The Thesis Committee for Rebecca Rose Morrison
Certifies that this is the approved version of the following thesis:

Purification of Feo Proteins and Analysis of Residues Important for Feo Protein Interactions

APPROVED BY
SUPERVISING COMMITTEE:

Supervisor:

Shelley M. Payne

Elizabeth E. Wyckoff

Purification of Feo Proteins and Analysis of Residues Important for Feo Protein Interactions

by

Rebecca Rose Morrison, B.S.

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Dedication

This work is dedicated to my parents. I want to thank you for your endless support and love. I also want to thank all my friends and family who have been instrumental in any success that I have had.

Acknowledgements

I first want to thank Shelley Payne for her guidance and encouragement throughout my time in the Payne lab. I also want to thank Elizabeth Wyckoff for allowing me to work on the Feo project, sharing reagents and limitless guidance. Lastly, I want to thank all current and former Payne lab members for their help and times of laughter.

Abstract

Purification of Feo Proteins and Analysis of Residues Important for Feo Protein Interactions

Rebecca Rose Morrison, M.A.

The University of Texas at Austin, 2012

Supervisor: Shelley M. Payne

Iron is an essential element for virtually all forms of life. Complicating matters, it is present in the insoluble ferric form in aerobic environments, while the more soluble ferrous form is found in anaerobic or reducing environments. *Vibrio cholerae*, the causative agent of the disease cholera, requires iron to survive. In order to meet the need for iron, *V. cholerae* expresses a variety of iron acquisition systems. One of these systems, Feo, is highly conserved among bacterial species as well as archaea and transports ferrous iron. The Feo system consists of three proteins: FeoA, FeoB, and FeoC. Previous work using the bacterial adenylate cyclase two hybrid system has shown that FeoC interacts with the cytoplasmic N-terminal domain of FeoB. However, the significance of this interaction is not known. In this study, *V. cholerae* Feo system proteins were analyzed for residues important for the interaction between FeoB and FeoC. In addition, FeoA and FeoC were purified for antibody production. It was found that a residue in the G protein domain of FeoB was not necessary for interaction with FeoC. However, a conserved residue in FeoC did abolish the interaction with FeoB. These results indicate that there is at least one residue important in the interaction of

FeoB and FeoC, although further characterization will most likely reveal more. Antibodies to FeoA and FeoC were generated to use them for further characterization of the Feo system.

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I. INTRODUCTION

IRON ACQUISITION IN BACTERIA

Iron is an essential element for nearly all forms of life; however, it can be toxic and the levels of intracellular iron require strict control. Iron can react with oxygen species to create destructive oxygen free radicals through the Fenton reaction, causing damage to lipids, DNA, and proteins (121). However, when insufficient iron is present, key cellular processes cannot function. Complicating matters further, iron is found in two oxidation states with insoluble ferric (Fe^{3+}) iron present in oxidative and aerobic environments. The more soluble ferrous (Fe^{2+}) iron is present in reducing and anaerobic conditions. In order to meet the requirement for iron, bacteria use several strategies to regulate iron intake.

Bacteria must first acquire iron from their surrounding environment, and they have numerous systems to accomplish the task of scavenging iron. Many bacteria secrete molecules called siderophores, which are low molecular weight (500-1000) high-affinity iron chelators that bind ferric iron (84). More than 500 siderophores have been described, and the *Escherichia coli* siderophore, enterobactin, was one of the first siderophores discovered and has been well studied(2, 91, 99). Enterobactin consists of three distinct parts: the triacetone backbone, the amide linkage, and the metal binding area(103). In Figure 1, the hydroxyl groups shown in red on the metal binding areas interact with the iron molecule shown in green through hydrogen bonds. The model on the lower left

shows enterobactin without iron and the model on the lower right shows enterobactin in complex with iron.

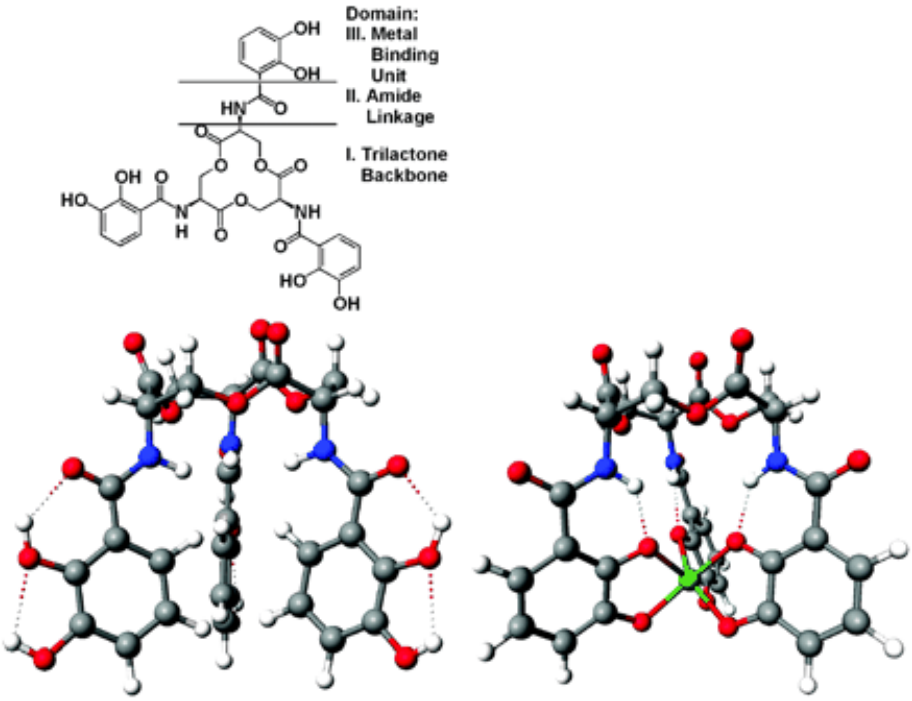


Figure 1. Structure of the *E. coli* siderophore, Enterobactin.

Figure from Raymond et al.(103), used with permission.

The siderophore enterobactin binds ferric iron with high affinity, which enables it to bind free ferric iron from the environment(103). Producing siderophores is a common strategy to acquire iron used by bacteria; however, secreting molecules into the extracellular environment can be costly to the cell. In order to make use of the iron bound to siderophores, bacteria use receptors to transport the siderophores bound to iron into the cell. Receptors for siderophores bind to the ferri-siderophore with high affinity and help

maximize the amount of iron that can be brought into the cell. Ferri-siderophore complexes are thought to be too large to pass through the porins that allow small solutes to enter through Gram-negative outer membranes and must use a receptor to enter the cell(2).

In Gram-negative bacteria, such as *E. coli* and *V. cholerae*, there are two lipid membranes and a periplasmic space that the siderophores must traverse. The outer-membrane consists of an asymmetrical bilayer: the outermost layer consists of lipid-anchored oligosaccharides and the inner layer consists of phospholipids. Next is the aqueous periplasmic space that contains many enzymes, chaperone proteins, and transport factors as well as a thin layer of peptidoglycan, which confers rigidity to the cell and maintains the cell shape(100). Lastly, the cytoplasmic membrane is composed of a phospholipid bilayer that is rich in proteins that harvest ion gradients for energy conversion(100).

The outer-membrane receptors for siderophores have high affinity for their ligands, which enables efficient uptake of the molecule. The enterobactin receptor in *E. coli*, FepA, as well as other receptors has been crystallized. All of these proteins are β-barrels that cross the outer membrane and have an N-terminal globular domain that can open the channel in response to siderophore binding (11). Figure 2 below illustrates the catechol- type siderophore-mediated ferric iron transport system of *E. coli*.

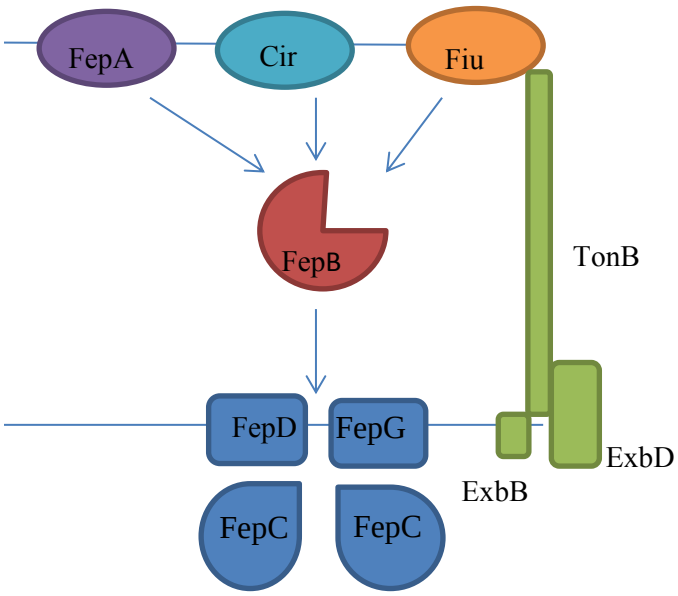


Figure 2. *E. coli* K-12 siderophore-mediated ferric iron transport.

The outer membrane receptors for catechol-type siderophores are FepA, Cir, and Fiu. The periplasmic binding protein is FepB. The cytoplasmic permease consists of FepD and FepG. The ABC proteins are composed of FepC. Note that TonB-ExbB-ExbD interacts with all of the outer membrane receptors, not just Fiu. Adapted from Andrews *et al.* (2), used with permission.

Bacteria often have receptors for both the siderophore they produce as well as for siderophores produced by other bacterial species. For example, *Vibrio cholerae* produces the siderophore vibriobactin, but it also produces receptors for enterobactin and ferrichrome (40). By having receptors for several different siderophores, the bacteria can take advantage of siderophores produced by other bacteria in the environment, lowering the energy expenditure needed to acquire iron.

Binding of the ferri-siderophore to the receptor induces conformational changes believed to prime the interactions with the TonB system(2). TonB, ExbB, and ExbD use energy generated from the proton motive force of the cytoplasmic membrane to power the transport of siderophores through their receptors (56, 100). TonB spans the periplasmic space, and is stabilized in the cytoplasmic membrane by ExbB and ExbD as noted in figure 2 (52, 109). Receptors that interact with TonB have a conserved sequence, called the TonB box, where TonB physically interacts with the receptor and provides the energy needed to drive transport (2).

The interaction of TonB with an outer-membrane receptor allows the siderophore to be transported into the periplasmic space. Transporting the siderophore across the periplasm and the cytoplasmic membrane involves a periplasmic binding protein and an associated cytoplasmic permease. These proteins follow a general configuration: the periplasmic binding protein ferries the siderophore to the appropriate cytoplasmic permease, while the permeases in the cytoplasmic membrane have an ATP- binding cassette to power transport across the cytoplasmic membrane. It is not clear whether the periplasmic binding protein directly interacts with the outer-membrane receptor or if it is merely close to the receptor(64). The best characterized of the siderophore periplasmic binding proteins is FhuD from *E. coli*, and it interacts with iron hydroxymate siderophores(25). As with most other periplasmic binding proteins that shuttle iron ligands, it is a bilobal protein. The protein or proteins that allow the siderophore through the inner membrane may be composed of a homodimer, a heterodimer, or one large unit.

In the figure above, FepB is the periplasmic binding protein, while FepD and FepG compose the cytoplasmic permease(85). The ABC unit is generally made of two ATPase proteins, which may be identical. In the figure above, FepC is an ATPase that powers transport of the siderophore across the cytoplasmic membrane and is associated with FepD and FepG(66). As outlined in figure 2, Cir and Fiu are also outer-membrane receptors that transport Fe^{3+}-dihydroxybenzoic acid and Fe^{3+}-dihydroxybenzoyl serine respectively(2). Nikaido *et al.*(86) proposed that these receptors may help *E. coli* recover the hydrolytic products of enterobactin from the environment(86). Siderophores brought in through Cir and Fiu are transported by FepBCDG into the cytoplasm(2).

