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Molecular Mechanism of Ferricsiderophore Transport via the Outer Membrane Receptor FhuA in *Escherichia coli*.

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Molecular Mechanism of Ferricsiderophore Transport via the Outer Membrane Receptor FhuA in *Escherichia coli*

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Jennifer K. Cooke

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ABSTRACT

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Iron is essential for life and growth in most organisms. Although it is abundant, iron exists mostly as insoluble iron-oxyhydroxide. Bacteria secrete siderophores to chelate iron and transport it into the cell via specific outer membrane receptors. The FhuA receptor protein transports ferrichrome, a siderophore produced by *Ustilago sphaerogena*. We determined the binding affinity of variants from the conserved 'lock region' of FhuA and also created and characterized variants of the highly conserved R452 to determine its role in ferrichrome transport. We hypothesize that during transport the plug domain of FhuA does not leave the barrel; rather it undergoes a conformational change to form a channel. We mutated selected amino acids to cysteine to form disulfide bonds to tether the plug, preventing its displacement or unfolding during transport. The tetra-cysteine mutant 72/615/109/356C was able to bind and transport radiolabeled ferrichrome. One double-cysteine mutant, 104/149C, was purified for crystallization.

DEDICATION

To Graham

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CHAPTER 1

INTRODUCTION

The Importance of Iron

Iron is the 26th element in the periodic table and has a formula weight of 55.85. It is the fourth most abundant element in the earth's crust, following oxygen, silicon, and aluminum (Hem 1959). Most of the earth's core is made up of metallic iron. Iron readily participates in chemical oxidation and reduction reactions and forms both organic and inorganic complexes that affect its solubility (Hem 1959).

In the environment, iron commonly occurs in igneous, metamorphic, and sedimentary rocks and in soil. In sedimentary rocks, ferric iron (Fe^{+3}) is more abundant, whereas ferrous iron (Fe^{+2}) exists more often in igneous and metamorphic rocks (Hem 1959). Weathering of these rocks at a neutral pH in aerobic conditions oxidizes most ferrous iron, converting it to the ferric state. Ferric iron is then incorporated into clay minerals or hydrolyzed to ferric oxyhydroxides such as goethite (α-FeOOH), akaganeite (β-FeOOH), and lepidocrocite (γ-FeOOH) (Oades 1963). In soil, iron exists primarily in the ferric state, although ferrous iron may be more abundant in soils that are rich in organic matter. Ferric oxyhydroxides are the most common iron-containing compounds, especially amorphous oxyhydroxide, goethite, and hematite (Oades 1963).

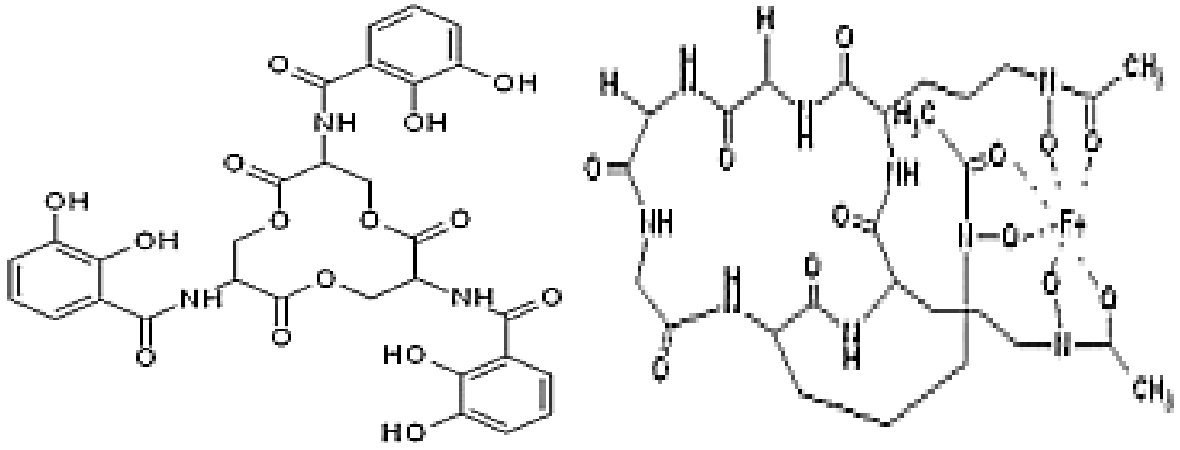
Iron can be found in all living organisms and is essential for many biological processes including ATP synthesis, electron transport, and the reduction of DNA precursors (Litwin and Calderwood 1993). Bacteria require an internal iron concentration of 1μM or higher in order to support growth and reproduction (Raymond

et al. 2003). However, the free ferric iron concentration in equilibrium with environmental ferric oxyhydroxide is approximately 10^{-17} M, far below what bacteria need in order to persist (Raymond et al. 2003). Iron acquisition is an important virulence factor during bacterial infection. Bacteria have developed several efficient mechanisms to overcome the problem of iron availability. One of these systems involves the secretion of small organic molecules known as siderophores (Braun et al. 1999; Braun and Braun 2002).

Siderophores

Siderophores (Greek for “iron carrier”) are small molecules produced by bacteria and fungi that chelate ferric iron from the environment with high affinity and transport it into the cell using an energy-dependent transport system (van der Helm et al. 1987). They have a molecular weight ranging from 500-1500 Da. Many are nonribosomal peptides from a class of secondary peptide metabolites produced by bacteria and fungi that are typically synthesized independently of mRNA by nonribosomal peptide synthetases (Ecker et al. 1986). One of their defining characteristics is their charge; after chelation of iron, they have a net charge of -3. Despite the incredible diversity in their structures, most siderophores can be classified as either catecholate or hydroxamate types, based on whether they use a catechol or a hydroxamate group to chelate iron. Enterobactin (Fig. 1), which is produced by *Escherichia coli*, is an example of a catechol type siderophore (Ecker et al. 1986). Ferrichrome (Fig. 1), which is produced by the fungus *Ustilago sphaerogena*, is an example of a hydroxamate type siderophore (van der Helm et al. 1980). Siderophores have also been reported with carboxylate groups or mixed groups such as schizokinen, pseudobactin, and

pyoverdine (Cobessi et al. 2005; Storey et al. 2006). It is not uncommon for bacteria or fungi to produce more than one type of siderophore and the same type of siderophore may be produced by more than one species.



A. Enterobactin **B. Ferrichrome**

Figure 1 Examples of typical siderophores: A. Enterobactin is an example of a catechol type siderophore; B. Ferrichrome is a hydroxamate siderophore

Siderophore Transport

Outer Membrane Transporter Proteins

In Gram negative bacteria, iron transport is achieved via the binding of the ferricsiderophore complex to proteins in the outer membrane that function as

transporters (van der Helm 1998). Protein sequences have been reported for more than 30 such outer membrane proteins transporting chemically diverse ferricsiderophores (van der Helm 1998, van der Helm and Chakraborty 2002 and references therein). The binding sites of ferricsiderophore outer membrane transporters are known to be highly selective for only one siderophore (Schalk 2007). After binding to the transport protein, the ferricsiderophore is released from the binding site and vectorally transported across the outer membrane into the periplasm (Braun and Braun 2002). Multiple alignment studies on 19 different iron transport proteins have shown 20% similarity or greater when the protein sequences are aligned. In cases where they transport the same siderophore (e.g., FepA, BfeA, and PfeA which all transport ferricenterobactin), the similarity is approximately 40% (Chakraborty et al. 2003 and references therein).

The crystal structures of the outer membrane ferricsiderophore transport proteins FecA, FepA, and FhuA from *Escherichia coli* and FpvA and FptA from *Pseudomonas aeruginosa* have been solved (Ferguson et al. 1998; Locher et al. 1998; Buchanan et al. 1999; Ferguson et al. 2002; Cobessi et al. 2005). FhuA, FecA, FpvA, and FptA have also been solved in their ligand-bound forms (Ferguson et al. 1998; Locher et al. 1998; Ferguson et al. 2002; Cobessi et al. 2005). The structures of these proteins are very similar (Fig. 2); they consist of a 22 strand β-barrel composed of approximately 600 C-terminal residues and a plug domain formed inside the barrel of approximately 150 N-terminal residues (Chakraborty, et al. 2006 and references therein). All ferricsiderophore outer membrane receptor proteins have 8-10 conserved residues in their N-terminus known as the ‘TonB box’, which interacts in the periplasm with the C-terminus of TonB

at the glutamine at location 160 in the protein sequence (Lundrigan and Kadner 1986; Sauer et al. 1990; Howard et al. 2001).

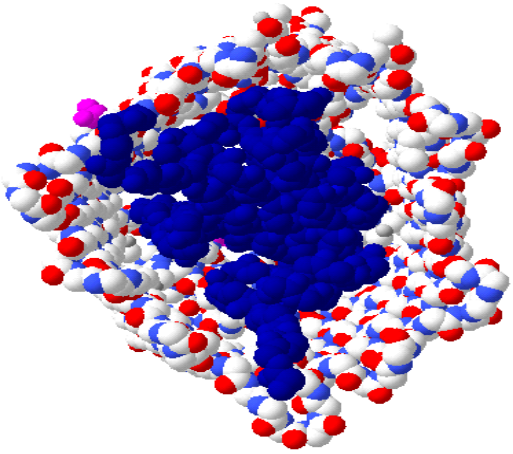


Figure 2 Space-filled model of the outer membrane receptor protein FepA as viewed from the periplasm. The blue area represents the plug domain blocking access from the extracellular medium (Chakraborty et al. 2006, used with permission).

The TonB-ExbB-ExbD Complex

Transport across the outer membrane is an energy-consuming process that requires the transport protein to interact with the TonB-ExbB-ExbD complex to provide the necessary proton motive force (Wandersman and Delepelaire 2004 and references therein). The TonB-ExbB-ExbD complex can be found in all siderophore-mediated iron transport systems in Gram negative bacteria. It also plays an important role in other non-siderophore types of iron transport such as the heme transport system

(Wandersman and Delepelaire 2004). In *E. coli*, TonB-dependent receptors also transport vitamin B12 and other nutrients that are too large, too scarce, and too important to diffuse across the outer membrane through porin proteins (Devanathan and Postle 2007).

TonB can be divided into three domains. Residues 1-33 form the amino terminal domain and are anchored to the inner membrane to serve as the site of interaction with ExbB-ExbD. Residues 34-154 form the central domain with repeating Pro-Glu and Pro-Lys located in the periplasm, and residues 155-239 form the carboxy-terminal domain, which is also located in the periplasm (Chakraborty et al. 2006). Residues 155-239 interact with the N-terminus of ferricsiderophore receptors in the outer membrane (Wiener 2005). Under iron-limiting conditions, TonB expression is increased (Wiener 2005 and references therein).

TonB-dependent energy transduction requires interactions with two additional proteins, ExbB and ExbD (Postle and Kadner 2003). ExbB is a cytoplasmic membrane protein (~26 kDA) with a periplasmic N-terminus. It is composed of a cytoplasmic domain consisting of approximately 90 residues and two transmembrane domains (Postle and Kadner 2003). ExbD is a cytoplasmic membrane protein with an identical topology to TonB and a periplasmic domain of approximately 90 residues (Postle and Kadner 2003). ExbB and ExbD are homologues of MotA and MotB proteins of the bacterial flagellar motor, and they energize TonB by coupling with the proton motive force of the cytoplasmic membrane (Braun and Braun 2002; Chakraborty et al. 2006). In the absence of any interactions between the TonB complex and the TonB box, ligands

like siderophores continue to bind to transport proteins with high affinity but are not transported across the outer membrane (Devanathan and Postle 2007). A requirement for ATP hydrolysis would be consistent with the participation of an additional undiscovered protein in the TonB system (Postle and Larsen 2004). It has been shown that TonB activity decreases rapidly in the presence of protein synthesis inhibitors. Chromosomally encoded TonB, ExbB, and ExbD have all been shown to be stable in the presence of these inhibitors, indicating the existence of one or more different short-lived protein(s) in the system (Postle and Larsen 2004 and references therein). It is possible that the short half-life of TonB activity under these conditions is indicative of the contribution of one or more as yet uncharacterized proteins.

Efforts to isolate and purify the whole TonB protein have been unsuccessful due to its instability, but the structure of the C-terminus has been solved in complex with FhuA and BtuB (Pawelek et al. 2006; Shultis et al. 2006). In both structures, the C-terminus is replaced by the TonB box of the receptor protein in the beta sheet, but this interaction does not result in any conformational change of the outer membrane receptor beyond those directly involved in the movement of the TonB box (Chakraborty et al. 2006). The mechanism by which TonB transduces energy to the outer membrane receptor protein is not well understood and is still a subject of investigation.

Periplasmic Binding Proteins

Once it enters the periplasm, the ferricsiderophore complex must make its way to an ABC (ATP binding cassette) transporter protein in order to cross the cytoplasmic membrane. Because these membrane proteins do not necessarily have a strong binding affinity for the substances they transport, Gram negative bacteria use a number of soluble periplasmic binding proteins (PBPs) (Koster 2001; Borths et al. 2002). PBPs act as high-affinity carriers that bind and escort nutrients to the membrane transporters so they may be taken into the cytoplasm of the cell. They are usually required for nutrient uptake and may also be involved in chemotaxis and energy transduction (Berendsen and Hayward 2000). PBPs are homologous to many eukaryotic receptors including the glutamate-gated ion channel (NMDA) receptors, binding domains from G-protein-coupled receptors (GPCRs), and guanylate cyclase-atrial natriuretic peptide (ANP) receptors (Bruns et al. 1997).

PBPs are bilobal, with the lobes joined either by 2-3 β-strands or by a long backbone helix. Each lobe has a mixed αβ structure (Duan et al. 2001; Lee et al. 2002). FhuD is the only periplasmic siderophore binding protein (PSBP) to have its structure reported and is used as a model to understand the mechanism of siderophore binding (Clarke et al. 2000). FhuD has a resemblance to ExbD and binds a variety of hydroxamate siderophores: ferrichrome, coprogen, ferrioxamine B, and rhodotorulic acid (Clarke et al. 2002). The structure of gallichrome-bound FhuD had also been solved (Clarke et al. 2000). Gallichrome was used instead of ferrichrome to reveal a lesser degree of specificity in FhuD that allows it to bind to a number of hydroxamate-

type siderophores. Only 45% of the molecular surface of gallichrome is buried in the complex, bound by four hydrogen bonds (Clarke et al. 2000). The interactions between FhuA and FhuD that result in acquisition of the ferricsiderophore complex from the outer membrane transporter are not well understood. It is possible that FhuD may simply bind to the ferricsiderophore in the periplasm after it has been released from the transporter (Clarke et al. 2000).

