ROLE OF CUSF IN COPPER HOMEOSTASIS IN *ESCHERICHIA COLI*

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Abstract

Escherichia coli cells have developed at least two systems that maintain copper homeostasis as this metal is toxic to the cells when in excess. The copper efflux (*cue*) system is the primary copper resistance system and functions in anaerobic conditions and under moderate copper concentrations. The copper sensing (*cus*) system gets activated under anaerobic conditions and higher copper concentrations. The Cus system includes a tripartite CBA pump, and an additional periplasmic protein, CusF. Although CusF contributes to copper resistance of the cell, experiments have shown that this protein is not necessary. This project examines the role of CusF in the Cus system in an attempt to understand how the system works as a whole. Growth assays and functional analysis show that CusF is not absolutely required for copper resistance and that there are functional effects when the two methionine residues (M47 and M49) that contribute to copper binding by the protein are altered. Crosslinking experiments show that CusF interacts with CusA and CusB. This may be evidence that supports the "switch" model of copper efflux through the CBA pump.

Introduction

Copper is an essential micronutrient in most living organisms. It is required by many metalloenzymes involved in electron transport, redox, and other important reactions (2). Examples of such enzymes include terminal oxidases, monooxygenases, and diooxygenases. Copper is also required for the transport of electrons in several photosynthetic and respiratory pathways (10). The importance of copper in redox reactions arises from its ability to transit between its oxidative states, Cu(I) and Cu(II) depending on the surrounding chemical environment (2). Although copper ions are useful to the cell, they can also be toxic even at low concentrations, even as low as 10uM (2). Excess copper competes with other metals for their binding sites in proteins. For example Cu(I) displaces iron from iron-sulfur clusters of various enzymes such as isopropylmalate dehydratase, which is responsible for the biosynthesis of amino acids (6). With increased cytoplasmic copper levels, other iron-sulfur cluster enzymes such as fumarase A are inactivated (6). Free copper ions can also form hydroxyl radicals that may damage cellular components including proteins, lipids, and nucleic acids (8). All these interferences with regular cell activities eventually lead to death of the cell. Thus, copper has been widely used as bactericides, algaecides, fungicides, veterinary food additives, anti-fungal and anti-microbrial agricultural applications, anti-fouling paint addictives, and for preservation of natural and manmade materials (2, 11).

The wide use of copper to eliminate microorganisms has caused microbes to evolve mechanisms that keep intracellular copper at low enough levels that are not toxic to the cell but can still be useful for cell functions. In *Escherichia coli*, at least two systems have been discovered to maintain copper homeostasis: The *Cue* system (Cu efflux) and the *Cus* system (Cu sensing).   The *Cue* system, which is the primary copper homeostasis system in *E. coli*, consists of a copper-responsive metalloregulatory protein, CueR which up-regulates the expression of *copA* and *cueO* genes (11). CopA is a P-type ATPase which pumps excess copper out of the cytoplasm while CueO is a multicopper oxidase that protects periplasmic enzymes from copper-mediated damage (4). Previous studies suggest that CueO, converts periplasmic Cu(I) to the less toxic Cu(II) under aerobic conditions. Under anaerobic conditions, CueO loses its activity (11) thus the periplasmic concentration of Cu(I) increases which triggers the activation of the *cus* system (5).

The *cus* system, a CBA-type transporter, includes the *cusRS* and *cusCFBA* genes. The *cusRS* genes encode for a copper-responsive two-component system comprising of CusR a response regulator and CusS a histidine kinase (6). It is believed that CusS senses copper stress in the periplasm and relays the information to CusR which then acts as a transcription factor that activates the transcription of the *cusCFBA* genes. These genes code for CusA, CusB, and CusC which make up a tripartite efflux pump that spans both the inner and outer membranes of the *E. coli* cell (6). CusA, a member of the resistance-nodulation-division (RND) family, is an inner membrane substrate antiporter predicted to be 115-kDa (10). Like other RND proteins, it utilizes the proton motive force for efflux of substrates and forms trimers. Site-directed mutagenesis show that conserved residues M573, M623, and M672 in CusA are necessary for copper resistance and are believed to be in the metal binding site (4). CusB is a member of the membrane fusion protein (MFP) family. It is an adapter protein that connects CusA and CusC, an outer membrane factor (OMF). The metal binding site of CusB is shown to consist of three methionines residues - M21, M36, and M38 (6). Deletion of *cusA* and *cusB* genes led to complete loss of copper resistance in *E. coli*. While the deletion of *cusC* had less effects on copper resistance (4). Another component of the *cus* system is CusF. It is a periplasmic protein about 12-kDa and is proposed to bind Cu(I) and Ag (I). Three residues M47, M49, and H36 have been discovered to co-ordinate metal in CusF (9). W44 is hypothesized to shield bound metal from solvent. Deletion of the CusF genes led to diminished but not complete loss of copper resistance suggesting that it is not an essential component of the *cus* system (6). Most CBA transporters with an RND transporter do not have CusF homologs and so questions have arisen regarding the role of this protein in metal resistance (6). One possible role is that it is a metallochaperone that can transfer metal to CusB (6). Alternatively it could be a metal-activated regulator that stimulates export activity of the efflux pump (8).