Pathogenic bacteria take advantage of host iron sources, although the host iron is sequestered in an effort to limit the bacterial infection (12). Mammals lower iron levels to about 10^{-18} M in tissue fluids, which cannot support bacterial growth(12). The host proteins, transferrin and lactoferrin, are used by the host to solubilize and transport iron in normal conditions. Lactoferrin is also an antimicrobial protein produced by host cells to bind iron in extracellular compartments and can limit growth of organisms including *E. coli* and *P. aeruginosa* (4, 83). However, some pathogenic organisms such as *Neisseria meningitidis* are able to retrieve the iron from lactoferrin (95). Receptors for transferrin as well as lactoferrin have been identified in *Neisseria* and other organisms (102). These receptors strip the iron from lactoferrin or transferrin and release the surrounding protein to the external environment. They also utilize the TonB system and Fbp to ferry the iron to the cytoplasm(1).

As heme and hemoglobin are some of the most abundant sources of iron in the human body, it is not surprising that bacteria have evolved methods to obtain this store of iron. Access to host heme stores can be accomplished by hemophores secreted into the extracellular environment where they scavenge heme from assorted hemoproteins due to the hemophores higher affinity for heme, and return heme to their particular outer-membrane receptor(21). However, some bacteria bind heme directly at their surface(35). The heme molecule is bound and transported through an outer membrane receptor using the TonB system and an ABC type cytoplasmic membrane transporter.

FEO- A MAJOR FERROUS IRON TRANSPORT SYSTEM

Bacteria can also transport ferrous iron in addition to ferric iron. Ferrous iron (Fe^{2+}) is the more soluble oxidative state of iron, but it is only present in low oxygen and reducing environments. This makes is it likely that ferrous iron is the predominant form of iron in areas such as the human intestine. A conserved operon called *feo* (ferrous iron transport) is present in approximately fifty percent of bacterial genomes that have been sequenced and transports ferrous iron(45). It is present in organisms as diverse as cyanobacteria(22), archea(53), gram-negative bacteria(53) and gram-positive bacteria(57). The Feo system was discovered in 1987 in *E. coli* in a screen for mutants deficient in ferrous iron transport(44). The *feo* operon consists of *feoA*, *feoB*, and *feoC*. FeoB is thought to be the ferrous iron permease, while the functions of FeoA and FeoC are uncertain. FeoA and FeoC are both small, hydrophilic proteins of approximately 8kDa. *E. coli* FeoB is 773 amino acids in length and contains several integral membrane

domains(124) . The configuration of this operon and the encoded proteins is illustrated below.

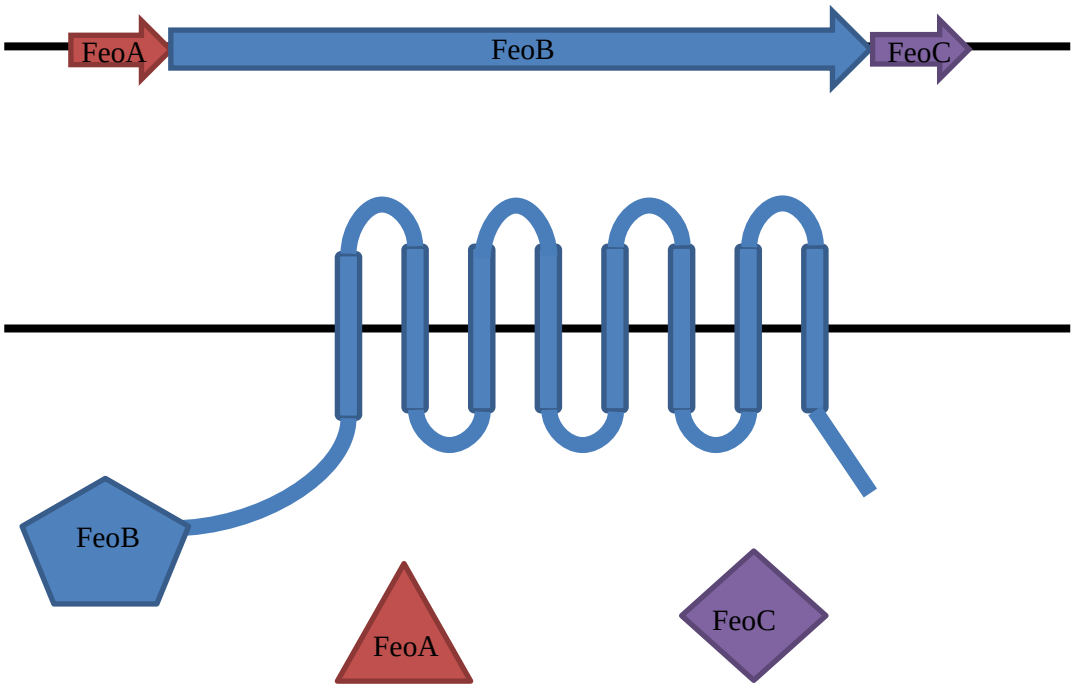


Figure 3. FeoABC operon and protein localization.

Adapted from Cartron *et al.*(20),used with permission.

In bacterial species that contain the *feo* operon, 80% have *feoA* immediately upstream of *feoB*(20). FeoA has weak homology with the SH3-like domain of DtxR of *C. diptheriae*, which suggests that FeoA might participate in facilitating interactions with other proteins (20). A structural study of FeoA has confirmed it contains an SH3-like domain, consistent with the hypothesis that it is involved in interactions with other

proteins(116). In addition, a *feoA* mutation prevented the uptake of Fe^{2+} through the Feo system, suggesting that FeoA is necessary for ferrous iron acquisition(63). Recently, FeoA was shown to interact with FeoB in *Salmonella enterica* using the bacterial adenylate cyclase two-hybrid system (BACTH) (63). The BACTH system is based on the *Bordetella pertussis* secreted adenylate cyclase (59). This molecule has two catalytic domains, which do not function when separated. However, if each catalytic domain is fused to an interacting protein then the adenylate cyclase activity will be restored (60). This process allows for screening for interactions between proteins. If two proteins interact, then the bacteria will turn blue when grown on plates containing the indicator X-gal and display increased activity in a beta-galactosidase assay. Thus, with the evidence from the BACTH and other studies, the prevailing hypothesis is that FeoA interacts with FeoB.

FeoB is a large, membrane spanning protein with an N-terminal G protein domain. G proteins normally function in cellular signaling and in regulation of diverse cellular processes. The G protein domains interact with guanine nucleotides as GTPases that hydrolyse GTP to GDP, with GTP "turning on" processes and GDP turning them off. In vitro, the G protein domain cycles through a nucleotide-free state, or "apo" form; however, a GTP molecule quickly binds to the nucleotide-free G protein domain(17). The loss of the GDP nucleotide from the G protein is a crucial part of regulating G proteins: the loss of GDP allows the cycling of the G protein from GDP to the apo form and back to the GTP-bound state. The amount of time that the G protein is bound to GDP correlates to the amount of time that the G protein is in the "off" configuration.

Critical elements that regulate whether GTP or GDP is bound to the G protein domain or if it is in the apo state include guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The GAP proteins increase the rate of GTP hydrolysis, while GEFs as increase the rate of GDP removal(8). The G protein domains consist of 5 G regions that contact the guanine-nucleotide as well as a magnesium ion. The binding of the GTP molecule causes a conformational change in two regions known as switch I and switch II which allows effector proteins to interact and initiate signaling(42).

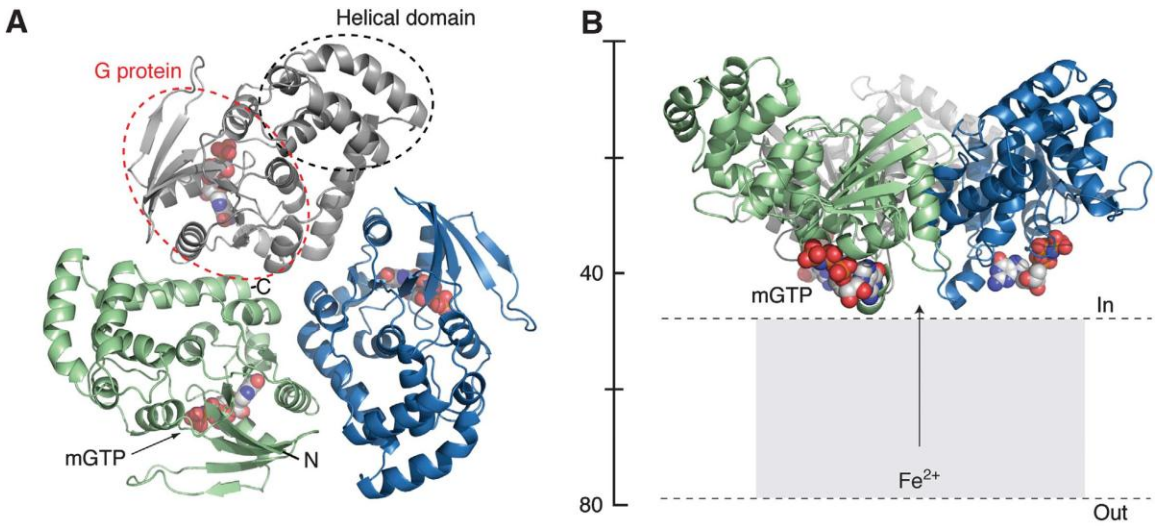


Figure 4. Crystal structure of FeoB and GTP.

Figure from Guilfoyle et al. (42) , used with permission.

Sequence analysis of the integral membrane domain of FeoB has predicted that it has between eight and twelve transmembrane domains, depending on the species and the prediction program used(20). Crystal structures of on the cytoplasmic N terminus of

FeoB have provided more insight into the workings of FeoB. A crystal structure of the portion of FeoB containing the G protein domain and the linker domain with GDP and a water molecule shows how FeoB positions the water molecule to initiate GTP hydrolysis(6). There are several other structures of the G protein domain of FeoB, which all show that FeoB has considerable homology to other G proteins. Crystal structures have been determined for the N terminus of FeoB in *Streptococcus thermophilus*(5), *E. coli* (42), *Thermotoga maritima*(47), *Klebsiella pneumoniae*(53), *Pyrococcus furiosus*(53), *Methanococcus jannaschii*(65), and *Legionella pneumophila* (94). However, the interpretation of some of the results are conflicting, as studies have claimed that FeoB is a monomer(47), dimer(94), and a trimer (42).

Other studies have revealed more consistent results. In vitro studies show that the G protein domain of cytoplasmic portion of FeoB has a slow rate of GTP hydrolysis and a fast release of GDP, which would suggest that the G protein is usually in the "on" position(70). The Unger group(31) has demonstrated that the linker domain between the N-terminal G protein domain and the membrane-spanning loops might act as a guanine-nucleotide dissociation inhibitor; therefore, it could act as a regulator on an otherwise unusual G protein domain causing FeoB to remain in the GDP-bound state longer (31). The Jormakka(5) group established that the GTPase activity of FeoB is potassium activated, since potassium leads to a 20 fold increase in the GTP hydrolysis rate of FeoB(5). These in vitro results suggest that although FeoB has a slow rate of GTP hydrolysis and a fast release of GDP on its own, it is most likely regulated so that it is not in the "on" position for as long.

Genetic investigation has revealed insights into the *feo* operon and confirmed its role in ferrous iron acquisition. The G protein domain of FeoB is necessary for ferrous iron uptake through Feo, which was determined by random insertional mutagenesis (68). The switch regions of FeoB were important for transport of ferrous iron through Feo as shown by *E. coli* mutants which were able to bind and hydrolyze GTP but were non-functional in vivo (31).