ABC Transporter Proteins

For iron to be released into the cytoplasm, a specific ABC transporter able to bind the ferricsiderophore is required for passage across the inner membrane (Fig. 3). In *E. coli*, the transporters involved in this process are FhuBCD for ferrichrome and FepBCD for ferricenterobactin (Koster 2001). FhuD, as previously discussed, is a PBP that binds to the ferricsiderophore and escorts it to the cytoplasmic membrane. FhuB is located in the cytoplasmic membrane, and FhuC (an ATPase) is associated with the inside of the cytoplasmic membrane. Peptide-mapping studies have shown that the FhuD-ferricsiderophore complex interacts with the transmembrane region and cytoplasmic segment 7 of FhuB (Braun et al. 2004 and references therein). A negatively charged Glu111 and Asp225 on the apex of each lobe of FhuD may contact with positively charged arginine pockets of FhuB, causing the transmembrane region of FhuB to form a pore through which FhuD inserts itself and transfers the ferricsiderophore to the cytoplasm in a process driven by ATP hydrolysis from FhuC (Locher et al. 2002; Mademidis et al. 1997).

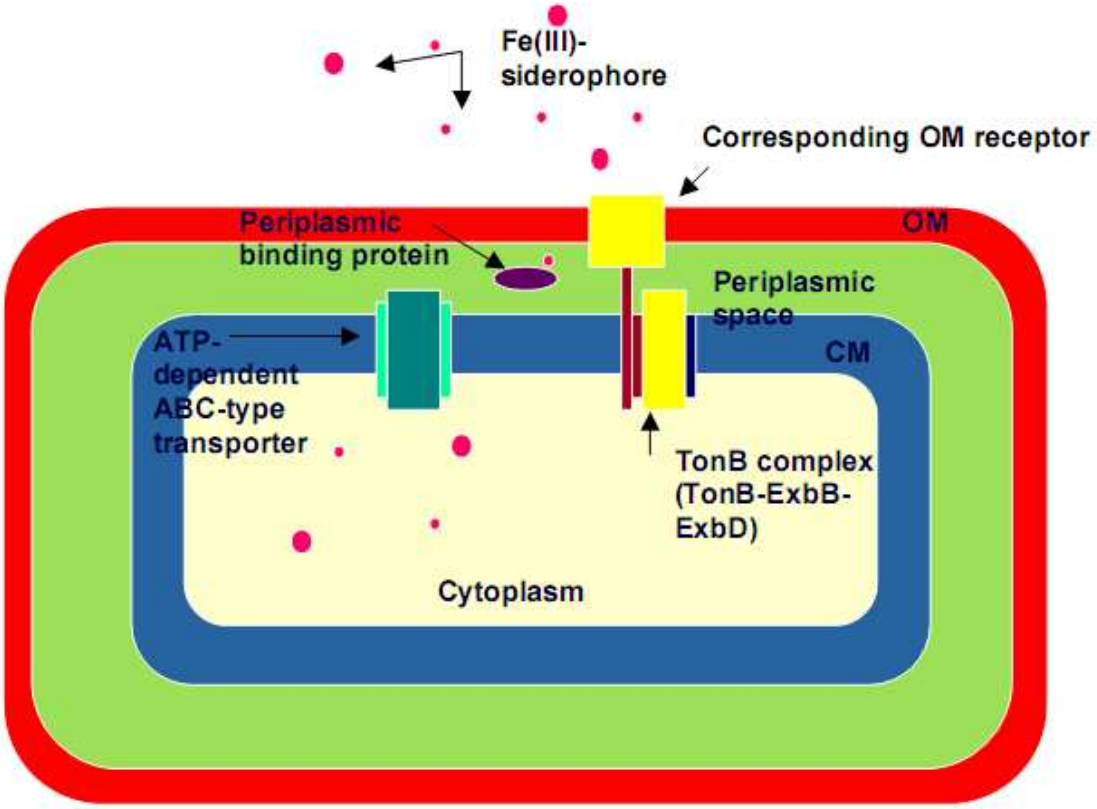


Figure 3 A summary of the iron uptake process in gram-negative bacteria (Unpublished, used with permission).

The Fate of Ferricsiderophore Complexes Inside the Cell

Once it reaches the cytoplasm, the ferricsiderophore complex dissociates. Two mechanisms for the release of iron from the ferricsiderophore complex have been described in Gram negative bacteria. The first involves hydrolysis of the complex, such as the degradation of ferricenterobactin in *E. coli*, in which the cyclic triester is hydrolyzed by esterases (Langman et al. 1972). The second involves the reduction of

ferric iron to ferrous iron, which has a much lower affinity for the siderophore. The siderophore may then be recycled to the extracellular medium (Matzanke et al. 2004 and references therein). This is the mechanism described in *E.coli* for ferrichrome, coprogen, and ferrioxamine B (Matzanke et al. 2004). Iron that will not be used immediately is taken up by bacterial iron storage proteins.

Bacteria possess two types of iron-storage protein, the heme-containing bacterioferritins and heme-free ferritins (Andrews 1998). Bacterioferritins and ferritins are not closely related but have shown similar structural and functional properties. Both are composed of 24 identical subunits that form a spherical complex with a large hollow center (Bruns et al. 1997; Andrews 1998). The hollow center acts as an iron-storage cavity with the capacity to accommodate at least 2000 iron atoms in the form of a ferric-hydroxyphosphate complex. The role of the heme is unknown, although it may be involved in mediating iron-core reduction and iron release (Andrews 1998). Some bacteria contain two bacterioferritin subunits, or two ferritin subunits, while others have both a bacterioferritin and a ferritin, and some show no evidence that they possess any type of iron-storage protein (Andrews 1998 and references therein). The reason for these differences is not understood. Studies on ferritin mutants have shown that ferritin facilitates growth during iron starvation and is also involved in iron accumulation during the stationary phase of growth.

Regulation of Ferricsiderophore Transport Systems

If intracellular iron concentration is sufficient, bacteria shut off the synthesis of siderophores and transport proteins by inhibiting the transcription of the related genes via binding of ferrous iron to two proteins: Fur and DtxR, which act in the iron-loaded form as transcriptional repressors (Hantke and Braun 2000). The Fur protein of *E. coli* is a 17 kDa DNA-binding protein that interacts with target sequences of the genome based on the presence or absence of intracellular ferrous iron. Fur is an abundant protein, with approximately 5,000 copies per cell during the logarithmic growth phase and 10,000 copies during stationary phase (de Lorenzo et al. 2004 and references therein). It has been estimated that more than 90 genes in *E. coli* are regulated by Fur (de Lorenzo et al. 2004 and references therein). Fur is capable of polymerizing along the target strand of DNA, which may strengthen the repressive signal (de Lorenzo et al. 2004). Fur may also function as a storage protein for free ferrous iron, protecting the cell from redox damage (de Lorenzo et al. 2004). Fur target sites, also known as the Fur box, are highly conserved in many bacteria (Wee et al. 1988; de Lorenzo et al. 1988). The Fur box consists of a 19 bp inverted, AT-rich sequence. The mechanism of Fur binding is not entirely understood; the existence of the Fur box coupled with the dimeric structure of the protein seem to suggest that Fur interacts with DNA in the manner of classical bacterial repressors, which a protein dimer recognizes and binds to a palindromic sequence (de Lorenzo et al. 1988). However, more recent studies indicate that multiple Fur molecules wrap around the DNA helix in a screw-like manner, extending into regions that do not appear to match the Fur box (de Lorenzo et al. 2004).

Other Systems for Iron Acquisition in Bacteria

Siderophore acquisition systems are not the only mechanism employed by bacteria to gain iron. Many Gram negative bacteria also use the Feo system, which transports ferrous iron and is typically active under anaerobic conditions (Kammler et al. 1993). Heme is also used as a potential iron source. Some organisms are able to bind heme directly and transport it across the membrane via a TonB-dependent mechanism. In *Shigella*, ShuA is the receptor for heme (ChuA in *E. coli*) (Burkhard and Wilks 2007) and these receptors appear to share some similarity with ferricsiderophore transporters (Wandersman and Stojiljkovic 2000). Once inside the cell, iron is extracted from heme by heme oxygenases that break the tetrapyrrole ring (Debarbieux and Wandersman 2004).

Bacteria may also secrete hemophores that chelate iron from a variety of sources. The hemophore HxuA has been identified in *Haemophilus* influenza (Cope et al. 1995). HxuA is secreted by a signal peptide-dependent pathway. It acquires heme from hemopexin and presents it to the HxuC receptor in the outer membrane (Cope et al. 1995; Cope et al. 2001). Another hemophore system has been described in several bacteria including *Serratia marcescens, Pseudomonas aeruginosa, Pseudomonas fluorescens,* and *Yersinia pestis*. These hemophores, called HasA ("heme acquisition system"), do not demonstrate homology to any other known proteins (Letoffe et al. 1994; Letoffe et al. 1998; Debarbiex and Wandersman 2004). HasA hemophores are secreted by ABC transporters and require the SecB chaperone for efficient secretion (Arnoux et al. 1999).

Iron as a Virulence Factor

Thanks to the evolution of hemoglobin for oxygen transport, the required concentration of iron in vertebrates is three times that of lower life forms (Bothwell and Finch 1962). However, most of this iron is sequestered away by storage and transport proteins like transferrin and lactoferrin. The ability to sequester iron in these storage proteins is a primary defense mechanism against bacterial infection. To establish a successful infection, bacterial pathogens must overcome host iron limitation (Braun and Braun 2002). This selective pressure has led to the development of iron uptake systems specific to host iron sources such as heme and transferrin (Bullen and Griffiths 1999). These mechanisms can take the form of specific receptors or siderophores that are capable of removing iron from human transferrin (Bullen and Griffiths 1999). Transferrin iron accounts for less than 1% of the human body's total iron, while heme iron accounts for more than 80%. The abundance of heme iron over other sources may provide a favorable environment for pathogens such as *Staphylococcus aureus* that preferentially acquire iron from heme (Skaar et al. 2004). Other indications that iron can play a role in virulence are demonstrated by the fact that several virulence factors are iron-regulated. For example, shiga toxin (*Shigella)*, shiga-like toxin (enterohemorrhagic *E. coli*), diphtheria toxin (*C. diphtheriae*), and exotoxin A (*Pseudomonas aeruginosa*) are regulated directly by iron concentration or by an iron-responsive regulator (Litwin and Calderwood 1993). Additionally, the genes for hemophore expression in *E. coli* are located on the pColV virulence plasmid, suggesting that it may have a role in virulence (Otto et al. 1998).

Surprisingly, some bacterial pathogens have overcome this host defense mechanism by eliminating the need for iron. Analysis of the membranes of *Borrelia burgdorferi* indicated a lack of any metalloproteins commonly found in bacterial membranes (Bledsoe et al. 1994). Analysis of the complete genome sequence confirmed that *B. burgdoferi* does not possess genes encoding these proteins (Fraser et al. 1997). Even the Fur homolog of *B. burgdorferi* does not appear to require iron as a cofactor; it has shown a 54.3% similarity to the *Bacillus subtilis perR* gene product that uses manganese as a cofactor (Bsat et al. 1998 and references therein). The *B. burgdoferi* SodA has demonstrated >50% identity with manganese-dependent enzymes from *Thermus thermophilus, Thermus aquaticus,* and *Bordetella pertussis* (Graeff-Wohlleben et al. 1997 and references therein). Therefore, *B. burgdorferi* may be manganese-dependent rather than iron-dependent. The intracellular iron concentration in *B. burgdorferi* has been shown to be less than 10 atoms per cell, well below any physiologically relevant concentration (Posey and Gherardini 2000).

Other Applications for the Study of Iron Transport Systems

The study of iron acquisition systems in bacteria has many potential benefits. There is a growing threat of antibiotic resistance in many dangerous pathogens, most notably in Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium tuberculosis*. Siderophore-mediated iron transport systems present an intriguing new target for antibiotics. A significant decrease in virulence has been demonstrated in uropathogenic *E. coli* double mutants unable to synthesize the outer membrane

receptor proteins FepA and IutA when introduced into the mouse kidney (Torres et al. 2001), because of the organisms' decreased ability to transport iron. This suggests that therapeutic agents that target outer membrane receptors may be an effective way to treat bacterial infection. It is also notable that some outer membrane siderophore receptor proteins also transport antibiotics (Braun and Braun 2002 and references therein). This dual function may be useful in bypassing some antibiotic resistance mechanisms by conjugating antibiotics with low diffusion rates to ferricsiderophore complexes (Braun and Braun 2002). Another potential target is siderophore biosynthesis. In 2005 Ferreras et al. identified a small molecule called salicyl-AMS that inhibits siderophore biosynthesis and, subsequently, growth of *M. tuberculosis* and *Y. pestis* in vitro (Ferreras et al. 2005). A synthetically designed nucleoside antibiotic has recently been shown to inhibit siderophore biosynthesis in *M. tuberculosis* by inhibiting the activity of the adenylate-forming enzyme MbtA that is involved in the secretion of mycobactins (Somu et al. 2006).

The study of iron transport may also yield more options for the treatment of diseases associated with iron overload. The most popular treatment currently in use is Desferal, a synthetic siderophore of the ferrichrome family (Hussein et al. 1976). Desferal chelates excess iron and is an effective agent in the treatment of patients with iron poisoning or β-thalassemia, a condition in which patients produce defective hemoglobin and must undergo periodic blood transfusions, leading to an accumulation of iron in the body (Hussein et al. 1976). This therapy can be cost-prohibitive and is only available by injection, requiring patients to follow a strict regimen (Cao et al. 1997). It is

possible that a greater understanding of siderophores and siderophore transport might yield less costly iron-chelating treatments with a more efficient system of delivery.