The aim of this project is to understand the role of CusF in metal resistance in *E coli*. This will help answer questions on how the CusCFBA system exports metal from the cell. Two mechanisms have been proposed: The funnel model and the switch model (7). The funnel model suggests metal is transferred from the metal binding site of one protein to that of the next. In this model CusF binds periplasmic cu(I), transfers the metal to CusB, which then transfers metal to CusA (7). The switch model predicts that when CusB binds metal, it switches conformation to the "on" conformation. In this model, metal is not transferred from CusB to CusA. However, CusF may be able to transfer metal to both CusA and CusB. This project will determine if CusF interacts with CusA and CusB through cross-linking experiments. The importance of the metal binding residues of CusF to copper resistance will also be investigated. This will be accomplished through functional analysis and growth assays of mutants of these residues in relation to the wild-type.

Materials and Methods

Functional Analysis

Cloning- CusF mutants were cloned into pBad24, an L-arabinose inducible promoter. The variants include, Wild-type, empty vector, W44A, W44M, M47C, M47I, M47K, M49C, M49I, M49K, H36A, M47K/H36A, and M47I/M49I. See table 1 for complete details of vectors and strains.

Transformation- 1 uL of pBAD plasmid containing *cusF* genes/mutants was added to 200 uL of EC933 cells and incubated on ice for 15 minutes. The cells were heat-shocked at 42˚C for 90 seconds and then placed on ice for 5 minutes. 300 uL of sterile Luria-Broth (LB) media was added and then incubated in a shaker at 37˚C for one hour. 150 uL of cells was spread on plates containing ampicillin (100 ug/mL) and incubated at 37˚C overnight (~ 15 hours).

Overnights- One colony of each CusF mutant was inoculated into 5 mL of LB containing amp100 and 0.02% glucose. The cells were incubated in a 37˚C shaker overnight (15 hours).

Subculture- 100 uL of overnight culture was inoculated into 5 mL LB containing Amp100 and incubated in a shaker at 37˚C until the optical density at 600 nm reached ~ 1. The cells were then induced with L-arabinose to a final concentration of 0.2 % (50 uL of 20 % L-arabinose added to 5 mL cells). The cells were allowed to shake for 20 minutes at 30˚C.

Plating- The cells were streaked with inoculating loops on copper-containing LB plates (0 mM, 1 mM, 1.25 mM, 1.375 mM, 1.5 mM, 1.625 mM, 1.75 mM, 2.0 mM, 2.25 mM, 2.5 mM, 2.75 mM, and 3.0 mM) containing amp100 and 0.2 % L-arabinose. The plates were incubated overnight at 30˚C.

Survival Rates

Overnight cultures of *cusF* mutants were made and sub-cultured as in functional analysis.

Normalization – The OD600 of each mutant was taken. Then the cells were normalized into 2 mL with sterile LB so that each strain had the same number of cells. 1 OD600 ~ 5 *10^8 cells. A five-fold and six-fold series dilution was done so that total number of cells in 1 mL were ~5,000 and 50,000 respectively.

Plating- 100 uL of cells were spread on copper plates (1.5 mM, 1.75 mM, and mM) containing Amp100 and L-arabinose. The plates were incubated overnight at 30˚C and then the number of colonies in each plate was counted.

Growth Assay

Overnights of *cusF* mutants were made as in functional analysis. 200 uL of overnight culture was inoculated into 8 mL LB containing amp100 and allowed to shake at 37˚C until the cells reached an OD of 1.2 (4 hours). The cells were normalized into 10 mL with sterile LB-amp100. 1 mL of normalized cells was innoculated into 10 mL of sterile LB containing different concentrations of $CuSO_4$ (0 mM, 0.5 mM, 1.0 mM, 1.5 mM, 1.75 mM, 2.0 mM, and 2.5 mM) , amp100 and 0.2 % L-arabinose in a 25 mL culture tube. The tubes were then placed in a shaker at 37˚C. The OD600 at different time points for each mutant in each concentration was measured.