FeoC is less conserved than either FeoA or FeoB and has been associated with γ-proteobacteria(20). Like FeoA, it is a small, hydrophilic protein, but it has been proposed from sequence analysis to have a winged-helix structure within its N-terminus. This structure normally binds DNA, leading to the speculation that FeoC is a transcriptional regulator of the *feoABC* operon(10, 20). Guo *et al.*(43) showed that a *feoC* deletion in *Y. pestis* increased resistance to cationic antibiotics. They suggest that as FeoC has been reported to be a repressor of *feoAB*, then the increase in resistance to cationic antibiotics would be due to derepression of *feoAB*. Once *feoAB* has been derepressed, then the amount of ferrous iron in the cell would increase. Indirectly, the increase of ferrous iron could turn on genes needed for resistance to cationic antibiotics. However, a deletion of *feoC* in *Y. pestis* done by the Perry group had no effect on Feo function(93). Recent evidence has also shown that it binds the G protein domain of FeoB in *K. pnuemoniae*(54). The role of FeoC remains unclear and further work is needed to elucidate its function.

The *feo* operon has a different genetic organization in some organisms. Interestingly, there are cases where multiple *feoA* genes are associated a single *feoB* in an

operon. In these cases, the two FeoA homologs are evolutionary distant by sequence analysis (20). The evolutionary distance may suggest that each FeoA protein has developed functional specialization. Remarkably, there are also two FeoB sequences in some species. When multiple *feoB* genes are present, they shown signs of genetic divergence based on sequence analysis. This may signify that the evolutionary pressure is likely lessened on the duplicated gene, which then can more freely evolve a new function. As this operon is present in such diverse species, it is logical that the organization may have diverged in some of them.

The Feo system and ferrous iron acquisition have also been implicated in disease. It is probable that the reducing, low oxygen environment inside the host intestine makes ferrous iron the more abundant form; therefore, ferrous iron acquisition is an important process for bacterial life inside the intestine. In *S. flexneri*, the *feoABC* operon was induced in anaerobic conditions, by the anaerobic regulators ArcA and Fnr as shown in vitro (9). In *Salmonella* species, the two-component response regulator, PhoP/Q, turns on *feoB* transcription in response to acidic pH(24). This may help *Salmonella* sense when it is entering a host as the bacteria must pass through the human stomach, which is highly acidic. This further supports that Feo is important under oxygen-limiting conditions such as those seen inside a host. Specifically, Feo has been linked to virulence with *Streptococcus suis*(3), *S. typhimurium*(123), *Yersinia pestis*(32), *Helicobacter pylori*(124), *Legionella pneumophilia*(104), *Campylobacter jejuni*(82), and is even associated with virulence in plant pathogens such as *Xanthomonas oryzae*(92). The fact

that this conserved operon is also responsible for virulence in many different host systems makes further study and characterization important.

OTHER FERROUS IRON ACQUISITION SYSTEMS

Other means of transporting ferrous iron include the metal ABC type transporters. These include SitABCD from *S. typhimurium* and YfeABCD from *Y. pestis.* The Sit system was discovered within a pathogenicity island in *S. typhimurium* and is required for murine infection(131). However, further research has shown that although Sit does transport ferrous iron, its major function may be in Mn^{2+} transport in *S. typhimurium* (131). In *Shigella flexneri,* Sit has a minor role in ferrous iron acquisition when inside a host epithelial cell, but a mutant in *sitA* showed reduced growth in vitro (106). Sit may also be important for *S. flexneri* to survive in macrophages, as a *sitA* mutant was defective in surviving exposure to hydrogen peroxide, one of the bacteriocidal mechanisms of macrophages(107). Unlike *S. typhimurium, S. flexneri* Sit is more involved in iron transport than manganese (33). The Yfe system of *Y. pestis* is required for murine infection, and a mutant that had two genes in the *yfeABCD* operon deleted showed reduced growth in the presence of an iron chelator(93).

Another ferrous iron acquisition system is the EfeUOB system. The genes are arranged in a three gene operon which is expressed during iron starvation. EfeU shows homology to the high-affinity yeast iron permease, Ftr1p, suggesting that it acts as the permease in the EfeUOB system(18). The functions of EfeO and EfeB are unclear, although a crystal structure of EfeO suggests that is involved with metal binding(101).

The *efeUOB* operon is repressed at high pH and is de-repressed at low pH as evidenced by the examination of transcriptional fusions(18).

The EfeUOB system was discovered in the *E. coli* Nissle 1917 strain(41). In many laboratory strains such as *E. coli* K12, the first gene in the operon (EfeU) has a frame-shift mutation that abolishes this system's function(41). This frame-shift mutation prevented the discovery of the *efeUOB* operon until 2006 (41). However, pathogenic strains of *E. coli* such as O157:H7 display a functional system(18).

Other ferrous iron acquisition systems may be involved in the transport of other metal ions, but can be used during times of iron starvation to maximize the amount of iron brought in to the cell. One of these systems is the ZupT system of *E. coli*. It was initially described as a metal permease responsible for the uptake of zinc but has since been shown to display a broad substrate specificity for divalent metal cations(38, 39). ZupT transports zinc and ferrous iron as well as cobalt, manganese, and cadmium. *zupT* expression does not seem to be induced or repressed by any metals but is instead transcribed at a low constitutive level(120).

E. coli mntH encodes a protein believed to be membrane associated and is similar to the eukaryotic NRAMP family, which transports divalent metal cations(69). MntH was found to have a higher affinity for manganese and ferrous iron in transport studies, but can transport many divalent cations as evidenced by competition assays(69). In *S. typhimurium*, MntH was found to be more important in manganese transport, and *mntH* expression is regulated by hydrogen peroxide as well as ferrous iron or manganese when

present(62). It has been implicated in virulence in the mouse as well as H_2O_2 resistance(62).

BACTERIAL IRON REGULATION

Iron is important for almost all forms of life and yet can be toxic in large quantities. Therefore, the regulation of iron transport is just as imperative as its acquisition in order for the cell to survive. The master regulator of iron acquisition in many bacteria is Fur (ferric uptake regulator). Fur was first found in *E. coli* in a screen investigating the regulation of ferric iron uptake systems, and the Δ*fur* mutant showed constitutive expression of iron transport genes such as *fhuA* and *fepA*(46). Fur is a 17kDa protein and acts as a homodimer that represses transcription of iron acquisition systems in iron-replete conditions (26). In high iron conditions, one ferrous iron molecule binds per subunit of the dimer, changing the Fur dimer's confirmation and allowing it to bind to conserved Fur boxes in the DNA preventing transcription(26). Structural studies have also shown Fur to bind zinc at a distinct site, most likely for structural purposes(98).

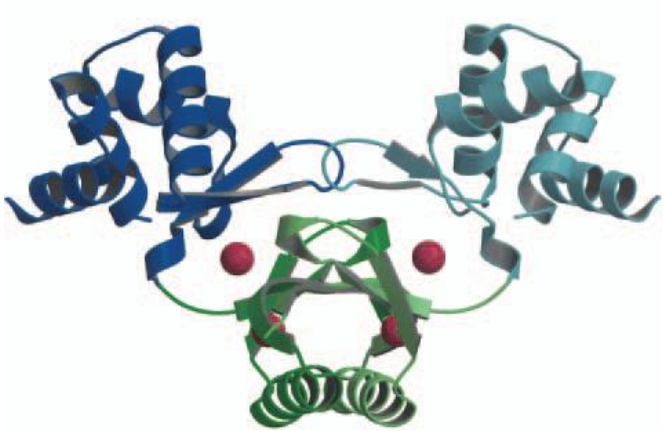


Figure 5. Crystal Structure of *P. aeruginosa* Fur dimer

Figure from Pohl et al. (98) The DNA binding domains are shown in blue and the dimerization domains in green. In red are the zinc ions.

The majority of genes repressed by Fur involve iron acquisition; however, genes not associated with direct iron acquisition system function are also repressed by Fur. *yqiH*, the gene in *E. coli* responsible for reducing siderophore-bound ferric iron coming from siderophores, is regulated by both Fur and a divergently transcribed gene, *yqjI*(80, 125). The product of this gene may not be directly involved in acquiring iron, but it does have a role in iron usage in the cell. In *S. typhimurium*, Fur was required for the activity of *sodA*, which is a superoxide dismutase that contains Mn^{2+} and is responsible for protecting the cell from superoxide damage(122). The regulation of *sodA* by Fur may result in turning on mechanisms to protect the cell in times of high intracellular iron levels, when damage is most likely to occur. Fur also plays a role in virulence in pathogens, as the shiga-like toxin of *E. coli* is negatively repressed by Fur(16).

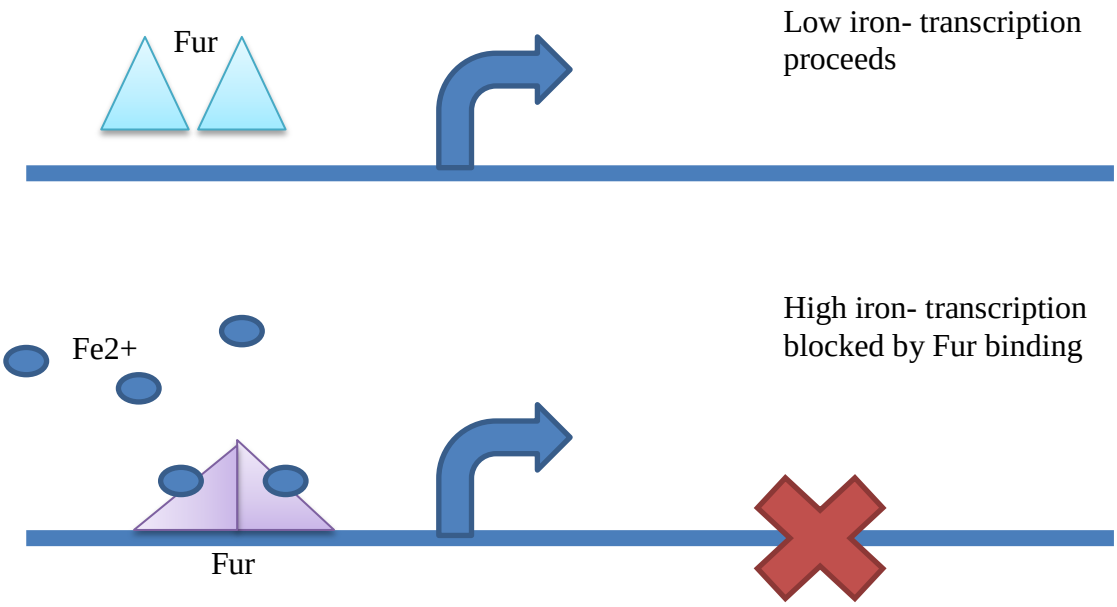


Figure 6. Action of Fur on target gene transcription

There are also some genes induced in the presence of Ferri-Fur. It was later found that this induction does not happen as a result of Fur, but is rather provided by another layer of iron regulation mediated by the small RNA, RyhB. This sRNA is responsible for some genes being indirectly induced by Fur because Fur represses *ryhB*, which encodes a post-transcriptional negative regulator of genes whose products use iron. RyhB accomplishes the negative regulation of these transcripts by binding to its target mRNA in an anti-sense fashion along with the chaperone, Hfq. Both RyhB and the target mRNA are degraded by RNaseE. RyhB controls around 20 mRNAs (~60 proteins) whose products are non-essential and bind iron (73). Thus, when the intracellular iron level is high, iron acquisition genes are turned off and genes whose products use iron are turned on, including *sodB* and *fumA* in *E. coli*(72). Therefore, RyhB restricts iron usage only to essential proteins during times of limited iron availability. The presence of both Fur and

RyhB allow the bacterium to fine-tune the iron usage and acquisition, maximizing the efficiency of the cell.