FhuA

FhuA transports ferrichrome and also serves as a transporter for the antibiotics albomycin, rifamycin CGP 4832, and microcin J25 and as a receptor for colicin M and bacteriophages T1, T5, and Φ80 (Ecker et al. 1986; Braun and Braun 2002). Residues 6-11 serve as the TonB box (Braun and Braun 2002). The crystal structures of the protein appear to show that transport from the binding site to the periplasm requires either a major conformational change in the plug domain or complete dislodgement of the plug to allow the siderophore complex to move through the barrel (Chakraborty et al. 2003). It is thought that ligand-induced movement of the N-proximal region of FhuA facilitates its interaction with the TonB protein (Braun and Braun 2002). Interaction with TonB opens a channel in the β-barrel, turning FhuA into a porin-like protein that allows the ferricsiderophore complex to diffuse into the periplasm. Ferrichrome may diffuse along amino acid side chains that line the channel, as has been shown for the facilitated transport of maltodextrins through the LamB porin (Van Gelder et al. 2002). It is less likely that the entire plug domain is removed from the channel to allow transport, because this would require significantly more energy (Braun et al. 1998), and there are more than 60 hydrogen bonds involved in binding the plug to the β-barrel (Braun and Braun 2002).

Conserved Regions in FhuA

Certain amino acid residues, which are highly conserved in the family of ferric siderophore transporting outer membrane receptors, have been shown to have an important purpose during the internalization of ferricsiderophore complexes (Chakraborty et al. 2003). One such interesting residue is the arginine located on beta strand 11 in all ferricsiderophore receptors (Lopez et al. 2007). This arginine is conserved in all ferricsiderophore transporters and also among heme transporters. In FatA, it has been shown that mutation of this arginine to alanine reduces transport without affecting receptor binding (Lopez et al. 2007). If arginine 452 has a common role in the transport of ferricsiderophore complexes into the cell, its mutation to alanine or glutamine in FhuA should show similar results. There are several conserved regions and amino acid residues that have been identified and mutated in the ferricenterobactin transporter FepA such as Gly127, Arg75, and Glu567 (Chakraborty et al. 2003). In the N-terminal and beta barrel domains of FepA, there is a 'lock region' that includes a cluster of 10 highly conserved amino acids. There is a central component made up of four charged residues: Arg75, Arg126, Glu 511, and Glu567 (Chakraborty et al. 2003). These four residues form hydrogen bonds with other amino acids in the lock region that may play an important role in maintaining its structural integrity (Chakraborty et al. 2003).The center of this cluster connects to two arginines from the plug and two glutamates from the barrel, forming a quadrupole. This lock region is structurally the same in FepA, FhuA, and FecA (Fig. 4) (Buchanan et al. 1999; Ferguson et al. 2002; Chakraborty et al. 2003). It is possible that the binding of ferrichrome to the receptor, followed by its interaction with TonB, induces a conformational change in the lock region

leading to channel formation. It has been shown that the mutation of conserved residues from this region, both in FepA and FhuA, selectively affects the transport without affecting binding. In FhuA, Glu522Gln, Glu522Arg, Glu571Gln, Glu571Arg, Gly141Arg, and Arg93/133Gln (Fig. 5) have all been identified as mutants that show defective transport and normal binding (Chakraborty et al. 2006).

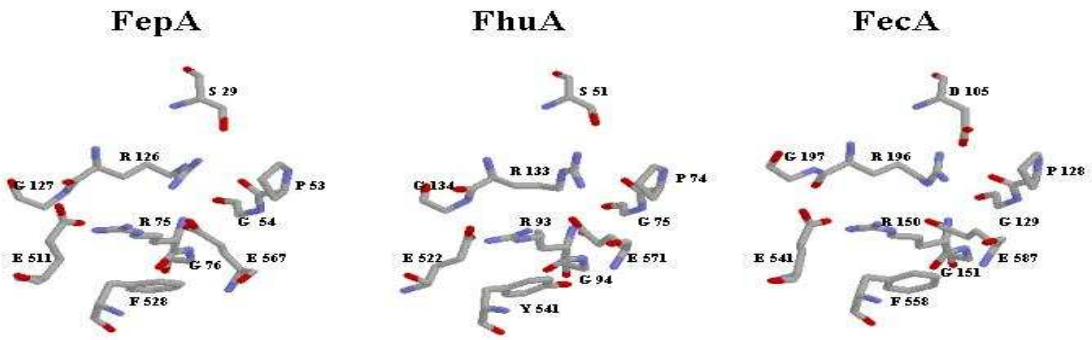


Figure 4 Structural configuration of the conserved residues of the 'lock region' in three outer membrane receptors in *E. coli* (Chakraborty et al. 2006, used with permission).

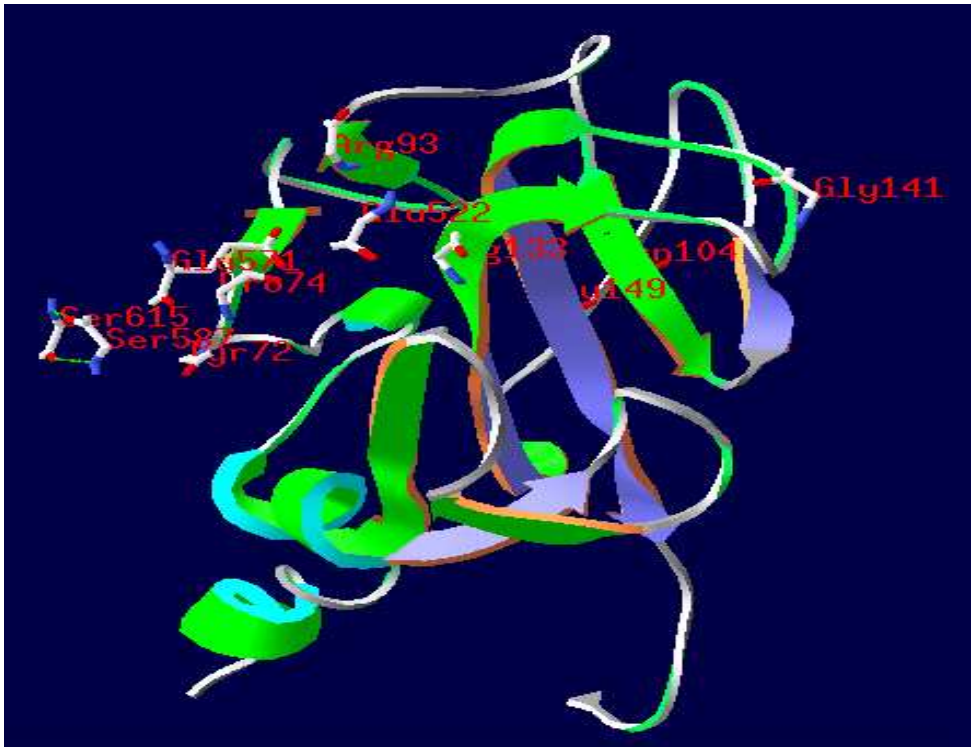


Figure 5 Locations of some residues of interest in FhuA (Unpublished, used with permission).

Conformational Changes During Transport

As previously discussed, the plug domain of FhuA is held in position inside the β-barrel by approximately 60 hydrogen bonds (Chakraborty et al. 2006). Analysis of the ligand-bound structures of FhuA and FecA and the recently solved structures of FpvA and FptA have shown that the binding of the ferricsiderophore induces conformational changes in the extracellular loops, barrel, and in the plug, but it does not induce the formation of any pore large enough for the ferricsiderophore to pass through (Chakraborty et al. 2006). In order for this to occur, the plug would either have to be

dislodged from the barrel, or it would have to undergo conformational changes within the barrel to form a channel. Dislodgement of the plug would explain the transport of larger molecules like colicin, but due to the abundance of intramolecular polar contacts between the surfaces of the plug and β-barrel, this scenario does not present an efficient use of energy (Chakraborty et al. 2006). Recent studies have also indicated that colicin may use the outer membrane receptor protein as a binding site and then enter via a nearby porin (Kurisu et al. 2003).

Present Work

Experiments were designed to show whether during transport of the siderophore the N-terminal plug domain is dislodged or if it remains in the barrel and goes through a conformational change that either forms a channel or causes the plug domain to partially unfold. Double- and quadruple-cysteine mutants were created to either tether the plug or prevent its unfolding. Nonconserved residues at locations 72, 109, 356, and 615 in the plug and beta barrel were mutated to cysteine in order to tether the plug in place so that it could not be dislodged during transport. By using nonconserved residues, any deleterious effects on iron transport that might result from mutating an amino acid that is essential to the structure and function of the protein can likely be avoided. Another cysteine mutant, 104/149Cys (Fig. 6), had been previously created by graduate student Erin Storey with a disulfide bridge connecting two strands within the plug to prevent its unfolding. This variant FhuA has shown normal binding with

completely diminished transport, and was purified using high performance liquid chromatography (HPLC) for crystallization so that its structure can be determined.

In addition, the K_D and K_M were determined for mutants R93A, E571A, E571Q, E522/571A, E522/571Q, and R133A that were previously created by Chakraborty et al. but had not been characterized. These values were compared to those of the wild type to determine if any of these mutants are defective in binding. Site-directed mutagenesis was used to mutate the arginine 452 to alanine and glutamine. There are two reasons why FhuA was chosen as a model for this project. First, the crystal structure of FhuA is available both in native form and bound with ferrichrome (Ferguson et al. 1998; Locher et al. 1998). Second, ferrichrome is a very stable siderophore and is therefore easy to handle and can be used for co-crystallization with FhuA.

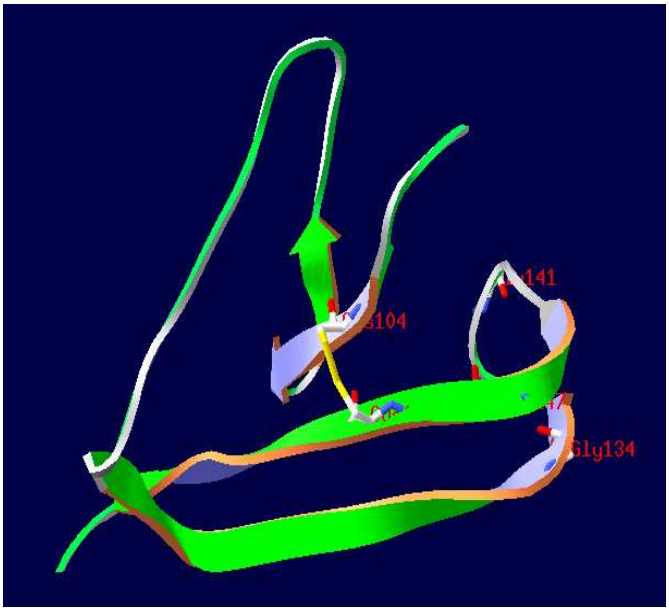


Figure 6. The disulfide bridge created by the double-cysteine mutant 104/149C (Unpublished, used with permission).

CHAPTER 2

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

All materials and reagents, unless otherwise indicated, were purchased from Fisher Scientific. Mutations in the FhuA gene cloned in pAlter plasmid (Fig. 7) were carried out using *E. coli* JM109 cells. The open reading frames of variant FhuA were cloned into the plasmid pUC18 under the *lac* promoter and transformed into *E. coli* KDF541, which is genetically engineered to knock out all iron transporting systems (Rutz et al. 1992). Variants were grown in Luria-Bertani (LB) broth at pH 7.5. Ampicillin or Carbenicillin was added for a final concentration of 100 µg/ml. For plates, 15 g/L agar was added before autoclaving. Glycerol stock cultures of cells containing all wild type strains and variants were prepared and stored at -80˚C. Cultures were grown in LB broth with 100 µg/ml carbenicillin for 12-14 hours and used as inoculum in fresh media and allowed to grow to OD_{600} 0.5-0.6 for the preparation of stock cells. Bacterial culture aliquots of 0.8 mL were added to 0.2 ml sterile 75% glycerol in 1.5 ml Eppendorf tubes and frozen at -80˚C.

Because siderophores are only produced under iron-limiting conditions, MOPS minimal medium (Table 1) was used as an iron-restricted media. *E. coli* was grown in LB broth with 100 µg/ml carbenicillin on a rotary shaker at 37˚C for 12-14 hours prior to inoculating iron-restricted media. *E. coli* was grown in LB broth with 100 µg/ml

carbenicillin on a rotary shaker at 37˚C for 12-14 hours or until OD_{600}= 0.4-0.6. Then, a 1 µM concentration of 1 M IPTG was added, and the culture was returned to the shaker for 1.5-3 hours. Finally, the culture was removed from the shaker and could be used for further experimentation.

