 g
	2mM EDTA		0.760 g
	10mM Benzamidine		1.566 g
	2% Triton-X100		20 mL
	ddH_2O to		1 L
Mobile Phase B:	50mM Tris, pH 7.5		6.06 g
	3mM Sodium Azide		0.195 g
	2mM EDTA		0.760 g
	10mM Benzamidine		1.566 g
	2% Triton-X100		20 mL
	500mM NaCl		29.22 g
	ddH_2O to		1 L

TonB C-Terminal Extraction/Purification Buffers

Buffer T:	100mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.9	26.8 g
	5mM Benzamidine	0.783 g
	ddH_2O to	1 L
Buffer U:	100mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.9	26.8 g
	5mM Benzamidine	0.783 g

500mM NaCl	29.22 g	
ddH_2O to	1 L	

10x Phosphate Buffered Saline (PBS), pH 7.2:	Na_2HPO_4	10.9 g
	NaH_2PO_4	3.2 g
	NaCl	90 g
	ddH_2O to	1 L

Ni-NTA Equilibration Buffer:	10x PBS	20 mL
	2M Imidazole (supplied)	1 mL
	ddH_2O to	200mL

Ni-NTA Wash Buffer:	10x PBS	10 mL
	2M Imidazole (supplied)	1.25 mL
	ddH_2O to	100mL

Ni-NTA Elution Buffer:	10x PBS	2 mL
	2M Imidazole (supplied)	2.5 mL
	ddH_2O to	20mL

MES Buffer:	20mM MES	2.172 g
	100mM NaCl	2.922 g
	pH 5.0	
	ddH_2O to	500 mL

<u>TonB C-Terminal Cloning Mixtures</u>

PCR:	DMSO	0.6µL
	5x Reaction Buffer	5µL
	10mM dNTP	0.5µL
	20µM Primer	0.6µL (x2)
	100ng Template	1µL
	ddH_2O	16.35µL
	High fidelity polymerase	0.25µL

PCR Digest:	PCR Reaction	20µL
	NdeI Enzyme	1µL
	HindIII Enzyme	1µL
	NEB Buffer	2.5µL
	ddH_2O	0.25µL
	100X BSA	0.25µL

Ligation:	pET17b Vector	3µL
	TonB Insert	5µL
	Ligation Buffer	1µL
	T4 DNA Ligase	0.5µL
	ddH_2O	0.5µL

VITA

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