VIBRIO CHOLERAE

Virtually all forms of life require iron. One particular organism that has many systems to acquire iron and is implicated in human disease is *V. cholerae*. The diversity of its iron acquisition systems and its role in human disease makes it an interesting and important organism to study. *V. cholerae*, the causative agent of cholera, is a gram-negative rod that has been implicated in global pandemics and caused many fatal infections. According to the World Health Organization, there are an estimated three to five million cases of cholera a year resulting in 100,000 to 120,000 deaths(90). This organism lives in several different environments, including both marine habitats and in the human gastrointestinal tract. In the marine environment, *V. cholerae* may associate with copepods and other marine organisms(55, 118).

It is introduced to humans by drinking contaminated water. In studies using human volunteers, about 10^{11} organisms were required to cause disease(58), but that number is lowered to between 10^3 and 10^4 when the organisms were delivered with an antacid to decrease the acidity of the stomach(58). Once ingested and passed through the stomach, *V. cholerae* colonizes the small intestine using its toxin co-regulated pilus, TcpA(51). Once the organism has adhered to an intestinal epithelial cell through the toxin-coregulated pilus, then cholera toxin binds to a receptor on the epithelial cell surface, GM1, and following a cascade of events causes disregulation of the second

messenger cAMP(58). This causes the epithelial cell to pump out chloride ions releasing vast amounts of water and electrolytes which cause the rice water stools characteristic of cholera(58).

The symptoms of cholera in its milder form are generally the same as gastroenteritis including vomiting and abdominal discomfort, but can become life-threatening in the severe form, *cholera gravis*(58). Even if no symptoms are perceived by the host, bacteria are spread through the stool, potentially infecting many more people by contaminating water supplies. Merrell *et al*.(74) proposed that passage of the bacteria through the intestine of the host results in a hyper-infectious state that is maintained once outside the host, which may contribute to epidemic spread(74). Symptoms of cholera usually begin within 5 days and are accompanied by sudden diarrhea. The volume of stool is large in cholera patients and especially with the more severe form of the disease. Dehydration is the most clinically relevant effect of cholera and most treatment consists of oral or intravenous rehydration. In *cholera gravis*, hypotension and cardiac arrest can occur because of the profound loss of fluid and electrolytes.

V. cholerae possesses many virulence factors, including the toxin coregulated pilus and cholera toxin. Many of the factors are regulated by ToxR, a trans-membrane protein responsible for binding to the promoter of at least 17 genes. These genes include, *ctxA*, the cholera toxin gene and for the gene encoding a transcriptional activator, ToxT, causing a cascade of virulence factors to be produced(29). Later studies have confirmed the role of ToxR and ToxT but they have also shown that there are other regulators that

increase expression of virulence factors encoded by *tcpPH*(7). These virulence factors may respond in part to oxygen levels, as anaerobiosis increased proteins involved in biofilm formation and virulence factors(71).

Different biotypes of *V. cholerae* can account for the severity of the disease to some degree. Pandemic forms of *V. cholerae* are split into two major serogroups: O1 and O139, which are responsible for most epidemics, while other serogroups only occasionally result in outnbreaks(110). O1 can be further broken down into two biotypes: classical and El Tor, and into two major serotypes, Inaba and Ogawa(34). Classical biotypes are more often associated with *cholera gravis* than the El Tor biotypes(58, 96). However, new strains are developing in Asia and Africa which seem to cause a much more severe disease and are associated with higher fatality rates. These new strains have a mix of classical and El Tor characteristics, resulting in a more virulent organism(89). Furthermore, there is evidence that strains normally endemic in certain regions of the world are evolving and mixing characteristics from Inaba and Ogawa serogroups(23, 34).

There is also evidence that cholera is seasonal, with increases in bacteria corresponding to algal bloom in marine and estuary habitats(58). This seasonality may in fact reflect higher temperatures and higher nutrient availability in the marine environment rather than the presence of algae species. There is also evidence that cholera has occurred in waves of pandemics throughout modern history with the 7th pandemic occurring right now(81).

The most recent epidemic has been hard on the poverty-stricken nation of Haiti. In 2011, Haiti had more than 250,000 cases and 4,000 deaths within the first six months of the epidemic, making it one of the most deadly in recent history(30). It is thought that *V. cholerae* had such a large impact in Haiti due to the presence of a population naïve to the organism, the lack of clean drinking water, and unsanitary conditions (30, 97). The outbreak in Haiti brings to light the need of proper sanitation and access to clean drinking water to prevent *V. cholerae* from infecting humans.

VIBRIO CHOLERAE SPECIFIC IRON ACQUISITION

V. cholerae is an excellent model organism for iron acquisition due to the ease of study and the importance of understanding the basic science of *V. cholerae* to better design treatments and preventative measures. Since iron is required for its life, studying iron acquisition and the conserved Feo operon could provide vital information in fighting the organism and the disease it causes. As a result of the many iron acquisition systems, *V. cholerae* is able to live in many different environments.

V. cholerae has many genes that code for several different iron acquisition systems. *V. cholerae* makes the siderophore vibriobactin(40) but contains receptors for both vibriobactin(13) and two for enterobactin(77). It is thought that *V. cholerae* would not encounter *E. coli* in the human to make use of enterobactin, but enterobactin may be more important for growth in the environment(127). Vibriobactin consists of a norspemidine backbone and three dihydroxybenzoate moieties attached to the backbone(40). The pathway for converting dihydroxybenzoate (DHB) from chorismate is

identical as that for enterobactin and involves VibABC(130). Vibriobactin assembly involves DHB, threonine, and norspermidine and requires VibBDEFH(14, 61).

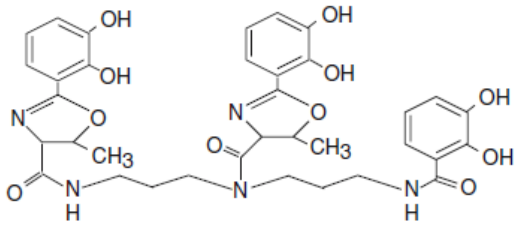


Figure 7. Structure of Vibriobactin

Figure from Griffiths et al(40), used with permission.

The gene encoding the vibriobactin receptor is *viuA*. Downstream of *viuA* is *viuB* which is most likely functioning in the removal of iron from the ferri-siderophore as it suppressed a *fes* mutation in *E. coli* (13). *fes* is a gene in *E. coli* that is known to encode a protein that removes iron from the ferri-siderophore. The genes for the receptors for enterobactin transport are *irgA* and *vctA*. IrgA and VctA show sequence similarity to CirA of *E. coli*(37) and FetA of *Neisseria gonorrhoeae*(19), respectively. In addition to enterobactin, *V. cholerae* is able to use agrobactin(40) and a siderophore made by *Vibrio fluvialis*(127), which is expected to be fluvibactin(112). *V. cholerae* is most likely able to transport these siderophores because their structure is similar to vibriobactin and are able to use the vibriobactin receptor(127). In addition, *V. cholerae* can use ferrichrome as a source of iron(40). The genes involved in this are *fhuABCD*(105).

V. cholerae has two periplasmic-binding protein dependent ABC transport systems for the transport of catechol siderophores called ViuPDCG and VctPDGC (128). Mutants with defects in either system are still able to transport both siderophores indicating that there is some flexibility in their binding capacity and they are not limited to either enterobactin or vibriobactin(79).

V. cholerae also uses heme and hemoglobin as iron sources(114). There are three genes for receptors for heme in *V. cholerae* which are *hutA*, *hutR*, and *hasR*(75). Less is known about the usage of heme once it has been transported into the cell. It was found that the gene *hutZ* was required for efficient heme usage(50, 75, 129). *hutZ* is found in the *hutWXZ* operon, which is divergently transcribed from the TonB1 system genes. Functions have not been assigned for *hutWX*. A crystal structure of HutZ showed a similarity to the HugZ protein of *H. pylori*, which is a heme oxygenase. The authors of this study, Liu *et al*.(67), noted that HutZ was closely related to HugZ, but no heme oxygenase activity could be described for HutZ in that study. They proposed that a divergence in one of the conserved regions of both proteins could account for the structural and functional mismatch. However, a recent study by Uchida *et al*.(117), found that HutZ bound to heme in the presence of ascorbate may act as a heme oxygenase to degrade heme(117). Further study of HutZ will elucidate the function of the *hutWXZ* operon and its relationship with heme.

Interestingly, *V. cholerae* contains two TonB-ExbB-ExbD systems. One of these operons, designated *tonB1*, is cotranscribed with *hutBCD*, which encodes an inner

membrane heme transport system. The second operon, *tonB2*, has low homology to *tonB1* operon but is able to restore function in a *tonB* mutant in *E. coli* when introduced on a plasmid(88). Further characterization has shown that the two TonB systems have over-lapping as well as specific functions(109). For example, transport though HasR is TonB2 dependent but transport using either HutA or HutR was TonB1 dependent(75) .

V. cholerae also has TonB-independent transporters. *fbpABC*, which encodes a periplasmic binding protein, a cytoplasmic permease, and an ATPase(126). The FbpABC proteins show a 50% sequence similarity to the FbpABC proteins of *Mannheimia haemolytica*, which is the most closely related species for which functional information is obtainable(108). To test whether the *V. cholerae* Fbp system is functional for iron transport, it was cloned on a plasmid and transformed into a *Shigella flexneri* mutant, SM193w, which is severely defective for iron transport and cannot grow without the addition of either an added functional iron transport system or exogenous siderophore. The addition of *fbpABC* on a plasmid to SM193w supported the growth of this strain, indicating that it encodes a functional iron transport system. The transport of ^{55}Fe SM193w with *fbpABC* was inhibited in the presence of the reducing agent ascorbate, indicating that this system is involved in the transport of ferric iron (126).

V. cholerae also has the Feo system for ferrous iron acquisition. This system consists of all three *feo* genes: *feoABC*. *feoA* and *feoB* are 40% identical to the *E. coli* homologs, but FeoC contains only 11% identity to the *E. coli* version. This may suggest a different function for FeoC in *V. cholerae* (126). To test whether *feoABC* encoded a

functional iron transport system, it was transformed into SM193w. Supplying either the *E. coli feo* operon or the *V. cholerae feo* operon allowed SM193w to grow without supplemental siderophore, which suggests that they are functional iron transport systems(126).

In previous work done by Emily Helton in her doctoral dissertation(49), it was shown that all three proteins, FeoA, B, and C, are needed for ferrous iron transport using the Feo system(49). Helton showed, using the bacterial adenylate cyclase two hybrid system (BACTH), that FeoB and FeoC interact. The interaction between FeoB and FeoC was greatest using the first 272 amino acids of FeoB, which includes the G protein domain and the linker domain, with the full length FeoC. A mutation in FeoC changing glutamate 29 to glycine abolished that interaction. This glutamate is conserved between *Vibrio* species, but is not conserved in *E. coli*. This residue may be important for either FeoC's function or structure. Further study of important residues in both FeoB and FeoC may help elucidate the function of FeoC.

Helton also found that the *feoABC* operon is induced under anaerobic and acidic conditions. These conditions closely match those seen in the human host; supporting the idea that ferrous iron and the Feo system may be important for *V. cholerae* survival in the human(49).

In addition to iron acquisition genes, *V. cholerae* also has the global iron acquisition regulator, Fur. Fur acts much the same way in *V. cholerae* as it does in other

bacteria: it represses iron acquisition genes in iron-replete conditions. *V. cholerae* Fur regulates almost all iron acquisition genes as well as other genes involved in pathogenicity, such as those encoding the toxin-coregulated pilus(78). The effect on the toxin coregulated pilus was confirmed because the *V. cholerae fur* mutant weakly auto-agglutinates. Supporting the link between Fur and virulence is the fact that the *fur* mutant was not able to compete with the wild-type strain for mouse colonization(78). *V. cholerae* Fur was crystallized which revealed some differences to Fur in *E. coli*(111). There are two zinc binding sites, but the second zinc binding site (Zn2) is thought to be a regulatory iron binding site and the first zinc binding site (Zn1) is thought to play a secondary role and not participate in DNA binding. There is also no evidence for metal binding to cysteine residues as there is in *E. coli*(111). However, all five potential metal binding domains remained conserved between *V. cholerae* and *P. aeruginosa* Fur, indicating that these residues are important for Fur function(111).