Table 1 Formulas used in preparation of MOPS growth medium

10X MOPS Buffer Concentrate		
	250 mL	**1 L**
1M MOPS, pH 7.4	100 mL	400 mL
1M Tricine, pH 7.4	10 mL	40 mL
0.01M $FeSO_4$	2.5 mL	10 mL
1.9M NH_4Cl	12.5 mL	50 mL
0.276M K_2SO_4	2.5 mL	10 mL
0.0005M $CaCl_2$	2.5 mL	10 mL
0.528M $MgCl_2$	2.5 mL	10 mL
5M NaCl	25 mL	100 mL
10X Micronutrient Solution	0.25 mL	1 mL
ddH_2O	90.75 mL	369 mL
10X Micronutrient Solution (500 mL)		
$3x10^{-6}$ M $(NH_4)_6(Mo_7)O_{24}$	18 mg	
$4x10^{-4}$ M Boric Acid	123.5 mg	
$3x10^{-5}$ M Cobalt Chloride	35.65 mg	
10^{-5} M Copper Sulfate	12.45 mg	
$8x10^{-5}$ M Manganese Chloride	79.15 mg	
10^{-5} M Zinc Sulfate	14.35 mg	
ddH_2O	To 500 mL	
Complete MOPS Growth Medium (1 L)		
10X MOPS Concentrate	100 mL	
0.132 M K_2HPO_4	10 mL	
100% Glycerol	4 mL	
1% Proline/1% Leucine/1% Tryptophan/0.1% Thiamine Mix	10 mL	
20% Casamino Acids	10 mL	
Sterile ddH_2O	866 mL	

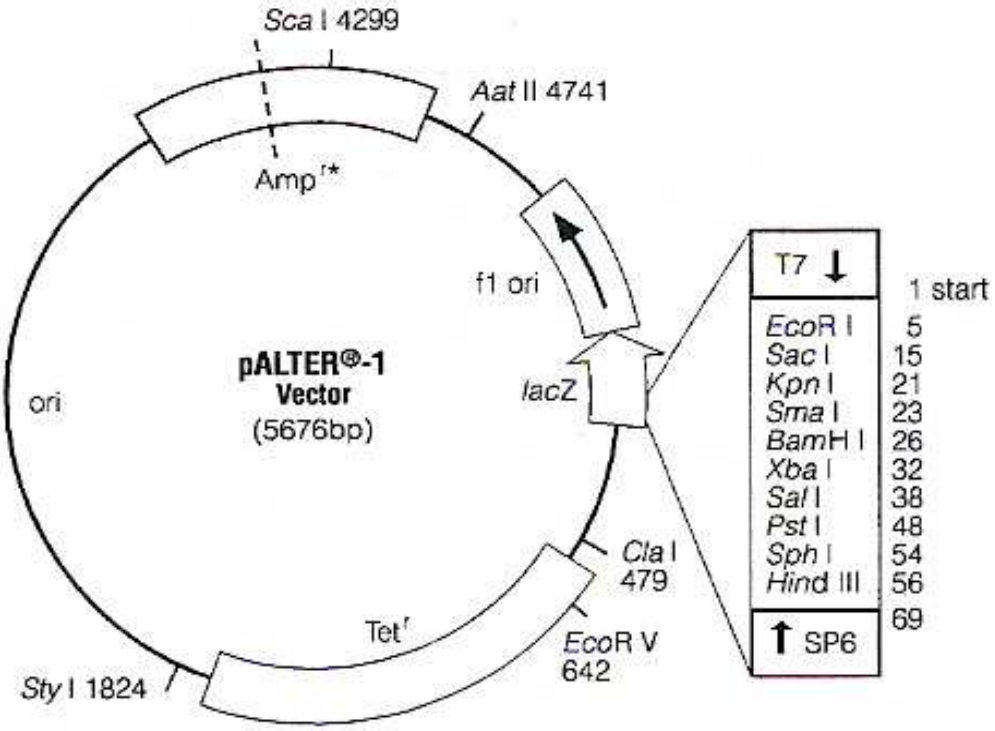


Figure 7. FhuA genes were cloned into pAlter using the XbaI/Hind III site (© Promega Corporation USA, used with permission).

Synthesis of Variant FhuA

Synthesis of Single-Stranded DNA

E. coli JM109 was grown to mid-logarithmic phase in LB broth (with 10 µg/ml Tetracycline) at 37℃. A 0.5 ml portion of this cul ture was used to inoculate 25 ml LB containing 10 µg/ml Tetracycline, which was then placed on a rotary shaker for 30 minutes at 37℃. Next, the culture was infected wit h 0.2 ml R408 Helper Phage and shaken for 6 hours at 37℃. This was centrifuged at 12,000 x g for 15 minutes, after which the supernatant was harvested and placed in a fresh tube to centrifuge again for 15 minutes. A total of 0.25 volume phage precipitation solution was added and the supernatant was left on ice for 1-16 hours and centrifuged for 15 minutes at 12,000 x g.

The pellet was resuspended in 0.4 ml TE buffer (pH 8.0) and transferred to a microcentrifuge tube. A 0.4 ml aliquot of chloroform:isoamyl alcohol (24:1) was added and the solution was vortexed for 1 minute to mix, then centrifuged at 12,000 x g for 5 minutes. The upper aqueous phase was transferred to a new tube to which additional Chloroform:isoamyl alcohol was added in the amount of 0.4 mL. This solution was vortexed for 1 minute and centrifuged at 12,000 x g for 5 minutes. Next, the aqueous phase was transferred to a new tube and the previous step was repeated until there was no longer any visible material from the lipid phase at the surface. The final aqueous phase was transferred to a fresh tube and 0.5 volume 7.5 M ammonium acetate and 2 volumes 100% ethanol were added. This solution was mixed and incubated at -20℃ for

30 minutes. After incubation, the solution was centrifuged at 12,000 x g for 5 minutes. The supernatant was removed and the pellet containing ssDNA was rinsed with ice-cold 70% ethanol and dried in a vacuum.

Site-Directed Mutagenesis

Primers were designed to mutate specific codons. A 20-25 bp long sequence of the FhuA gene was used, with the target codon as close to the center as possible. Only the target codon did not match the wild type sequence. Reaction mixtures of primers, single-stranded DNA, a tetracycline resistance knockout strand, and an ampicillin resistance repair strand were made in microcentrifuge tubes according to the instructions in the Promega Site-Directed Mutagenesis Kit (Fig. 8). Reaction mixtures were heated to 75℃ for 5 minutes and cooled to 45° C at room temperature, then to 22-25℃ on ice. Once the primers and the repair and kn ockout strands were annealed to the single-stranded DNA, the following components were added: 5 µl dH_2O, 3 µl 10X Synthesis Buffer, 1 µl T4 DNA Polymerase, and 1µl T4 DNA Ligase to complete the synthesis of the mutant strand using the single-stranded DNA as a template. This solution was incubated at 37℃ for 90 minutes to al low synthesis of the double-stranded variant plasmid. Ampicillin knockout primers and tetracycline repair primers were also included in the kit in order to aid in multiple rounds of mutagenesis by using alternating antibiotic resistance as an indicator of successful reactions. For this procedure, FhuA genes were cloned into pAlter using the Xbal/HindIII site (Fig. 7). Mutated plasmids were cloned into JM109 cells and grown on ampicillin plates. Next, colonies were

selected and grown overnight in LB broth so that plasmids could be harvested for sequencing. Plasmids were isolated in Qiagen mini-prep columns and sequenced at the molecular biology facility at University of Tennessee, Knoxville.

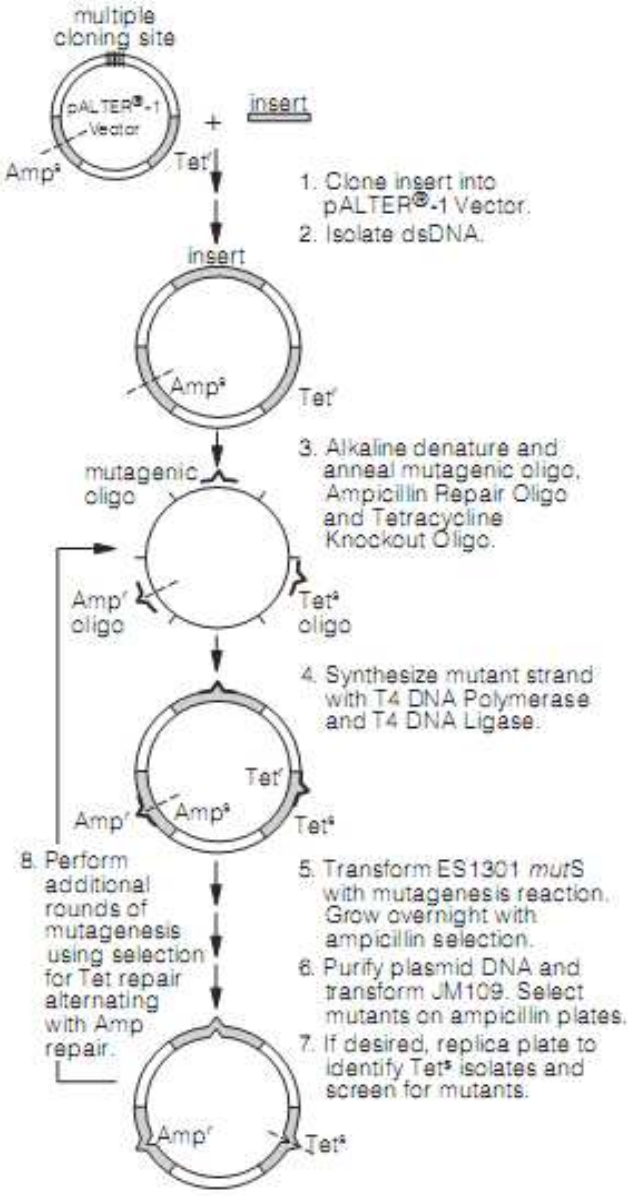


Figure 8. Schematic diagram of the Altered Sites II in vitro mutagenesis procedure using the pAlter vector (© Promega Corporation USA, used with permission).

Analysis of Variant Protein Expression

Preparation of Samples for SDS-PAGE

A 2 ml seed culture of the variant *E. coli* was used to inoculate 50 ml LB containing 100 µg/mL Carbenicillin. This culture was grown to mid-logarithmic phase (OD_{600}=0.4-0.6) and induced with 1 µM IPTG for 2-3 hours. Cells were harvested by centrifugation at 12,000 x g for 2 minutes and the supernatant was discarded. Equal volumes of 2X protein gel loading buffer (Table 2) were added to whole cell pellets and the pellet was resuspended. Samples and markers were boiled for 5 minutes prior to loading.

SDS-PAGE

Separating gels were prepared according to Table 3. A 30% Bis-acrylamide solution (Bio-Rad) was used. Gel components were deaerated for 10-15 minutes before the addition of ammonium persulfate. The separating gel was pipetted into a BioRad gel-casting unit and allowed to polymerize for 30 minutes. The stacking gel was prepared (Table 3) and deaerated for 10 minutes prior to the addition of ammonium persulfate. This gel was layered on top of the polymerized separating gel and a comb was placed. The stacking gel was allowed to polymerize for 20 minutes, then comb was removed and the gel was inserted into a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell system and the upper and lower chambers were filled with electrode buffer

(Table 2). Samples consisting of 10 µL homogenized protein were loaded onto the gel and the unit was connected to a power supply and run at 30 milliamps per gel for approximately 1 hour or until the dye front reached the bottom of the gel. Once the gel finished running, it was removed from the system. SDS-PAGE gels were stained in a 1% Coomassie blue solution, destained in 30% methanol, and preserved. Alternatively, once gels were removed from the electrophoresis system, they could also be electroblotted onto membranes and probed with antibodies against FhuA to verify protein expression.

Table 2 Recipes for buffers and reagents for SDS-PAGE.

4X Running Gel Buffer (1.5 M Tris-Cl, pH 8.8)	
Tris	36.3 g (Adjust pH to 8.8 with HCl)
ddH_2O	To 200 mL
4X Stacking Gel Buffer (0.5 M Tris-Cl pH 6.8)	
Tris	3.0 g (Adjust pH to 6.8 with HCl)
ddH_2O	To 50 mL
10% SDS	
SDS	50 g
ddH_2O	To 500 mL
10% Ammonium persulfate	
Ammonium persulfate	0.5 g
ddH_2O	To 5.0 mL
2X Sample Buffer	
Stacking Gel Buffer	2.5 mL
10% SDS	4.0 mL
Glycerol	2.0 mL
2-mercaptoethanol	1.0 mL
ddH_2O	To 10.0 mL
Electrode Buffer (4 L)	
Tris	12 g
Glycine	57.6 g
10% SDS	40 mL
ddH_2O	To 4 L

Table 3 Formulas used in preparing 10% SDS-PAGE gels.

	10% Separating Gel	Stacking Gel
30% Acrylamide Solution	6.66 ml	1.33 ml
Running Gel Buffer	5.0 ml	--------
Stacking Gel Buffer	--------	2.50 ml
10% SDS	0.2 ml	0.1 ml
ddH_2O	8.0 ml	6.1 ml
TEMED	10 µl	5 µl
10% ammonium persulfate	100 µl	50 µl

Western Blot

Western blots were performed using Millipore PVDF (polyvinylidene fluoride) membranes and a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell system. SDS-PAGE gels, Whatman #2 filter papers, PVDF membranes, and sponges were equilibriated in transfer buffer (Table 4) for 5-20 minutes prior to blotting. Then, the blotting chamber was assembled and the proteins were blotted at 25v for 4-16 hours at 4℃.

After the blots were removed from the system, they were soaked in 3% BSA (bovine serum albumin) blocking buffer (Table 4) for 1 hour at room temperature to reduce nonspecific binding of the primary antibody. Next, the blot was placed in a 1:100 solution of anti-FhuA antibody (ProSci) prepared from rabbit serum and diluted in blocking buffer for 3 hours at room temperature or overnight at 4℃. After incubation, the primary antibody was poured off, and the blot was washed five times in wash buffer

(Table 4) for 6 minutes per wash and rinsed briefly in plain tris-buffered saline (TBS) (Table 4). Then, the blot was incubated for 2 hours to overnight at 4˚C in a 1:5,000 solution of Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) diluted in blocking buffer. After incubation with the secondary antibody, the blot was washed in five 6-minute washes with wash buffer and rinsed briefly in plain TBS. Finally, the blot was placed in a freshly prepared HRP substrate buffer (Table 4) for 5-45 minutes to develop at room temperature, rinsed briefly in ddH_2O, and air-dried.

Table 4 Recipes for buffers and reagents used in Western blotting.

Tris-Buffered Saline (TBS) 2X Stock, pH 7.5 (2 L)	
Tris	9.7 g
NaCl	117 g
ddH_2O	To 2 L
Transfer Buffer, pH 8.3	
Tris	2.4 g/L
Glycine	11.26 g/L
Blocking Buffer	
Bovine Serum Albumin (BSA)	30 g
1X TBS	1 L
Wash Buffer	
Tween-20	0.5 mL
1X TBS	1 L
Substrate Buffer, pH 9.5	
Tris	6 g
NaCl	2.9 g
$MgCl_2$	1 g
ddH_2O	To 500 mL
Horseradish Peroxidase (HRP) Substrate	
4-Chloronapthol	60 mg
Methanol	20 mL
30% H_2O_2	60 µL
Substrate Buffer	100 mL

Analysis of Iron Transport in Variants

Siderophore Nutrition Assays

Siderophore nutrition assays were carried out using the method of Newton et al. (Newton et al. 1999). Commercially available (Sigma Co.) ferrichrome was used for these experiments. *E. coli* KDF541 with variant FhuA protein was grown to mid-logarithmic phase in LB broth. A 3 mL quantity of nutrient soft agar was poured into 4 cm petri dishes, with 0.2 mM dipyridyl to bind the free iron in the media, and 100 µl of the culture was added. A 10 µL quantity of a 50 µM solution of ferric siderophores was placed on filter disks on these plates. The plates were then incubated at 37℃ for 24 hours. Results were expressed as the area of visible growth around the filters.

Binding and Transport Assays

In vivo concentration-dependent binding assays were carried out for both the wild type and variant FhuA using ^{55}Fe-labeled ferrichrome. Bacterial seed cultures were grown in LB broth for 16 hours at 37℃ and used as 1% inoculum for 50 ml of MOPS (Table 1) medium with 4% glycerol as a carbon source. Cells were grown in this medium for 4-6 hours at 37℃ until OD_{600}= 0.4-0.6 and then induced with 0.1 mM of IPTG at 37˚C for 1.5 hours. The cells were then incubated on ice for 1 hour in order to drastically reduce cellular metabolism and then assayed at 0˚C for binding capacity

using increasing concentrations of radiolabeled ferrichrome, using time intervals of 1 minute. All binding assays were performed in triplicate.

In vivo concentration-dependent transport assays were conducted to determine the rate of transport, growing the wild type and variant FhuA in the same procedure as with the binding assays except without any incubation on ice. Transport assays were carried out at 37℃, measuring the rate of transpor t at increasing concentrations of ^{55}Fe-ferrichrome between 10 and 30 second intervals. The culture was maintained on a shaker at 37℃ while aliquots were drawn to measure transport at various concentrations. *In vivo* time-dependent transport assays were performed at 37˚C by measuring the amount of ^{55}Fe-ferrichrome transported at the end of time intervals ranging from 15 seconds to 15 minutes, using 1 nM labeled ferrichrome. Cellular radioactivity was measured in 10 mL ScintaVerse scintillation fluid using a 1-minute count in a Wallac 1409 Liquid Scintillation Counter. All concentration-dependent and time-dependent transport experiments were performed in triplicate.

Overexpression and Purification

Extraction

The variant protein 104/149C was over-expressed by cloning the open reading frame of the mutant genes of FhuA in pET21b vector under T7 promoter, and transformed into *E. coli*. A 6L culture was grown overnight at 37˚C from a 1% inoculum

and pelleted at 8000 x g. The pellets were resuspended in FhuA Extraction Buffer A (Table 5) with 2% benzamidine as a protease inhibitor and the cells were crushed in a French press at 35,000 psi. The French Press used for the extraction of FhuA 104/149C was provided by Dr. Yue Zou of the Biochemistry and Molecular Biology Department at the James H. Quillen College of Medicine. The ultracentrifuge in Equipment Room A107 of the James H. Quillen College of Medicine Biochemistry Department was used to spin the samples. The lysate was centrifuged at 8000 x g for 10 minutes at 4˚C, the pellet (P1) was discarded, and the supernatant (S1) was harvested and centrifuged in an ultracentrifuge at 104,000 x g for 90 minutes at 4˚C. The supernatant was discarded and the pellet was resuspended in lysis buffer and centrifuged for 90 minutes at 104,000 x g (B1). The previous step was repeated with an additional round of lysis buffer (B2). Next, the supernatant was again discarded, and the pellet was washed with distilled water (dH_2O) and spun for an additional 90-minute cycle at 104,000 x g and the supernatant (S2) was discarded. The pellet (P2) was homogenized in FhuA Extraction Buffer B (Table 5) with 1% octylglucoside and incubated at room temperature for 30 minutes on a rotary shaker. Then, it was centrifuged for 90 minutes at 104,000 x g. The supernatant was collected and the pellet was again homogenized in extraction buffer and spun for 90 minutes at 104,000 x g. The procedure was repeated and the second supernatant was collected and added to the previous and refrigerated overnight at 4℃.

Purification via HPLC

Protein was purified using a BioRad Biologic Duoflow HPLC system with a Waters 7.8 mm x 120 mm column as the stationary phase and deaerated, filtered low-salt Tris-Benzamidine anion exchange buffer and high-salt anion exchange buffer as mobile phases (Table 5). These were filtered using a Millipore filtration system with 0.45 µm membranes. The UV detector was set at 280 nm. The column was equilibriated with low-salt anion exchange buffer for 30 minutes prior to use. Sample volumes of 1 mL each were injected into the column. The program FhuA01.1 was used to elute the variant protein from the column. Fractions were tested for protein content using SDS-PAGE and Western blot. After the entire sample had been purified, any fraction containing pure variant FhuA was pooled and concentrated using a Centricon concentrator. SDS-PAGE was used to verify the presence of pure protein in 1 ml fractions.

Table 5 Formulas for buffers used in the extraction and purification of FhuA 104/149C

FhuA Extraction Buffer A (1 L), pH 8.0	
Tris	6.055 g
Benzamidine	1.566 g
ddH_2O	To 1 L
FhuA Extraction Buffer B (100 mL), pH 8.0	
Tris	0.605 g
Benzamidine	0.156 g
Octyl-Glucoside	1 g
ddH_2O	To 100 mL
FhuA Anion Exchange Buffer (4 L), pH 7.5	
Tris	24.2 g
Benzamidine	6.26 g
Sodium Azide	0.78 g
EDTA	2.92 g
Triton X	80 mL
NaCl (High Salt Buffer Only)	29.2 g
ddH_2O	To 4 L

Table 6 FhuA01.1 HPLC program for purification of FhuA protein

Step Number	Start (mL)	Step
1	0.0	Isocratic flow with 100% low-salt buffer at 1.5 mL/min
2	6.0	UV Zero Baseline
3	6.0	Isocratic flow with 100% low-salt buffer at 1.5 mL/min
4	16.0	Linear Gradient with 0%-100% high-salt buffer at 1.5 mL/min
5	42.0	Isocratic flow with 100% high-salt buffer at 1.5 mL/min
6	45.9	Isocratic flow with 100% low-salt buffer at 1.5 mL/min
7	52.4	End run

Data Analysis

The radioactivity in the samples gathered in the binding and transport assays was measured in terms of CPM. These readings were converted to DPM considering the efficiency of the scintillation counter. Binding capacity, K_D, K_M, and V_{Max} values were calculated using either the graphical plot or the GRAFIT 3.0 program from Erithacus. Binding and transport were measured and plotted against concentration and transport was measured and plotted against time in graphical presentations.

CHAPTER 3

RESULTS

Mutagenesis and Expression

Two single mutants and one tetra-cysteine mutant were created for this study. The basic, polar arginine 452 of FhuA was mutated both to neutral nonpolar alanine and neutral yet polar glutamine. Mutation to neutral polar glutamine will not allow salt bridge formation at that site within the protein but may allow hydrogen bond formation during transport of the ligand. Mutation to neutral nonpolar alanine will prevent hydrogen bond formation at that site (Chakraborty et al. 2006). Primers were designed using the *fhuA* sequence (Fig. 9). Based on previous data from the FatA iron transport protein (Lopez et al. 2007), R452 was expected to be involved in the transport process in FhuA but not in ligand binding.

Four additional primers were designed to mutate the residues at locations 72, 109, 356, and 615 to cysteine. The amino acids mutated in the tetra-cysteine variant were nonconserved amino acids whose mutation should not affect the function of FhuA protein. Creating a variant with four simultaneous mutations is difficult; previous attempts showed successful mutations in only two or three of the target codons. Two attempts were required in this study to create the tetra-cysteine mutant. Sequence data confirmed that all target codons in all three mutants were successfully changed via mutagenesis. The open reading frame of FhuA was then digested from pAlter plasmid

using EcoRI and Hind III, cloned into pUC18 plasmid, and transformed into *E. coli* iron transporter knockout strain KDF541.

SDS-PAGE and Western blot of each variant displayed clear bands at approximately 80 kDa, indicating that each mutant is expressing at a rate similar to the Wild Type (Fig. 10).

FhuA CC CTA GGC GGT CGT TAT GAC TGG GC

R452A CC CTA GGC GGT GCT TAT GAC TGG GC

R452Q CC CTA GGC GGT CAA TAT GAC TGG GC

Figure 9 FhuA sequence aligned with R452A and R452Q sequences. The target codon is highlighted in all three sequences.

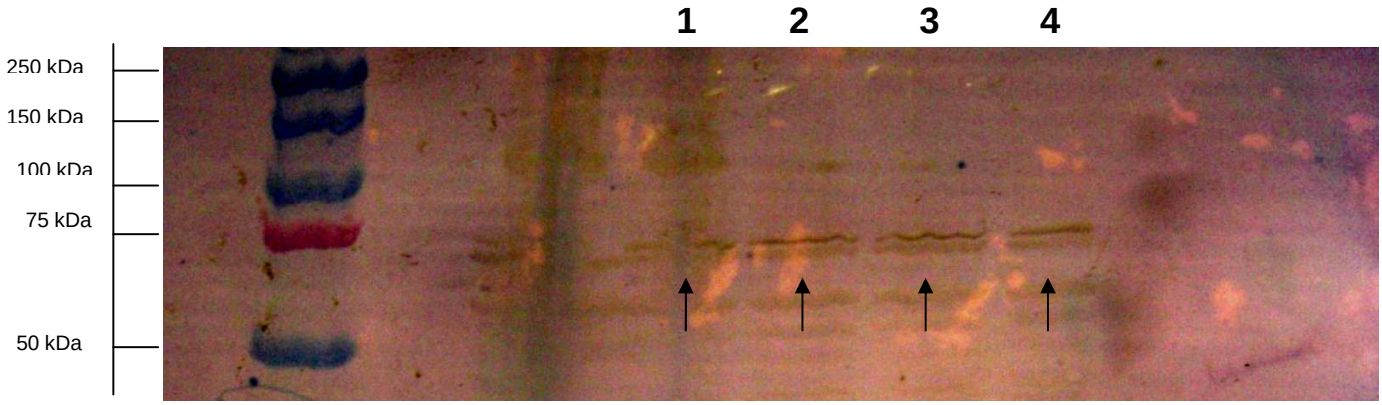


Figure 10 A Western Blot showing expression of FhuA variants: 1. 72/615/109/356C, 2. R452A, 3. R452Q, and 4. 104/149C. Protein expression was induced with IPTG for 3 hours prior to sample preparation and loading into SDS-PAGE. The pink band of the kaleidoscope marker indicates 75 kDa. Wild type not shown.

Siderophore Nutrition Assays

Siderophore nutrition assays were carried out for variants R452A, R452Q, and 72/615/109/356C as a rapid, inexpensive method of detecting deficiencies in ferrichrome transport (Table 7). After overnight incubation, a large zone of cell growth could be seen around the ferrichrome disk on the plate inoculated with 72/615/109/356C. This result was similar to that of the wild type FhuA, which was used as a control, and indicates that the tetra-cysteine mutant was transporting ferricsiderophores in the media. No zone of growth could be found on the plate inoculated with R452A, and only a small zone of growth appeared on the plate inoculated with R452Q. This assay was carried out twice, and the results were consistent.

Table 7 Results of the Siderophore Nutrition Assay for variants R452A, R452Q, and 72/615/109/356C

Variant	**Radius of the Zone of Cell Growth (mm) (Average of two experiments)**
Wild Type	22
R452A	0
R452Q	8
72/615/109/356C	20

In vivo Binding and Transport Assays, the Lock Region

In vivo concentration-dependent binding assays were carried out for R452A, R452Q, E571A, E571Q, R93A, R133A, E522/571A, and E522/571Q to determine if any of these variants were defective in binding. All of the mutant FhuA proteins except R133A showed ligand binding similar to that of the wild type, increasing with ferrichrome concentration until they reached the point of saturation. R133A assay samples showed almost no radioactivity and therefore K_D could not be determined (data not shown). E571A, E571Q, E522/571A, E522/571Q, R93A, R452A, and R452Q variants are fully capable of binding the ferricsiderophore complex (Fig. 11-14), while R133A concentration-dependent binding results indicate defective binding.

Time-dependent transport assays were performed with 1 nM ^{55}Ferrichrome with time intervals ranging from 30 seconds to 15 minutes in order to determine which variants were defective in transport. Time-dependent transport assays were performed first, because this is a much quicker and more cost-effective procedure than the concentration-dependent transport assay, in order to confirm transport capability. Any variant found to be transporting normally should then be assayed for K_M and V_{Max} using the concentration-dependent transport protocol. Data from these experiments revealed variably defective transport in mutants R452A and R452Q (Fig. 15). Data obtained previously also indicated that E571A, E571Q, E522/571A, E522/571Q, and R93A are defective in transport (Chakraborty et al. 2006).

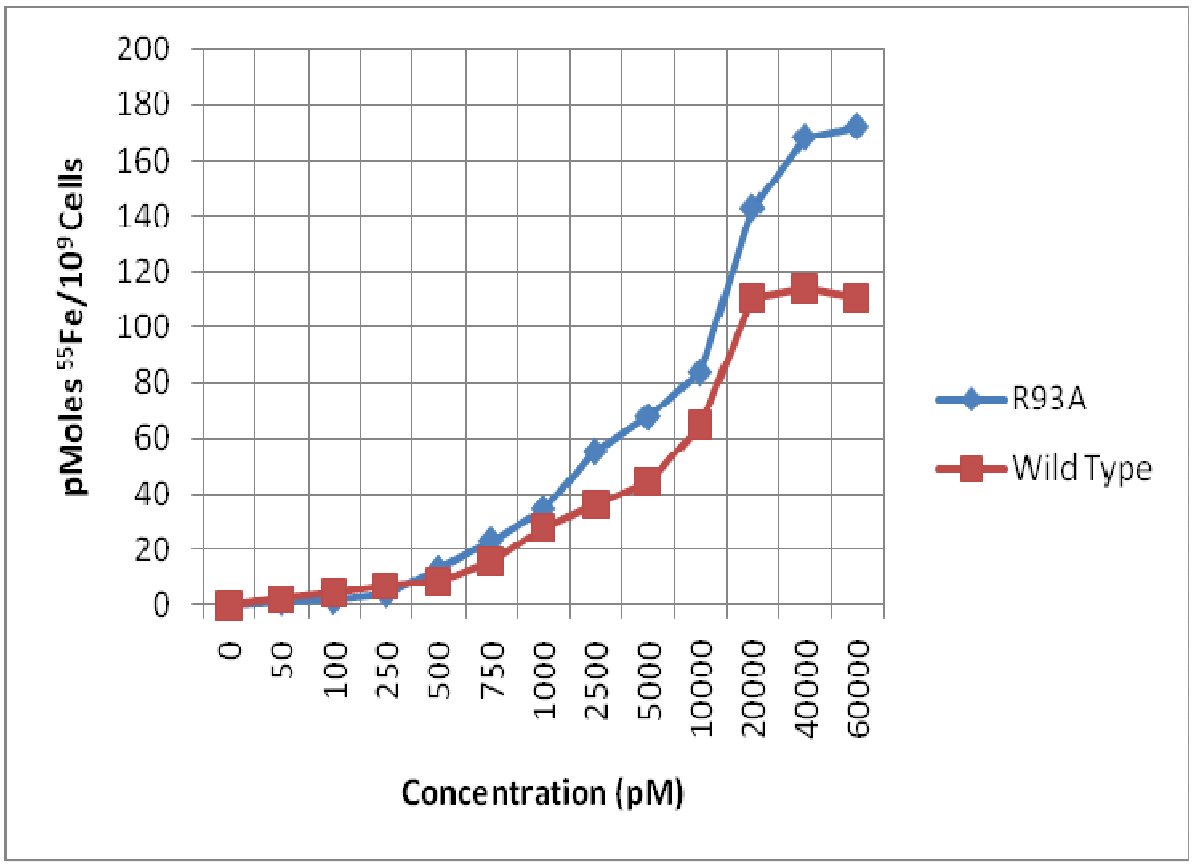


Figure 11 Concentration-dependent Binding data for FhuA variant R93A. Concentrations of ^{55}Ferrichrome ranged from 50 pM to 60 nM. Wild type data are included for comparison. Differences in binding capacity are likely due to variations in levels of FhuA expression. K_D= 18.4 nM.