V. cholerae also possesses the sRNA RyhB involved in iron regulation. *V. cholerae* RyhB is regulated similarly to *E. coli* RyhB as it is repressed by ferri-Fur, but *V. cholerae* RyhB is much longer than the *E. coli* RyhB (200 vs. 90 nucleotides in length) and it regulates an additional subset of genes in *V. cholerae,* including genes involved in motility, chemotaxis, and biofilm formation (28, 76). As all of these processes are needed for the bacteria to infect a host, these data further reinforce the role of iron in pathogenesis.

PURPOSE OF THIS RESEARCH

Although the functions of the Feo proteins have become clearer in recent years, the roles that they play have not been fully determined. Even the basics of Feo activity are not completely understood. The goal of this study is to provide better understanding of the function of all three genes, and that the functions of FeoA and FeoC will become clearer as they are the least understood. As ferrous iron acquisition has been shown to be an important part of life inside the human host, it may be noted that understanding this system could lead to a better understanding of pathogen iron acquisition inside the host and have far reaching effects on treatment of bacterial pathogens.

II. MATERIALS AND METHODS

BACTERIAL STRAINS AND PLASMIDS

Bacterial strains used in this study are listed in Table 1. Bacterial plasmids used in this study are listed in Table 2. *E. coli* DH5α was used for routine cloning procedures. *E. coli* BTH101 was used in the bacterial two-hybrid assays. *E. coli* BL21 (DE3) was used for protein expression.

MEDIA, REAGENTS AND GROWTH CONDITIONS

E. coli strains were grown in Luria-Bertini (LB) broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride) or on LB agar. Unless otherwise indicated, antibiotics were used at the following concentrations per milliliter: 25 µg ampicillin, 30 µg chloramphenicol, and 50 µg kanamycin. *E. coli* BTH101 was routinely grown at 30°C. To induce expression of two-hybrid constructs, isopropyl β-D-1-thiogalactopyranoside (IPTG) was used at a final concentration of 0.5 mM or as indicated. When used, bromo-chloro-indolyl-galactopyranoside (X-gal) was added to LB agar at a concentration of 40 µg/mL.

Table 1 Bacterial Strains Used in This Study

Strain	Relevant Characteristics	Source
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR [Φ80*d*lacΔ(lacZ)M15]*	
BTH101	F$^-$*, cya-99, araD139, galE15, galK16, rpsL1* (Str^r), *hsdR2, mcrA1, mcrB1*	Euromedex
BL21 (DE3)	*fhuA2[lon],ompT,gal,(λDE3)[dcm]ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	*New England Biolabs*

PLASMID DNA ISOLATION, RESTRICTION DIGESTION AND LIGATIONS

Plasmid DNA was isolated using GenElute Plasmid Miniprep Kit (Sigma) and eluted into either10 mM Tris, 1 mM EDTA buffer or MilliQ water and stored at -20˚C. Restriction digestion was performed according to New England Biolab's protocols. Following manufacturer's protocol, DNA was isolated from agarose gels using GenElute Gel Extraction Kit (Sigma). Ligation of DNA was performed using T4 DNA ligase (New England Biolabs). DNA molecular weight markers were purchased from New England Biolabs and included ΦX174 DNA-Hae III digest and lambda DNA-HindIII digest. All procedures using the commercial reagents were done according to the manufacturer's protocol.

Table 2	Plasmids Used in this Study	
Plasmid	Relevant Characteristics	Source
pKT25	Low-copy-number vector encoding T25 under control of lac promoter, MCS at 3'end of T25	Euromedex
pKNT25	Low-copy-number vector encoding T25 under control of lac promoter, MCS at 5'end of T25	Euromedex
pUT18	High-copy-number vector encoding T18 under control of lac promoter, MCS at 5' end of T18	Euromedex
pUT18C	High-copy-number vector encoding T18 under control of lac promoter, MCS at 3' end of T18	Euromedex
pKT25-zip	pKT25 carrying leucine zipper of GCN4	Euromedex
pUT18C-zip	pUT18C carrying leucine zipper of GCN4	Euromedex
pKT25FeoB	pKT25 carrying *V. cholerae feoB*	E. Helton
pKT25FeoB272	pKT25 carrying *V. cholerae feoB,* encoding amino acids 1-272	E. Helton
pKT25FeoB272- D72A	pKT25 carrying *V. cholerae feoB,* encoding amino acids 1-272 with the D72A mutation	This study
pUT18CFeoC	pUT18C carrying *V. cholerae feoC*	E. Helton
pUT18CFeoC-E29G	pUT18C carrying *V. cholerae feoC* with E29G mutation	E. Helton

Table 2 continued		
pUT18CFeoC-M35A	pUT18C carrying *V. cholerae feoC* with M35A mutation	This study
pET16b	T7 Expression vector including an N-terminal His•Tag and three cloning sites.	Novagen
pET16bFeoA	pET16b carrying *V. cholerae feoA*	This study
pFeo128	pET25b carrying *V. cholerae feoC*	E. Wyckoff
pFeo101	pWKS30 carrying *V. cholerae feoABC*	E. Wyckoff
VcFeoD72A	pFeo101 carrying *V. cholerae feoB* with D72A mutation	M. Walters

TRANSFORMATION

Cells were made competent by diluting overnight cultures 1:100 into LB broth(250 µL overnight culture into 25 mL LB broth) plus appropriate antibiotics and growing to mid-log (OD_{650}=0.5-0.8). Cells were centrifuged at 7000 rpm (Rotor - Sorvall SS-34) for 7 minutes and the pellets were resuspended in 10 mL of cold 100mM $CaCl_2$. Cultures were incubated on ice for 30 minutes. Cells were centrifuged at 7000 rpm for 7 minutes at 4°C and resuspended in 2 mL cold $CaCl_2$ solution (100 mM $CaCl_2$, 15% glycerol). Cells were divided into 150 µL aliquots and stored at -80°C. Heat-shock transformation was accomplished by adding DNA to $CaCl_2$-competent cells and incubating on ice for 1 hour. The cells were then heat-shocked at 42°C for 1 minute and incubated on ice for 2 minutes. The cells were then added to 1 mL LB broth and incubated at 37°C for 1 hour prior to plating on LB agar plus appropriate antibiotics.

POLYMERASE CHAIN REACTION

Oligonucletide primers used in this study are listed in Table 3. Primer design was completed using MacVector (MacVector, Inc, Cary, NC). Primers were obtained from Sigma. Primers arrived lyophilized and were resuspended in double distilled water to a concentration of 100 μM and stored at -20°C. All PCR reactions used in plasmid construction were performed using KOD Hot Start (Novagen) according to the manufacturer's protocol. PCR reactions used to confirm plasmids were performed using Taq polymerase (New England BioLabs), according to the manufacturer's protocol. DNA sequencing was performed by the DNA Sequencing Facility at the University of Texas Institute for Cellular and Molecular Biology on ABI 3730XL and ABI 3730 DNA sequencers.

Table 3. Oligonucleotide primers used in this study

Primer Name	Nucleotide Sequence (5' to 3')
pUT18C.497	CGGGCAACTGAAGGAATACACATTG
pUT18C.950Rev	GATGACGGTGAAAACCTCTGACAC
FeoA.Nde	GTTCATATGAAATTGTCACAAATGCAA
FeoA.Bam.rev	GCGGATCCTCATTGTGCTCTCTCCAC
Feo227B	ACTCTAGAGATGAAGTATCAACTACTCAC
Feo238	ATACCCGGGCTTTTTCGGTGAATTTATGG
FeoC.M35A.for	GAAGATGGCATCGATGCGGCGCTCGCGGTATGGATTAA
FeoC.M35A.rev	TTAATCCATACCGCGAGCGCCGCATCGATGCCATCTTC
For2	TGTAAAACGACGGCCAGT
Rev2	GGAAACAGCTATGACCATG

CONSTRUCTION OF PLASMIDS

1. pKT25FeoB272-D72A: The mutated form of FeoB was amplified from Vcfeo D72A plasmid acquired from Michelle Walters using the primer pair Feo227B and Feo238. The PCR product was then cut with SmaI and XbaI and ligated into pWKS30. The FeoB272-D72A fragment was removed from pWKS30 using SmaI and XbaI and ligated into pKT25, that had been digested with SmaI and XbaI.

2. pUT18CFeoC–M35A: The pUT18CFeoC plasmid was amplified using FeoC.M35A.for and FeoC.M35A.rev to introduce the M35A mutation according to the QuikChange manufacturer's protocol(115). The PCR product was then digested with DpnI to degrade the template.

3. pET16bFeoA: FeoA was amplified from pFeo101 using the primers FeoA.Nde and FeoA.Bam.rev. The PCR product was ligated into pBluescript, which had been digested using SmaI. pBluescript and FeoA and were digested using NdeI and BamHI. FeoA was ligated into pET16b, digested with NdeI and BamHI. pET16b contains a deca-His tag on the N terminal end along with a T7 promoter.

B-GALACTOSIDASE ASSAYS

A single colony from a plate was used to inoculate a culture of LB broth containing IPTG, ampicillin, and kanamycin. After 24 hours of growth at 30°C with aeration, β-galactosidase assays were performed as described by Miller (74).

PURIFICATION OF FEO A AND FEOC

To purify FeoC, BL21 (DE3) carrying pFeo128 was grown overnight at 30°C in LB plus ampicillin and then sub-cultured 1:100 into 100 mL of LB plus ampicillin. The cells were grown at 30°C to an OD_{650} of 0.5. IPTG was then added to a final concentration of 0.5 mM and growth continued for 1 to 2 hours. Initial experiments indicated that His-tagged FeoC was insoluble in cultures grown under these conditions, so it was purified from the pellet fraction. The culture was then centrifuged (Rotor-Sorvall SS-34 for 10 minutes at 10,000 rpm), and the pellet was resuspended in 4 mLs of 20 mM Tris-HCl pH 8.0. The cells were disrupted using sonication, centrifuged, and the pellet was resuspended in 3 mLs of isolation buffer (2M urea, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 2% Triton X-100). The pellet containing FeoC was sonicated, centrifuged, and resuspended in isolation buffer a total of three times. The final pellet was resuspended in 5 mLs of binding buffer (6M guanidine HCl, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 20 mM Imidazole). This was stirred for one hour at room temperature and centrifuged for 15 minutes at 15,000 rpm at 4°C. The supernatant was filtered through a 0.45 µm syringe filter (Acrodisc by Pallo) and loaded onto an AKTA FPLC (GE Healthcare Life Sciences). A 1 mL HisTrap HP nickel column (GE Healthcare) was used. A gradient from 0 to 100% refolding buffer (20 mM Tris-HCl pH 8.0, 0.5M NaCl and 20 mM imidazole) was added over 20 mL to refold FeoC while on the column. The column was washed with 5 mL refolding buffer and then FeoC was eluted in a 5 mL gradient from 0 to 100% elution buffer (20 mM Tris-HCl pH 8.0, 0.5 M NaCl, and 0.5M imidazole). FPLC fractions were stored at -20° C. Protein concentration from FPLC fractions likely to contain FeoA or FeoC was determined according to the protocol developed by Bradford.

BL21 (DE3) cells carrying pET16bFeoA were used to produce FeoA. The same protocol was used to purify FeoA except for the following changes: 200 mL of culture was induced with IPTG at a concentration of 1 mM.

A SDS 15% polyacrylamide gel was loaded with either FeoA or FeoC with 5 µL of purified protein and 10 µL of SDS-PAGE sample buffer to assess the purity of the fraction. After, electrophoresis, the gel was stained with Coomassie Blue.