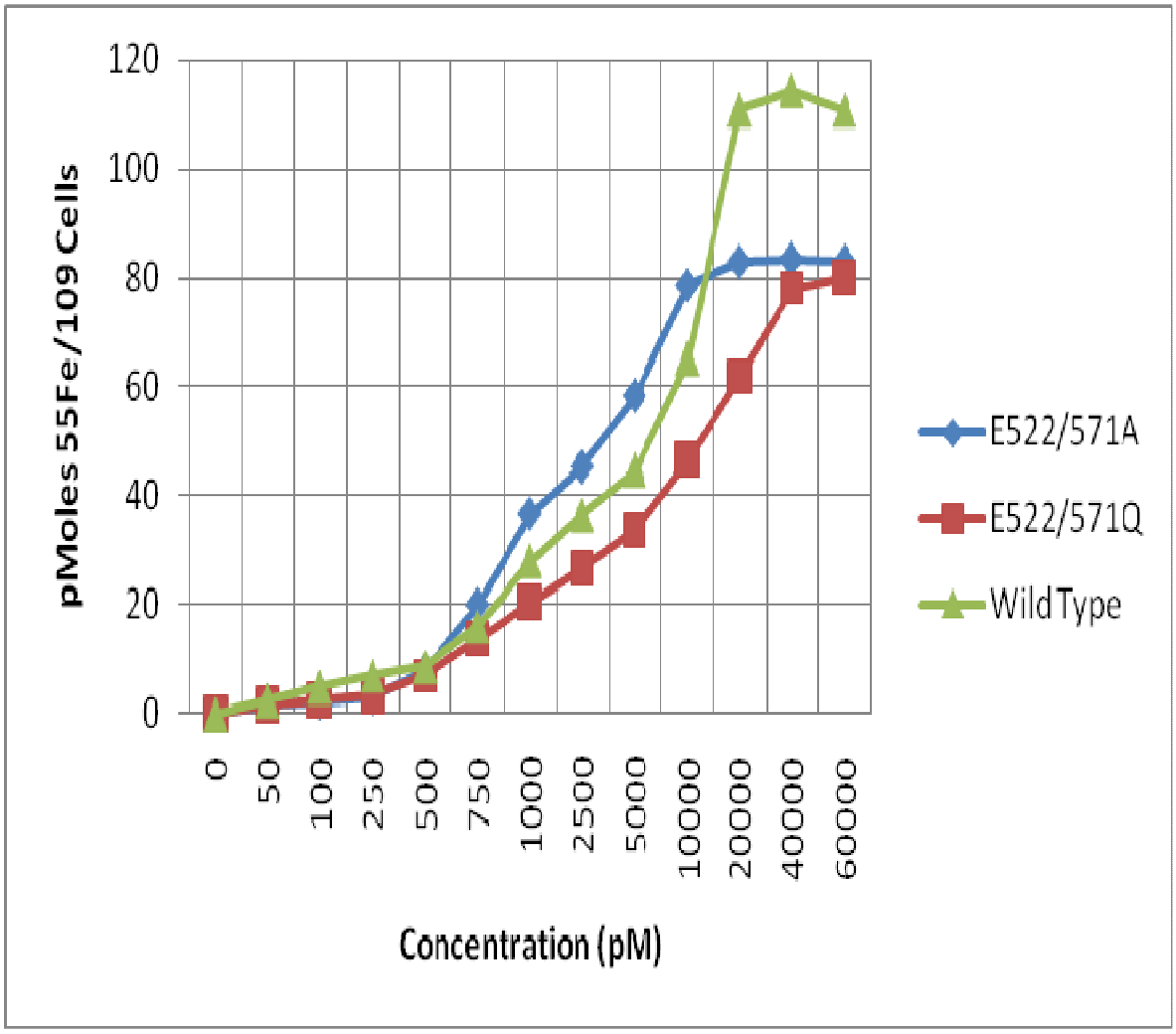


Figure 12 Concentration-dependent Binding data for FhuA variants E522/571A and E522/571Q. For E522/571A, K_D= 1.0 nM. For E522/571A, K_D= 5.0 nM.

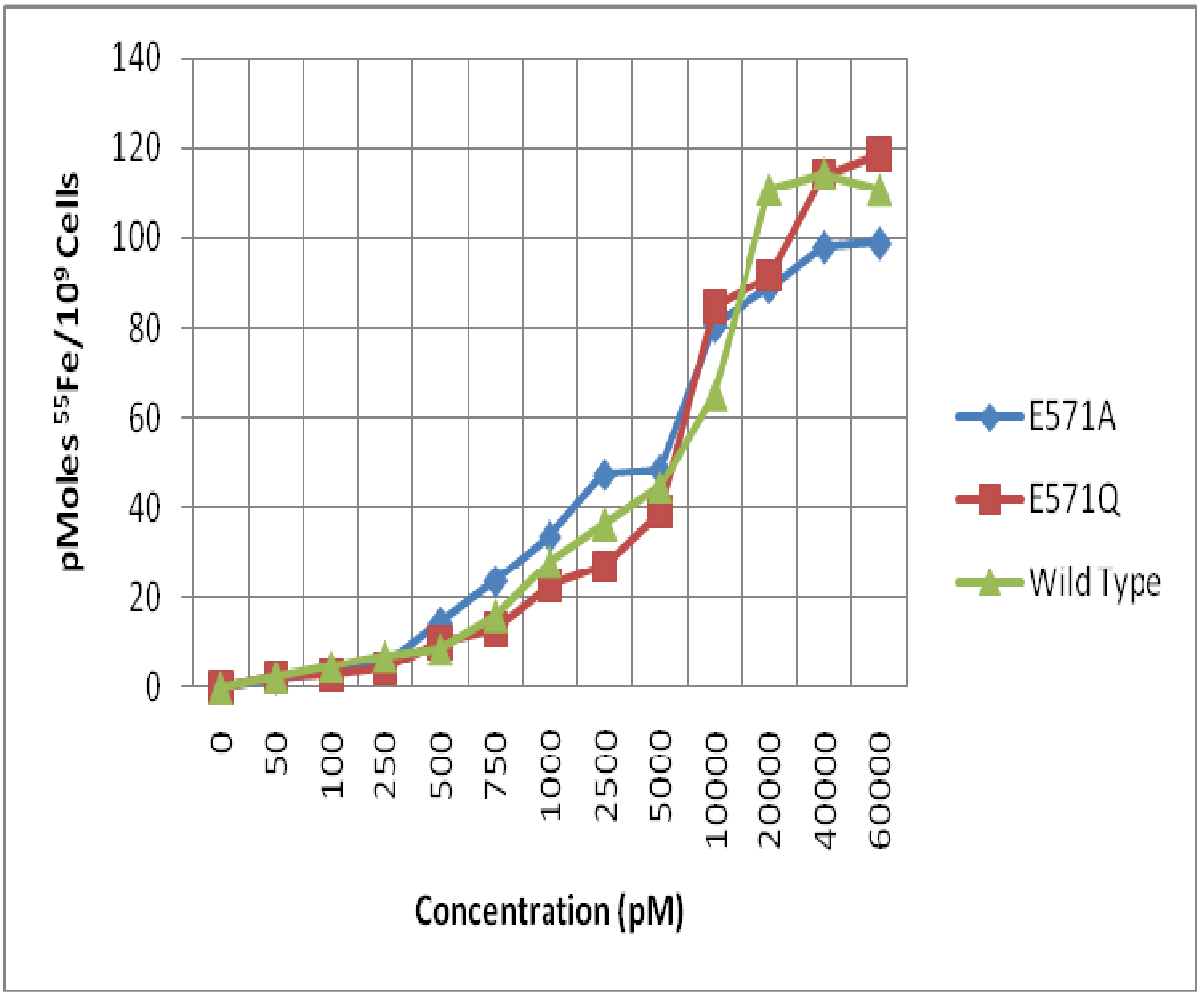


Figure 13 Concentration-dependent Binding data for FhuA variants E571A and E571Q. For E571A, K_D= 2.5 nM. For E571Q, K_D= 7.5 nM.

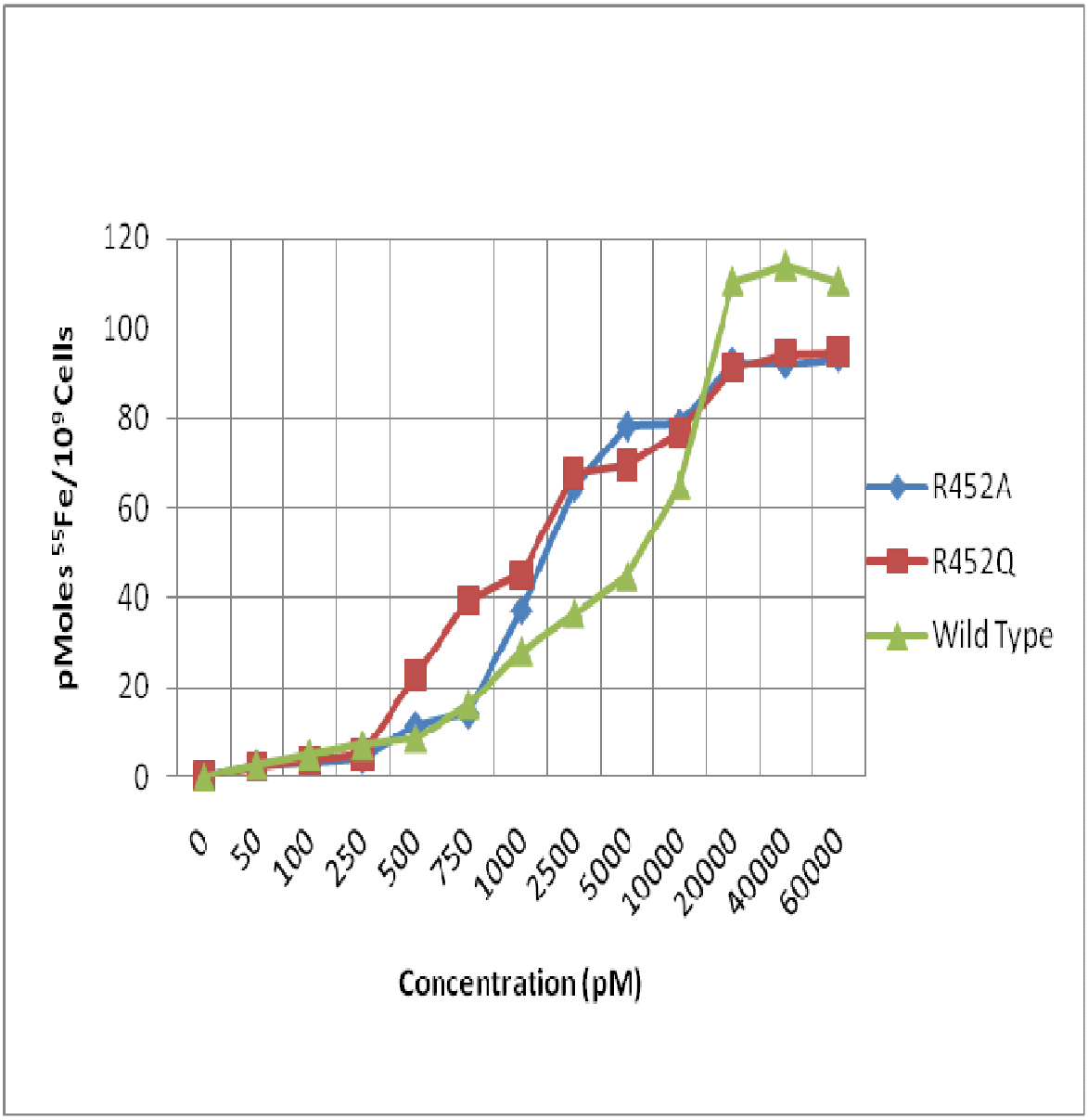


Figure 14 Concentration-dependent Binding data for FhuA variants R452A and R452Q. For R452A, K_D= 4.8 nM. For R452Q, K_D= 2.5 nM.

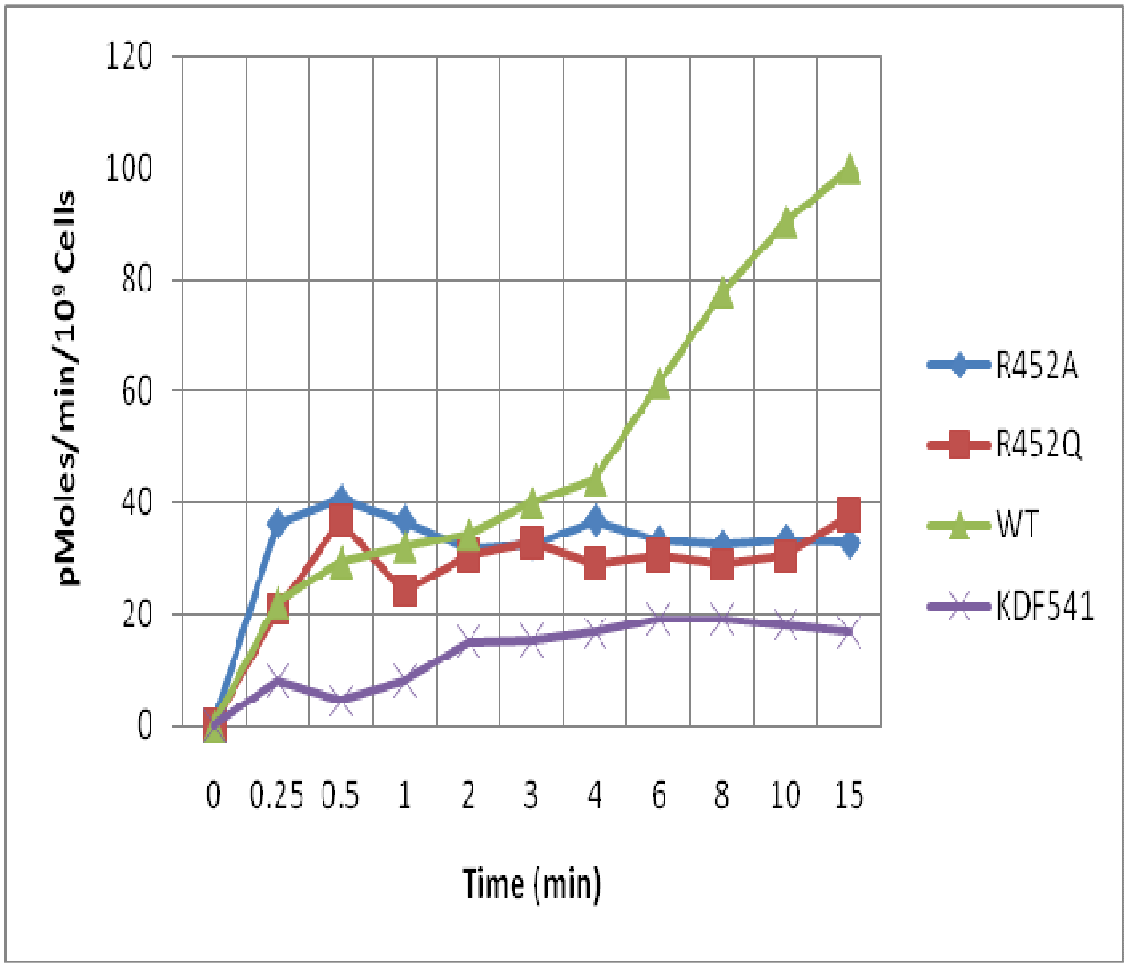


Figure 15. Time-dependent transport data for FhuA variants R452A and R452Q; data for the FhuA WT and empty KDF541 cells are also included. This procedure is performed solely to assess whether or not a mutant is defective in transport. Both R452A and R452Q were determined to be defective.

In vivo Binding and Transport Assays of the Tetra-Cysteine Mutant 72/615/109/356C

Variant 72/615/109/356C was assayed for K_D using the concentration-dependent binding procedure (Fig. 16); the tetra-cysteine mutant showed binding similar to the wild type, with increasing radioactive activity to the point of receptor saturation. A time-dependent transport assay (Fig. 17) determined that 72/615/109/356C is also capable of transport at levels similar to the wild type. Therefore, a concentration-dependent binding assay was performed in order to determine K_M and V_{MAX} for this FhuA variant (Fig. 18).

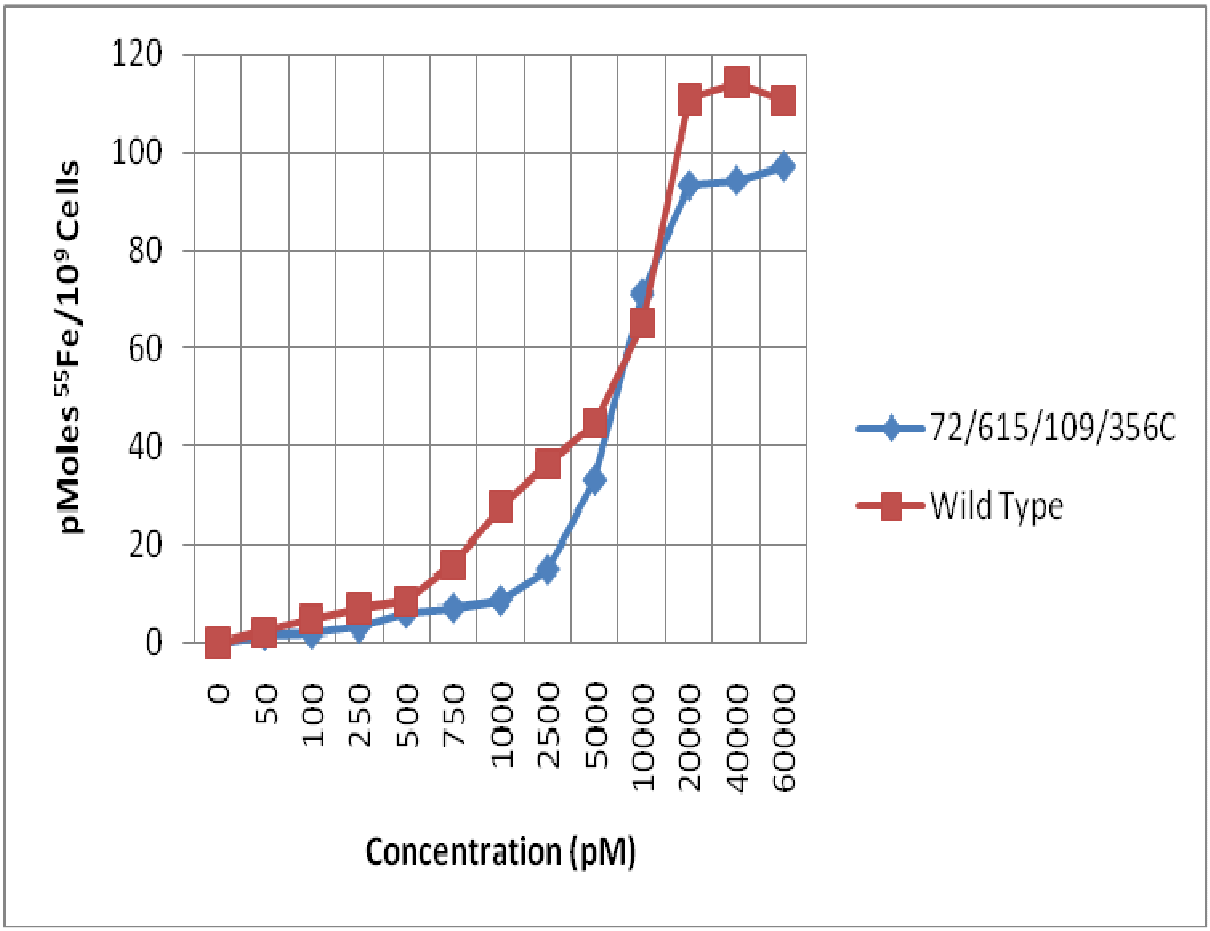


Figure 16 Concentration-dependent binding for FhuA variant 72/615/109/356C; K_D= 9.1 nM.

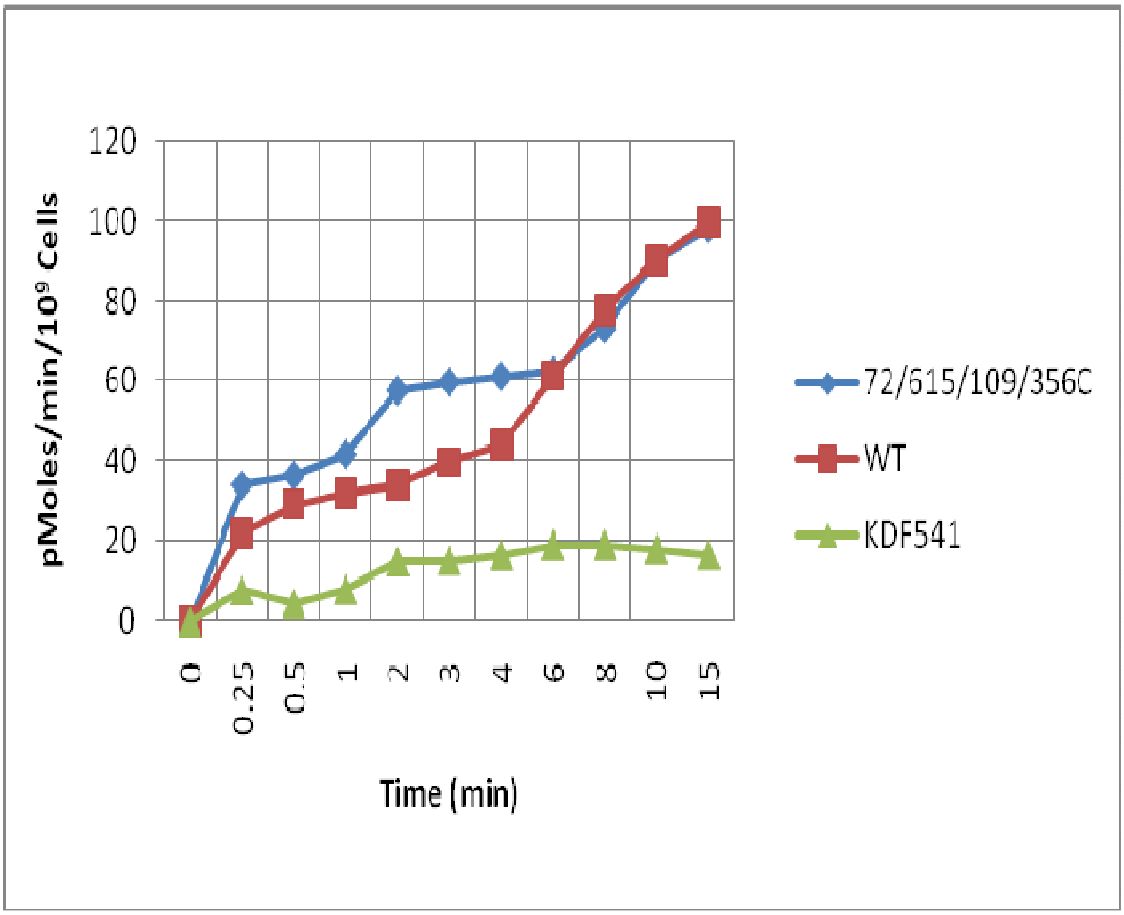


Figure 17 Time-dependent transport data for FhuA variant 72/615/109/356C. Data for FhuA WT and empty KDF541 cells are also included. Data indicate that this tetra-cysteine mutant is capable of transport.

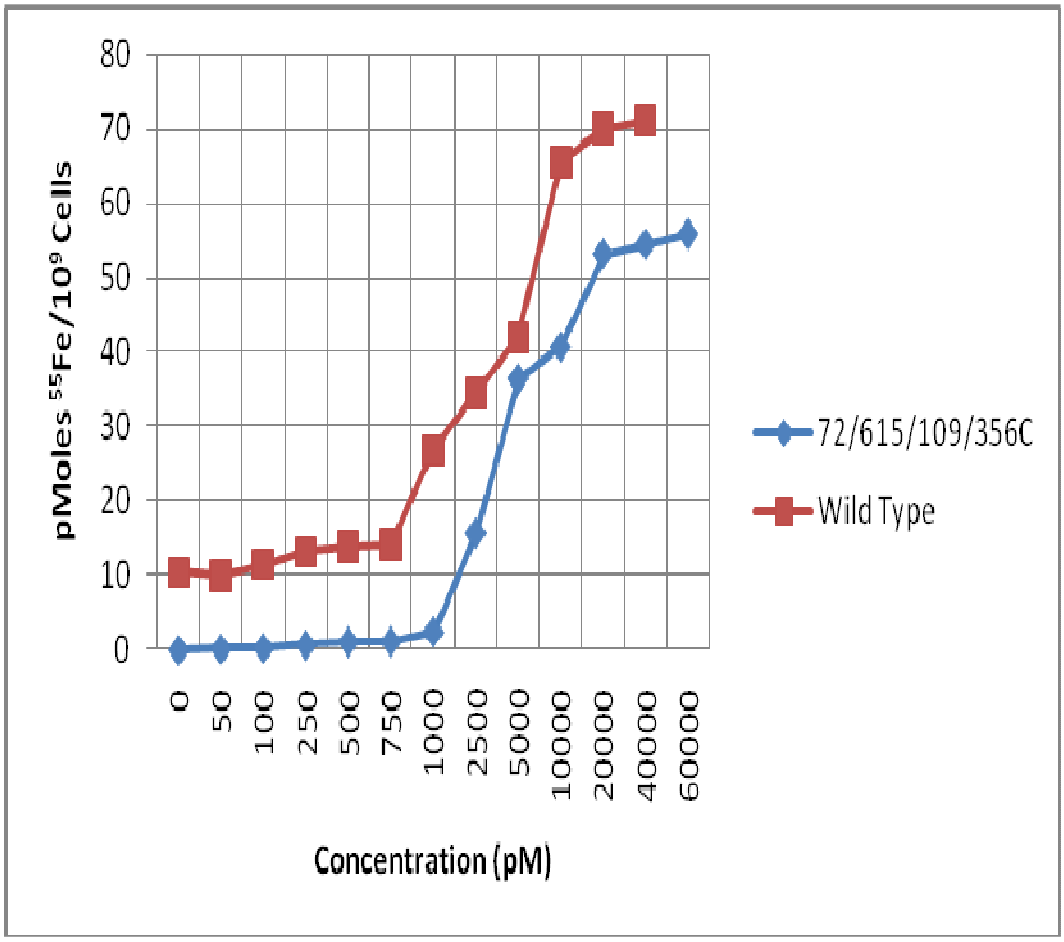


Figure 18 Concentration-dependent transport for FhuA variant 72/615/109/356C; K_M=5.0 nM, V_{MAX}=98.