ANTIBODY PRODUCTION

Purified FeoA (~1.2 mg) and FeoC (~1 mg) were sent to Cocalico Biologicals Inc. for antibody production. Two rabbits were immunized with a total of 5 injections (FeoA) or 6 injections (FeoC). The rabbits were exsanguinated after the final immunization.

WESTERNS FOR EVALUATING FEOA AND FEOC ANTIBODY ACTIVITY

To evaluate the antibody activity of FeoA and FeoC, samples were prepared from cells were grown in the following manner. DH5α cells containing either pET25b or pFeo101 were grown overnight at 30°C and sub-cultured 1:100 into 5 mL LB and antibiotics. The cells were grown to OD_{650}= 0.6. 1 mL of cells was centrifuged and the pellet was resuspended in 200 µl of SDS-PAGE sample buffer. BL21 (DE3) cells containing either pET25b or pET16bFeoA were grown overnight at 30°C and then diluted 1:100 into fresh media and grown until the OD_{650} reached 0.5-0.8. The cells were then induced with IPTG at a final concentration of 1mM for three hours. Equal numbers of cells (OD_{650}=1) were centrifuged and the pellet was resuspended in 200 µL of SDS-PAGE sample buffer. Samples were boiled for 3 minutes and loaded onto a SDS- 15% polyacrylamide gel. Samples of purified FeoC were prepared by mixing 5 µL of fraction C1 was mixed with 10 µL of SDS-PAGE sample buffer. After electrophoresis, one gel was stained with Coomassie Blue stain and proteins from an identically loaded gel were

transferred to a 0.45 μM nitrocellulose membrane. After blocking the membrane overnight in 5% powdered milk in Tris-buffered saline (TBS)-Tween (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20), the membrane was blotted with various dilutions of the appropriate serum. The membrane was then washed three times for 5 minutes in TBS-Tween and incubated with a goat anti-rabbit IgG HRP-labeled secondary antibody (Bio-Rad) at a 1:10,000 dilution in 5% milk (in TBS-Tween). The signal was detected by developing the blot using the Pierce ECL-detection kit (Thermo Fisher Scientific, Inc).

III. RESULTS AND DISCUSSION

A RESIDUE IN THE SWITCH REGION OF FEOB IS NOT NEEDED FOR INTERACTION WITH FEOC

G protein domains have long been known to regulate the function of diverse cellular processes in eukaryotes(36). G proteins have more recently been found to be present in prokaryotes where they also serve a regulatory function(15, 27, 119). In vitro studies have shown that FeoB has homology to known G proteins in eukaryotes and prokaryotes and that FeoB is a G protein(70). Studies by Marlovitz *et al.*(70) on the N terminal portion of FeoB showed that this protein hydrolyzed GTP as a GTPase (70). However, GTP hydrolysis by purified FeoB was slowed compared to other G proteins as discussed in the introduction(70). As the rate of GTP hydrolysis of FeoB is slow, it is probable that a protein may modulate FeoB to increase the rate of hydrolysis or to facilitate the binding of GDP. A potential candidate to modulate FeoB is FeoC, as it is part of the Feo system and has been shown to interact with FeoB.

The switch regions of G proteins are responsible for changing the conformation of the G protein when GTP is bound, allowing the G protein domain to interact with downstream effectors(113). To investigate the function of the switch region, Eng *et al.*(31) generated mutations in the switch region of the G protein, and the GTP binding affinity and hydrolysis rates of the mutant proteins were measured. FeoB containing a D73A mutation in switch II bound and hydrolyzed GTP with near wild-type affinity, but was non-functional for iron transport in vivo(31). These data suggest that GTP binding and hydrolysis by the G protein domain is not sufficient for FeoB activity(31).

The corresponding mutation was made in *V. cholerae* to test whether a mutation in the switch region of the G protein domain affected FeoB function in this organism. In a previous experiment, the *V. cholerae* FeoB D72A mutation prevented Feo-mediated iron acquisition in SM193w. Also a previous experiment showed that FeoC interacts with the G protein domain of FeoB(49, 54). To see if the FeoB D72A mutation interrupted the interaction with FeoC, the mutated FeoB and wild-type FeoC were tested for interaction using the bacterial adenylate cyclase two hybrid (BACTH) system in order to gain more insight into the function of FeoC.

The BACTH system is based on the secreted adenylate cyclase of *B. pertussis*, as described previously. This process allows for screening for interactions between proteins. If two proteins interact, then the bacteria will turn blue on media containing X-gal and display increased activity in a beta-galactosidase assay. When grown on media containing X-gal, the bacteria containing the first 272 amino acids of FeoB (FeoB272) and FeoC interact and display adenylate cyclase activity as evidenced by the blue color of the colonies, indicating the presence of β-galactosidase. Bacteria containing various combinations of either the vector alone or the vector with either FeoB272 or FeoC did not show adenylate cyclase activity and the colonies were white.

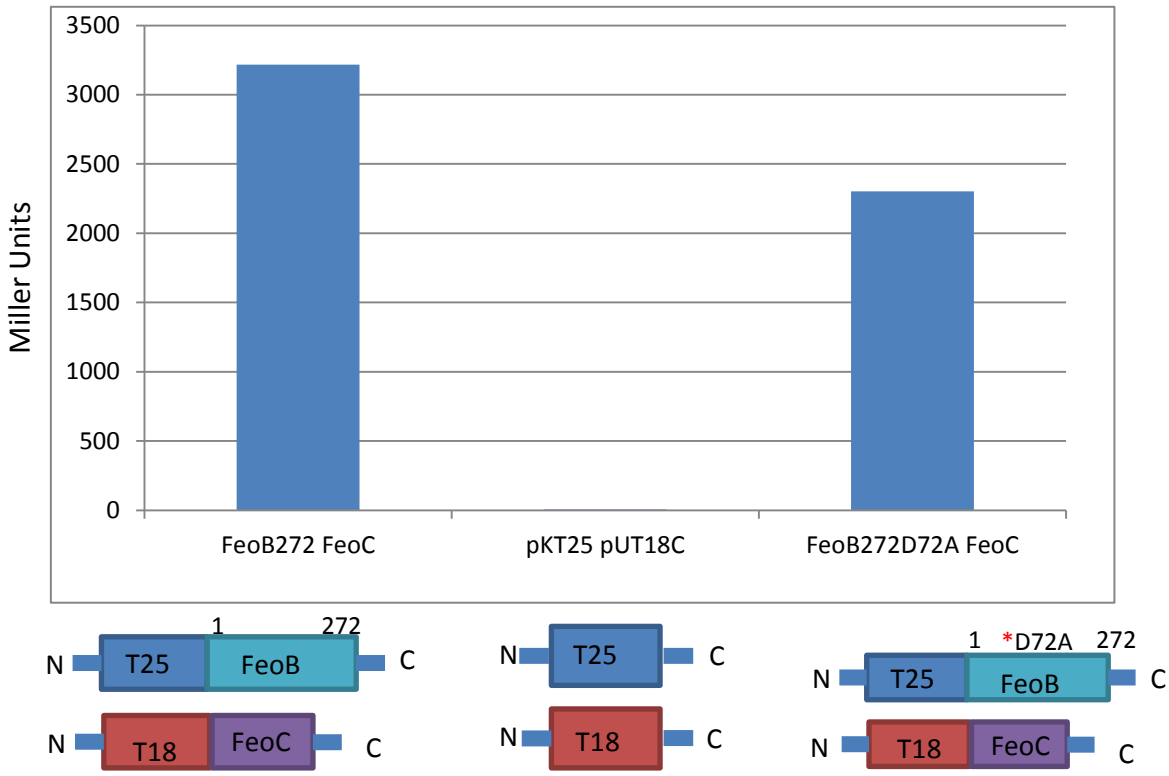


Figure 8. A mutation in the switch region of FeoB does not interrupt the interaction of FeoB and FeoC.

The FeoB switch region mutation D72A was introduced into FeoB fused to the T25 fragment of the BACTH system plasmid and tested for its ability to interact with FeoC fused to the T18 fragment. BTH101 was transformed with both plasmids and grown to OD_{650}=1. β-galactosidase activity was measured (Miller Units) as an indication of interaction between fused proteins. Wild-type FeoB272 fused to T25 and FeoC fused to T18 were included as a positive control, and empty vectors were the negative control. This figure represents the results of a single experiment.

As revealed by the bacterial two hybrid system in figure 8, the mutation in the switch region of *V. cholerae* FeoB did not affect the interaction with FeoC in this system in this single experiment. This might mean that FeoC does not modulate the activity of FeoB, as the switch regions are responsible for changing the arrangement of the G protein domain in response to GTP binding and are good candidates for modulation. However, FeoC may interact in this region primarily with different amino acids, it may interact in

another region of FeoB, or may act to regulate the function of FeoB in a different manner. Further work is needed to better understand the FeoB switch region's role in the Feo system.

A CONSERVED RESIDUE IN FEOC ABOLISHED THE INTERACTION WITH FEOB

FeoC is the least conserved protein between *V. cholerae* and *E. coli*, showing only 11% identity. An examination of the amino acid sequence of *V. cholerae* FeoC compared to the sequence from *E .coli* revealed a small region of conservation. This region represents most of the conserved residues between *V. cholerae* and *E. coli* in FeoC and in the middle of this conserved region is a methionine. The role of this conserved region in interaction was tested, and methionine 35 was focused on as it was in the center of the conserved region.

```
V. cholerae FeoC -MILNELKAAIESKNGATRQELARRFALSEDGIDAMLAVW
E. coli FeoC     MASLIQVRDLLALRGRMEAAQISQTLNTPQPMINAMLQQL

V. cholerae FeoC IKKGVLSRQQYINAEDEVVRVRYVMNQVGSLAVNVTM--- 76
E. coli FeoC     ESMGKAVRIQ--EEPDGCLSGSCKSCPEGKACLREWWALR 78
```

Figure 9. Alignment of FeoC from *V. cholerae* and *E. coli*.

Blue highlighted regions indicate conserved residues. The pink highlighted residue indicates methionine 35. The alignment was generated using ClustalW (European Bioinformatics Institute).

A point mutation was made changing the methionine at position 35 to an alanine residue and the point mutant was tested using the bacterial two hybrid system described above. Bacteria containing FeoB272 and FeoC with the M35A mutation were white and did not show adenylate cyclase activity as shown in figure 10.

1 – pKT25FeoB272 pUT18CFeoC
2 – pKT25 pUT18C
3 – pKT25FeoB272 pUT18C
4 – pKT25 pUT18CFeoC
5 – pKT25 pUT18CFeoCM35A
6 – pKT25FeoB272 pUT18CFeoCM35A

Figure 10.A mutation in FeoC abolishes the interaction with FeoB.

A mutation in the conserved region of FeoC, M35A, was introduced to FeoC fused to the T18 fragment of the BACTH system plasmid and tested for its ability to interact with FeoB272 fused to the T25 fragment. BTH101 was transformed with both plasmids and grown overnight on LB containing X-gal. β-galactosidase activity was measured by the blue colony appearance as indication of interaction between the fused proteins. In each area, the BTH101 cells contained the indicated plasmids at the bottom of the figure.

The white colony appearance and the loss of adenylate cyclase activity indicates that there was no measurable interaction between FeoB and FeoC when FeoC contained the M35A mutation. The loss of interaction suggests that the methionine in the 35th position is important for the interaction between FeoC and FeoB272. Bacteria containing FeoCM35A and FeoB272 were also tested using a beta-galactosidase assay to verify the results seen on the media containing X-gal. This assay confirmed that FeoCM35A no longer interacted with FeoB 272 as the Miller units were similar to those of the plasmids without any insert.

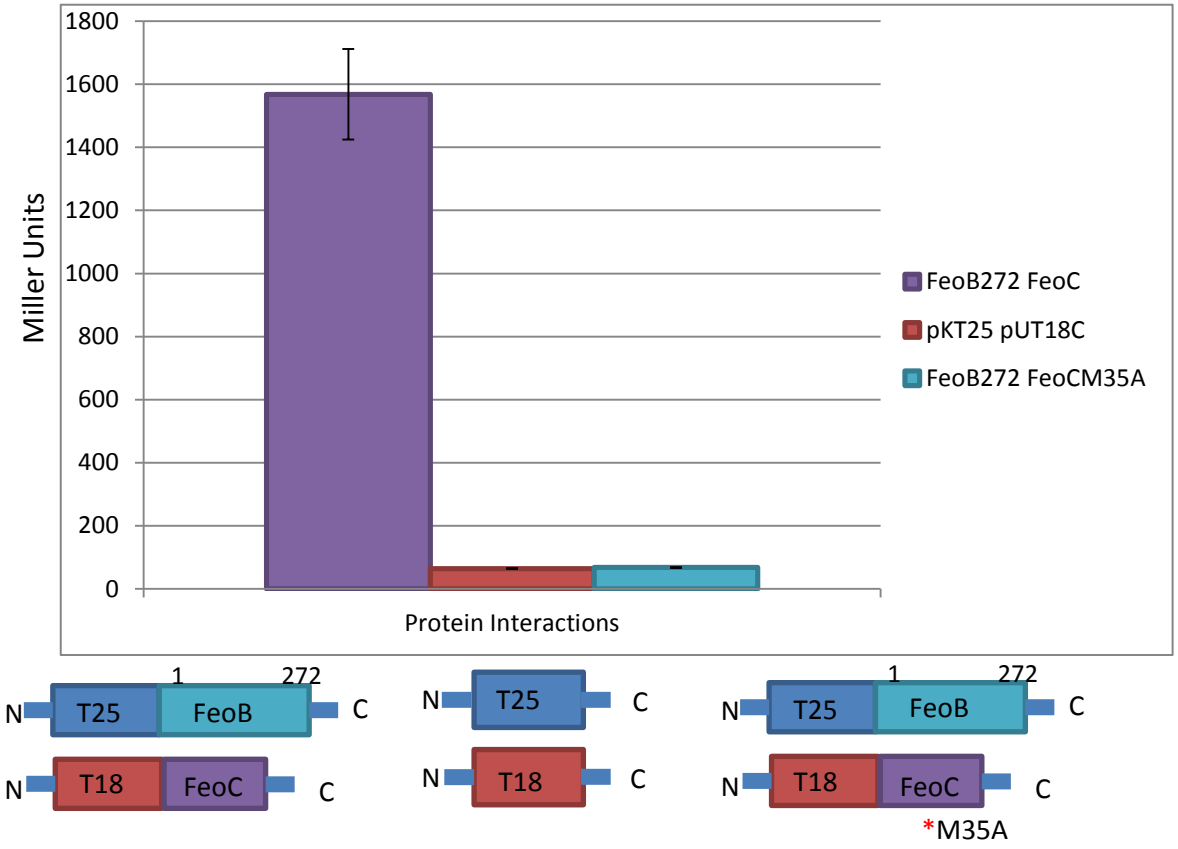


Figure 11. A mutation in FeoC abolishes the interaction between FeoB and FeoC.

A mutation in a conserved region of FeoC , M35A, was introduced into FeoC fused to the T18 fragment of the BACTH system plasmid and tested for its ability to interact with FeoB272 fused to the T25 fragment. BTH101 was transformed with both plasmids and grown to OD_{650}=1. β-galactosidase activity was measured (Miller Units) as an indication of interaction between fused proteins. Wild-type FeoB272 fused to T25 and FeoC fused to T18 were included as a positive control, and empty vectors were the negative control. This assay was done in triplicate. Error bars indicate one standard deviation.

One possible explanation for the white colonies on X-gal plates and the lowered β-galactosidase activity is that this conserved methionine 35 is important for FeoC contacting FeoB. There is evidence from *Klebsiella* species that FeoC binds to FeoB as evidenced by a crystal structure with FeoC bound to FeoB; however, the function of this binding has not been discovered(54). Another explanation for the loss of interaction between FeoB272 and FeoCM35A is that methionine 35 is important structurally. It is possible that when changed from methionine to alanine, the protein is misfolded and is degraded. Further work is being performed to examine whether FeoCM35A levels are comparable to wild-type FeoC levels.

ANTIBODY PRODUCTION FOR FEOA AND FEOC

Antibodies for the Feo system proteins in *V. cholerae* would be useful in assays that could provide a more detailed picture of the interaction between these proteins. To commence antibody production, *feoA* was cloned into the expression vector pET16b which contains an N-terminal His-tag, the bacteriophage T7 expression system, including the promoter, transcription start site, and the terminator of T7, for high levels of transcription and translation, as well as the lac operator region. Using pET16b allows for most of the bacterial cell's resources to be devoted to the expression of the target gene,

with the some expressed proteins encompassing more than 50% of the cell's total protein hours after induction using this system(87).

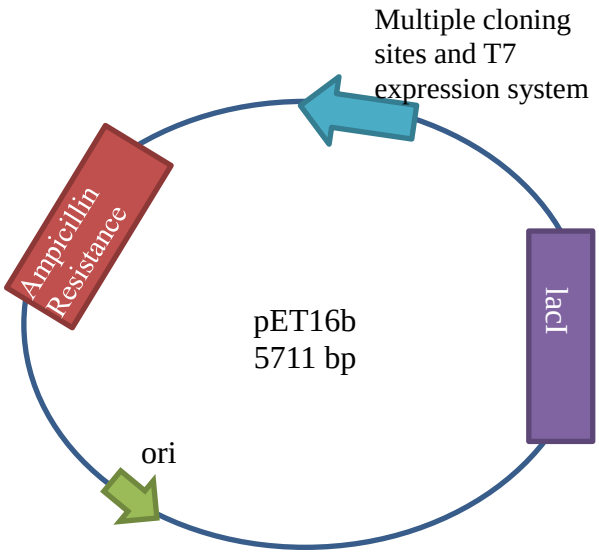
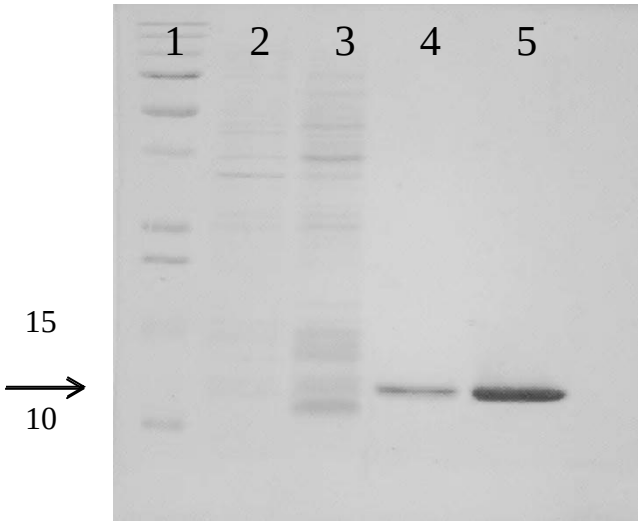


Figure 12. Plasmid map of pET16b with landmarks noted

The level of induction can be controlled by the concentration of IPTG used, as the host strain, BL21 (DE3), has the T7 RNA polymerase under the control of *lac* promoter. As IPTG is a molecular mimic of allolactose and binds to the lac repressor, it is able to induce the transcription of the T7 RNA Polymerase. The cells were induced using IPTG, and it was determined that FeoA was in the insoluble fraction of the cellular lysate. It is most likely that overexpressing FeoA caused it to misfold and aggregate into inclusion bodies.

This suggested that denaturation and on-column refolding of FeoA would be the best course of action. The cells were sonicated, filtered, solubilized in a denaturing solution as described in the methods section(48). The protocol used high concentrations

of urea and guanidine hydrochloride to denature and solubilize FeoA. Denatured FeoA was passed through a nickel column using an FPLC to separate the His-tagged FeoA protein from the other cellular proteins. Following the binding of the denatured FeoA onto the column, on column refolding was accomplished by decreasing the concentration of urea and guanidine hydrochloride. Finally, the refolded FeoA protein was eluted using a Tris buffer containing a high concentration of imidazole, which efficiently competes for binding to nickel and causes the His-tagged FeoA to elute from the column. In Figure 13, the second lane contains DH5α cells with the pET16b vector alone as a negative control that should not show a significant band in the area expected for FeoA. The third lane contains DH5α cells expressing pFeo128 as a positive control that should show a FeoA band. Lanes 4 and 5 contain fractions from the FPLC purification and they both contain FeoC at observable levels.



Lane 1 Molecular Weight Marker
Lane 2 pET16b
Lane 3 pFeo128
Lane 4 B12
Lane 5 C1

Figure 13. Coomassie-stained SDS-PAGE gel of FPLC purification of FeoA.

Arrow indicates expected FeoA position. In lane 1 is the molecular weight marker Presicion plus protein dual color standard (Bio-rad). The 10 kDa and 15 kDa positions are labeled. In lane 2 is DH5α containing the empty pET16b vector as a negative control. In lane 3 is DH5α containing the pFeo128 as a positive control. In lanes 4 and 5 are the FPLC fractions B12 and C1 containing purified FeoA.

The fractions likely to contain the protein based on UV absorption spectra generated by FPLC system (see Fig. 13) were analyzed and protein concentration was estimated using the Bradford assay. The amount of FeoA from two fractions was approximately 1.2 mg (Fig. 13 lanes 4 and 5). This was then sent to Cocalico Biologicals, Inc. for antibody production using two rabbits. A total of 5 immunizations against FeoA were performed in each rabbit.

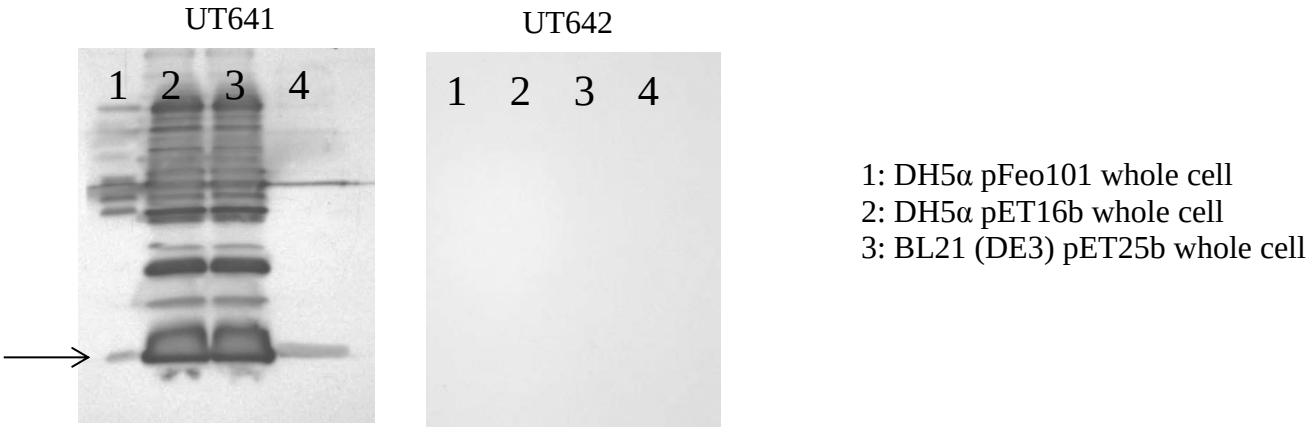


Figure 14. Pre-immune serum activity for FeoA.

Arrow indicates expected FeoA position. Serum from rabbits UT641 and UT642. In lane 1 is DH5α containing pFeo101. In lane 2 is DH5α containing pET16b. In lane 3 is BL21 (DE3) containing pET25b. In lane 4 is BL21 (DE3) containing pET16bFeoA.