K_D, K_M, and V_{max}

Using data obtained from the concentration-dependent binding and concentration-dependent transport assays, K_D and K_M values were calculated for variant proteins using the Grafit 3.0 program to characterize binding and transport (Table 8). K_D and K_M were used to compare the binding affinity and transport, respectively, of variant FhuA to that of the wild type. K_D was calculated from the data gathered from concentration-dependent binding assays. K_M is defined as the substrate concentration that yields a half-maximal transport rate, or ½ $V_{Max.}$ V_{Max} (Table 8) is the maximal velocity of the reaction at saturated substrate concentrations. K_M and V_{Max} were calculated from the concentration-dependent transport assay.

Table 8 K_D, K_M, and V_{MAX} values for FhuA variants; K_D values were determined from the curve generated by the concentration-dependent binding assay. K_M and V_{Max} were determined using data from the concentration-dependent transport assay.

FhuA Mutants	**K_D (nM)**	**K_M (nM)**	**V_{MAX} (pmoles/10^9 cells)**
Wild Type	0.5-5.0	0.1-1.0	50-150
R93A	18.4	TDT indicates no transport	
R133A	No binding	Cannot be determined	
E571A	2.5	TDT indicates defective transport	
E571Q	7.5	TDT indicates defective transport	
E522/571A	1.0	TDT indicates defective transport	
E522/571Q	5.0	TDT indicates defective transport	
R452A	4.8	TDT indicates defective transport	
R452Q	2.5	TDT indicates defective transport	
72/615/109/356C	9.1	5.0	98
TDT = Time-dependent Transport			

Extraction and Purification of 104/149C

FhuA variant 104/149C is a double-cysteine mutant created to assess whether or not the plug changes conformation within the β-barrel to form a channel during ferrichrome transport. As previously discussed, a disulfide bond connecting the β4 and β6 strands of the plug to prevent it from unfolding. Binding and transport data previously collected by Ms. Erin Storey indicated that 104/149C binds normally but is defective in

transport. As the final step in the characterization of the mutant, it should be purified and crystallized in order to observe structural changes.

SDS-PAGE and Western blot performed after the extraction process (Fig. 19) showed the presence of FhuA in Supernatant 2 (S2) and Pellet 2 (P2). P2 was homogenized in Extraction Buffer containing 1% Octylglucoside, which has been shown to elute FhuA protein. A total of 40 ml supernatant was collected from the extraction process. The resulting solution was purified via HPLC. A total of three HPLC runs were performed to purify the entire sample, with 53 1 ml fractions collected per run. SDS-PAGE of every fourth fraction showed small amounts of protein measuring at approximately 80 kDA in fractions 25-49. Unfortunately, due to the interference of Triton-X in the buffer, a graph of the protein peaks at OD_{280} could not be obtained.

Samples were taken in 50 µL quantities from various fractions and loaded onto SDS-PAGE gels to verify the presence of pure protein. Large quantities of FhuA protein were observed in fractions 30-33 from each run, although they were not entirely purified. These fractions were combined and dialyzed overnight to remove salt from the buffer. Then, they were injected into the HPLC column again to remove additional proteins. SDS-PAGE confirmed that the second round of HPLC yielded pure FhuA protein.

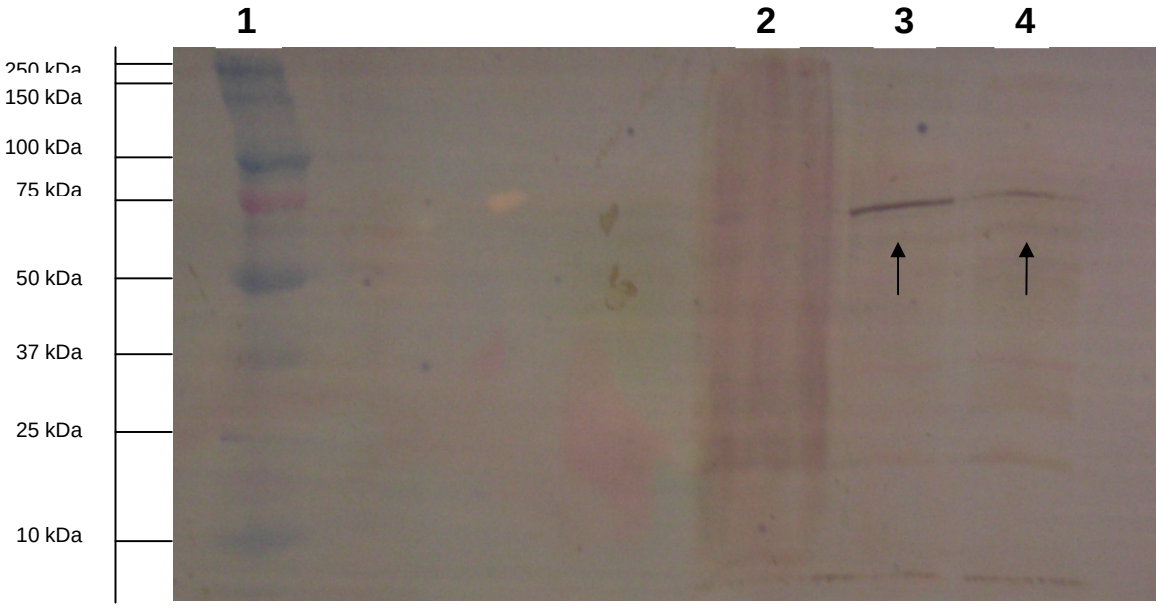


Figure 19. Western Blot of various samples taken during the process of extracting FhuA variant 104/149C. Lane 1. BioRad Broad Range Kaleidoscope protein markers; 2. Lysis Buffer Supernatant 2; 3. Supernatant 2; 4. Pellet 2.

CHAPTER 4

DISCUSSION

Ferrichrome transport was first studied to determine its role in microbial metabolism. In 1975, it was shown that *tonA* (now called *fhuA*) mutants resistant to phage T5 do not transport ^{55}ferrichrome and that ferrichrome inhibits the lethal action of colicin M. Purified FhuA from the outer membrane had previously been shown to bind T5 and colicin M (Braun 2004 and references therein), so ferrichrome transport was linked to the *fhuA* gene. FhuA became a model for the elucidation of active, energy-consuming transport across the outer membrane of Gram negative cells, especially in *E. coli* K-12 (Braun and Braun 2002).

As previously discussed, FhuA transports ferrichrome, the structurally similar antibiotic albomycin, the structurally unrelated antibiotic rifamycin CGP 4832 (a derivative of rifamycin) and acts as a receptor for phages T1, T5, Φ80, and UC-1, and for colicin M (a toxic protein) and microcin J25 (a toxic peptide) (van der Helm 2004). All functions except for phage T5 infection require TonB activity. Comparison of the solved structures of ligand-bound and unbound FhuA reveals major conformational changes that take place after ferrichrome binds to the protein. The switch helix (residues 24-29) unwinds and residues E19, S20, and W22 shift 17 Å from their original α-carbon position (Braun and Braun 2002). This movement exposes the TonB box to the periplasm.

All variant FhuA proteins demonstrated normal expression as detected by SDS-PAGE and Western blot, indicating that none of the mutations have deleterious effects on the expression of FhuA. Western blots were also performed on samples taken from each of the binding and transport assays to confirm expression of the target protein. All of these samples were positive for variant FhuA expression.

Mutants E571A, E571Q, R93A, E522/571A, E522/571Q, and R133A were all created previously (Chakraborty et al. 2006). Time-dependent transport analysis was performed on these mutants by Erin Storey and they were determined to be defective in transport to varying degrees. It was necessary to analyze the binding capabilities of these mutants in order to determine whether the lack of transport in each variant was due to the involvement of the target amino acid(s) in the transport process, or if the particular mutation had rendered the variant protein unable to bind the siderophore, in which case the effects on transport cannot be determined. The results of the binding and transport assays suggest that the residues E571, E522, and R93 may play critical roles in the transport function of FhuA but not in binding (Table 8). E571, E522, R133, and R93 are all part of the highly conserved lock region and may be part of the transport process of FhuA but not involved in binding. R93 and R133 from the plug make up the quadrupole which forms hydrogen bonds and electrostatic interactions with E522 and E571 on strands 14 and 16 in the barrel. It is possible that the mutation of R133 to nonpolar alanine changed the structure of the protein in a way that made it unable to bind the ferricsiderophore complex. A previously created mutant, R133Q, showed binding similar to the Wild Type and was defective in transport (Chakraborty et al. 2006). However, while glutamine is neutral like alanine, it is also polar like arginine and

therefore capable of forming hydrogen bonds. Mutation of the amino acid at location 133 from a polar to a nonpolar residue may have a drastic effect on the structural integrity of the FhuA protein.

The time-dependent transport data demonstrates that R452A and R452Q are indeed defective in transport, a conclusion that is further supported by the data obtained from the corresponding arginine in FatA, an outer membrane ferricsiderophore transport protein in *Vibrio anguillarum* (Lopez et al. 2007). It is thought that R452 is one of the residues lining the putative channel through which the ferricsiderophore passes, and that because of its positive charge and polarity, it induces the negatively charged ferricsiderophore complex to move from the binding site into the periplasm. It is not well understood whether the charge of the residue is the sole factor influencing its role in transport, or whether this is due to a combination of charge and its ability to form hydrogen bonds. To determine this, R452 was mutated to both alanine, a neutral nonpolar residue, and glutamine, a neutral polar residue.

In spite of the differences in polarity between alanine and glutamine, the degree of defective transport is largely similar in both variants. They transport similar levels of ^{55}Ferrichrome to the Wild Type until around the 2 minute time interval, when transport reaches a plateau (Fig. 15). It is possible that these variants are able to transport a small amount of ferricsiderophore initially because the channel is still able to open upon binding of ferrichrome, but due to structural changes caused by the mutation, the protein is unable to return to its original configuration. Crystallization of R452A and R452Q will be required to fully assess this. Transport in KDF541 cells was measured for

use as a baseline for these experiments. *E. coli* KDF541 is a knockout strain that was developed with all known outer membrane ferricsiderophore receptors removed. The minimal level of transport in KDF541 cells lacking FhuA genes is likely due to diffusion, although it could also be attributed to an as yet undiscovered iron transport mechanism. Minimal transport was seen in R452Q in the nutrition assay; this may be because polar glutamine continues to form hydrogen bonds that could contribute to a lesser degree of defective transport, while nonpolar alanine does not.

By selecting one amino acid in the β-barrel and a nearby amino acid in the plug domain and mutating them both to cysteine, a strong disulfide bond can be created to tether the plug to the inside of the barrel. Two double-cysteine mutants, 72/615C and 74/587C, were previously created to ascertain whether the plug domain is expelled during transport, or if it changes conformation to create a channel. The mutant 74/587C was able to bind ferrichrome; however, transport was significantly reduced. It was later determined that P74 is conserved in FhuA and may be involved in positioning the ferrichrome binding loop A (Eisenhauer et al. 2005; van der Helm and Chakraborty 2002). Variant 72/615C demonstrated normal binding and transport with regard to the Wild Type, with comparable K_D and K_M (17.2 nM and 0.3 nM, respectively). These results are corroborated by the data obtained by Eisenhauer et al. in 2005 using ferricrocin transport with FhuA. These results indicate that the plug may not need to be completely removed from the barrel in order for transport to take place. However, it could not be determined whether the plug was actually unfolding or if it was merely being pushed aside during transport.

The tetra-cysteine mutant 72/615/109/356C was made to tether the plug to the barrel on two sides so that it could not be displaced by the ligand during transport. Copper sulfate, an oxidizing reagent, was added to the media in which this mutant was grown to ensure that the disulfide bonds would not be reduced. In creating the tetra-cysteine mutant, it was necessary to choose nonconserved residues for mutagenesis. If conserved residues were used, it would be impossible to ascertain whether the resulting variant FhuA was defective in transport due to the plug being tethered in place, or due to a structural change in the protein that made it unable to carry out transport regardless. Great care was also taken to ensure that the target amino acids were located far from the TonB box, so that their mutation would not affect energy transduction. Time-dependent transport data for 72/615/109/356C indicates that the tetra-cysteine mutant transports the ligand normally with respect to the Wild Type. These results are largely corroborated by the results of the siderophore nutrition assays. FhuA mutant 72/615/109/356C is able to bind the ferricsiderophore and transport it into the cell. Thus, tethering the plug domain in place inside the β-barrel does not appear to diminish transport of the ligand. This supports the hypothesis that the plug unfolds within the barrel to form a channel during transport.

The double-cysteine mutant 104/149C was created in order to determine whether the core of the plug domain underwent conformational changes involving the movement of β-strands 4 and 6. Amino acids N104 and L149 were mutated so that they would form a disulfide bond tying these two strands together. Variant 104/149C demonstrated normal binding but defective transport, supporting the hypothesis that transport of the ligand requires partial disassembly of the core beta sheet of the plug. This supports the

formation of a channel rather than the expulsion of the plug during ligand transport. It is likely that only a partial disassembly is required because when the turn after β-strand 4 or the continuation of this strand is tethered to the barrel with a disulfide bond, the protein is still capable of normal transport (Eisenhauer et al. 2005). This variant was purified for crystallization in order to determine what structural changes may have been caused by the mutations, so that a better understanding of the transport process may be gained.

The other FhuA variants used in this study should also be crystallized as the final step in their characterization. Crystallization of the lock region mutants may lead to a better understanding of how their mutation has impacted their role in transport. Likewise, with the two R452 variants, having a three-dimensional image of the protein will better elucidate the relationships between this amino acid's charge and polarity and its possible interactions with the ferricsiderophore as it moves through the putative FhuA channel. It would be especially interesting to see these proteins crystallized in ligand-bound form as well.

The tetra-cysteine mutant 72/615/109/356C should also be crystallized. This should confirm the presence of disulfide bonds that tether the plug domain in place, while also providing insight into any minor structural changes. Additionally, crystallization of the ligand-bound form would likely yield further data to support the hypothesis that the plug domain of FhuA unfolds to form a channel during transport. There is also another tetra-cysteine mutant, 72/615/112/383C, which was created for this study; however, its expression has not been confirmed and it has not been the

subject of any further analysis. Characterization of this mutant would be beneficial to provide further corroboration for our hypothesis regarding conformational changes in the plug domain during ligand transport.

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