The pre-immune serum from one of the rabbits (UT641) showed a high level of reactivity to *E. coli* proteins. The western blot of the serum activity of UT641 in Figure

14 indicates that further purification will be needed to use that serum to isolate the antibodies specific for *V. cholerae* FeoA. The pre-immune serum from UT642 showed no activity however. In figure 13, the lanes containing the purified FeoA show only the band for FeoA, but the western blot of the pre-immune serum from rabbit UT641 shows multiple bands in each lane. The band of interest is indicated by the arrow in the figure, although all lanes show a band at this size. This indicates that this rabbit most likely encountered *E. coli* previously. It is of note that FeoA and FeoC bands are visualized as the same size. The serum from rabbit UT642 showed little background and had high specificity for the recombinant FeoA. The western blot in Figure 15 shows that the antibody from UT642 binds to FeoA isolated from BL21 DE3 cells containing pET16bFeoA with high affinity. Figure 15 also shows that the serum did not react to the cells containing only the vector as evidenced by the lack of band in lane 3. However, the serum did not react with pFeo101 which is puzzling, but this may be a result of poor expression in DH5α. Serum from rabbit UT641 showed multiple bands in each lane.

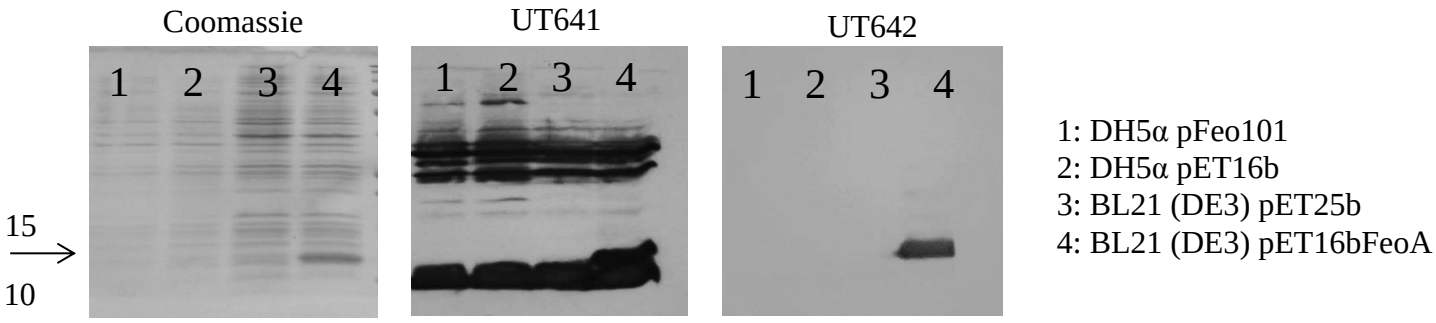
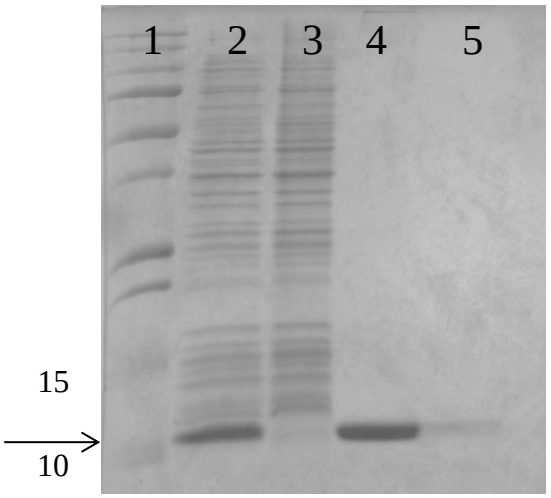


Figure 15. Antibody Activity against FeoA.

Arrow indicates expected position of FeoA. Serum is from rabbits UT641 and UT642 diluted 1:200 in 5% powdered milk and TBS-Tween. In lane 1 is DH5α containing pFeo101 as a positive control. In lane 2 is DH5α containing pET16b alone as a negative control. In lane 3 is BL21 (DE3) containing pET25b as a negative control. In lane 4 is BL21 (DE3) containing pET16bFeoA as a positive control.

Production of FeoC antibodies proceeded in a similar manner to that of FeoA. *feoC* was cloned into the expression vector pET25b which contains an N-terminal His-tag and the BL21 (DE3) cells were transformed with the pFeo128 plasmid. The cells were induced using IPTG and then passed over a nickel column to separate the His-tagged FeoC protein using an FPLC. Similar to FeoA, FeoC was in the insoluble fraction and a similar protocol was used to purify it.



1 Molecular Weight Marker
2 pFeo128 whole cell
3 BL21 (DE3) pET25b
4 FPLC fraction C1
5 FPLC fraction C2

Figure 16. Coomassie-stained SDS-PAGE gel of FPLC purification of FeoC.

Arrow indicates expected position of FeoC. In lane 1 is the molecular weight marker Presicion plus protein dual color standard (Bio-rad). The 10 kDa and 15 kDa positions are labeled. In lane 2 is DH5α containing the pFeo128 as a positive control. In lane 3 is

BL21 (DE3) containing pET25b as a negative control. In lanes 4 and 5 are the FPLC fractions C1 and C2 containing purified FeoC.

The fraction most likely to contain FeoC protein was analyzed using the Bradford assay and approximately 1 mg of protein was present (see Fig. 16 lanes 4 and 5). Figure 17 shows the pre-immune serum from the rabbits that were used to generate FeoC antibodies. Lanes 2 and 3 are used as controls. pFeo128 is the plasmid used to express FeoC in BL21 (DE3) cells. pET25b was used to create pFeo128 and is the vector alone. The FPLC fractions containing FeoC was sent to Cocalico Biologicals, Inc. for antibody production in two rabbits. A total of 6 immunizations against FeoC were performed in each rabbit. The western blot in Figure 17 shows that rabbit serum (UT639 and UT640) showed a high level of reactivity to *E. coli* proteins, so it was likely that the rabbits had an E. coli infection prior to immunization.

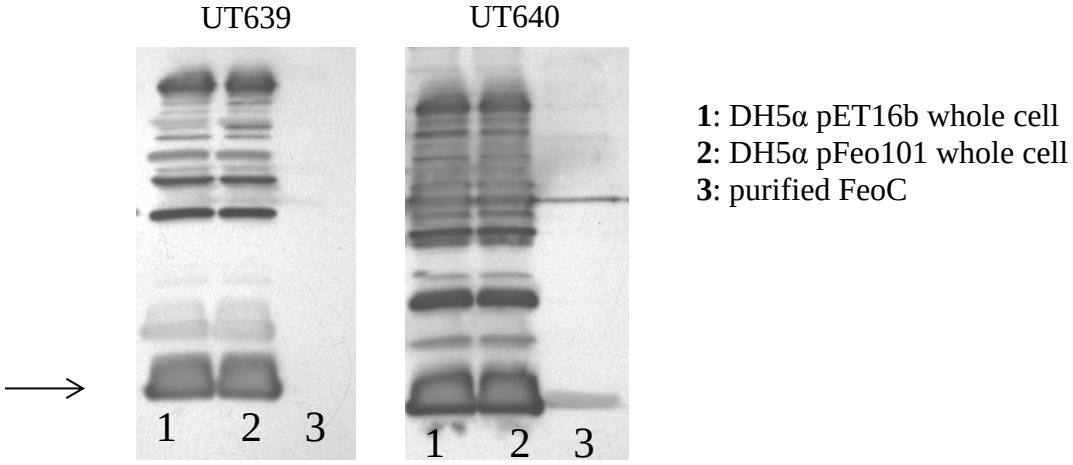


Figure 17. Pre-immune serum for FeoC activity.

Arrow indicates expected position of FeoC. Serum from rabbits UT639 and UT640. Serum was diluted 1:200 in 5% powdered milk and TBS-Tween. In lane 1 is DH5α containing pET16b alone. In lane 2 is DH5α containing pFeo101. In lane 3 is purified FeoC from the FPLC fraction C1.

As evidenced by the western blot in Figure 18, the antisera reacted similarly to cells expressing the Feo system and to cells that contained the expression vector only (Figure 18 Lanes 2 and 1 respectively). The bands in lanes 1 and 2 indicates that the rabbit is reacting with endogenous *E. coli* proteins found in the DH5α strain, and that they most likely encountered *E. coli* previously as evidenced by the activity of the pre-immune serum. However, the antibodies also react strongly with purified *V. cholerae* FeoC, indicating that antibodies do bind to *V. cholerae* FeoC strongly. Further purification will be needed to isolate the antibodies reacting to *V. cholerae* FeoC.

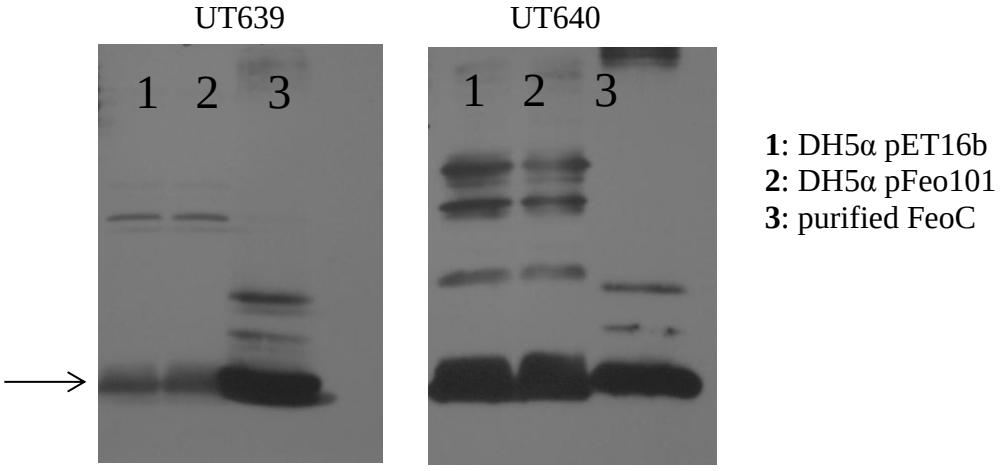


Figure 18. Activity of antibody against FeoC.

Arrow indicates expected position of FeoC. Serum from rabbits UT639 and UT640. Serum was diluted 1:1000 into 5% powdered milk and TBS-Tween. Lane 1 contains DH5α containing the pET16b vector as a negative control. In lane 2 is DH5α containing pFeo101 as a positive control. In lane 3 is purified FeoC from the FPLC fraction C1.

Conclusions

In light of the experiments performed in this work, it seems that certain amino acid residues are important for the interaction between FeoB and FeoC. A residue of FeoB reputed to be part of the switch region and responsible for nucleotide-dependent rearrangement in the G protein domain was not necessary for interaction between FeoB and FeoC as assayed in the BACTH system. However, a conserved residue of FeoC was important for this interaction. Further examination of the important residues in each protein may reveal the function of FeoC and whether it regulates the function of FeoB. The antibodies produced in the course of this study will expedite the study of the proteins in the Feo system. These antibodies can be used to probe protein-protein interactions in cross-linking studies, as well as other experiments to understand the Feo system. However, many questions remain about the Feo system. It remains to be determined whether FeoB functions as monomer, dimer, or trimer. The function of FeoA and FeoC still needs to be determined. It also needs to be determined whether FeoB is modulated by any other protein, such as FeoC.

Further characterizing this system may allow understanding of the ferrous iron transport in *V. cholerae* as well as many other organisms. Feo is conserved across bacteria and archaea, and seems to be important for iron-acquisition while in the micro-

aerophilic, reducing conditions of the human host. Understanding this system may lead to better treatment against pathogens such as *V. cholerae*. This study has helped identify amino acid residues that are important for interaction between proteins of the Feo system as well as provide tools for further study of this conserved system.

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