Expression and Purification of CusA

BL21 *pcusA* M623C cells were restreaked from glycerol stock on LB plate containing amp100. The plate was incubated at 37˚C overnight. One colony from restreak was inoculated into 200 mL LB containing amp100 and incubated in 37˚C shaker overnight. 50 mL of overnight was sub-cultured into four flasks containing 1L of LB media plus amp100 each. The flasks were then allowed to shake at 170 rpm, 37˚C until the cells reached an OD600 of 1 (4 hours). Each flask was induced with 20 uL of 10mg.mL anhydrotetracycline (AHT) and allowed to shake at 30˚C for 4 hours 40 minutes. The cells were spun down at 5000 rpm, 4˚C for 30 minutes and then pellets were frozen at – 20˚C. On a different day, pellet (from 2L) was thawed on ice and resuspended into 50 mL of buffer W (100mM Tris, 150 mM NaCl, pH 8.0). Protease inhibitors, Apoprotinin (50 uL, mg/mL), Leupeptin (50 uL, mg/mL), Pepstatin (50 uL, 2 mg/mL), PMSF (50 uL, 100 mM), Lysozyme, and DNaseI were added and the allowed to mix for 30 minutes at 4˚C. The cells were French Pressed three times at 1000 psi and centrifuged in rotor SS-34 at 16000rpm, 4˚C for one hour. The supernatant was transferred to a new tube and ultracentrifuged at 40000rpm, 4˚C for one hour. The pellet was resuspended in 5 mL buffer A (100mM Tris, 150 mM NaCl, 100 mM sucrose, pH 8.0) and homogenized in a homogenizer. 35 mL of Buffer B (100mM Tris, 500mM NaCl, 100mM sucrose, 0.05% CYMAL-6, pH 8.0) was added to bring the volume up to 40 mL. 1.5 mL of 10 % CYMAL-6 (detergent) was added for a final concentration of 0.56 mM, and allowed to mix at 4˚C for one hour or until lysate became clear. Lysate was ran on a Strep-affinity column four times, washed using buffer B and eluted with Buffer B containing 2.5 mM desthiobiotin. The fractions were ran on 10 % SDS-PAGE gel and those containing CusA were pooled together, concentrated using 10000 MWCO concentrator, and stored at -20˚C.

Expression and Purification of CusB

BL21 *pcusB* M64C cells from glycerol stock were retreaked on LB- amp100 plates and incubated overnight at 37˚C. Four colonies were innoculated into four flasks containing 60mL of

LB plus amp100 and allowed to shake overnight at 37˚C. The following morning, the overnight cultured were diluted each into 1L of LB containing amp100 and placed in a 37˚C shaker until the OD600 reached 1 (3 hours). The cells were induced with 20 uL of AHT (10 mg/mL) and incubated in 30˚C shaker for 5 hours. The cells were then centrifuged at 6000 rpm, 4˚C for 10 minutes and the pellets frozen at -20˚C. On a different day, pellets were defrosted and 60 mL of Buffer W was added. Protease inhibitors, Apoprotinin (60 uL, mg/mL), Leupeptin (60 uL, mg/mL), Pepstatin (60 uL, 2 mg/mL), PMSF (60 uL, 100 mM), Lysozyme, and DNaseI were added and the allowed to mix for 30 minutes at 4˚C. The cells were French Pressed three times at 1000 psi. 0.06 g CHAPS was added to lysate and mixed for 10 minutes at 4˚C. It was then centrifuged in rotor SS-34 at 16000rpm, 4˚C for 35 minutes. Lysate was ran on a Strep-affinity column four times, washed using buffer W and eluted with Buffer W containing 2.5 mM desthiobiotin. The fractions were run on 12.5 % SDS-PAGE gels and fractions containing cusB were pooled together, and dialyzed three times in into 25 mL of 50 mM Tris pH 9.0 and then run on a monoQ. The fractions from the monoQ were run on an SDS-PAGE gel and those containing purified cusB were concentrated using a 7500 MWCO concentrator, and stored at -20˚C.

Expression and Purification of CusF

pBAD-*cusF* M49C and M47C were transformed into BL21 cells. One colony of each strain was innoculated into 50 mL of LB media containing amp100 and 0.02 % glucose. The cultures were incubated in a 37˚C shaker overnight. The following day, the overnights were subcultured into 1L of LB media containing amp100 and shaked at 37˚C until the cells reached an OD of 1.2 (~3hrs). They were then induced with 0.2% L-arabinose and allowed to shake at 30˚C for 5 hours. The cells were spun down at 5000 rpm for 30 mins and pellets frozen at -20˚C. On a different day, pellet (from 1L) was thawed on ice and resuspended into 50 mL of buffer W

(100mM Tris, 150 mM NaCl, pH 8.0). Protease inhibitors, Apoprotinin (50 uL, 2 mg/mL), Leupeptin (50 uL, 2 mg/mL), Pepstatin (50 uL, 2 mg/mL), PMSF (50 uL, 100 mM), Lysozyme, and DNaseI were added and the allowed to mix for 30 minutes at 4˚C. The cells were French Pressed three times at 1000 psi and centrifuged in rotor SS-34 at 16000rpm, 4˚C for one hour. The supernatant was frozen at -20˚C. The following day, the supernatant was thawed, spun down at 16500rpm for an hour. The supernatant was dialyzed first for 2 hours in 2 L and then overnight in 4 L of 60 mM Lactate (pH 3.5). The white precipitate from dialysis was spun down at 10000rpm for 10 minutes in KA1.5 rotor and then loaded onto a cation exchange column (16/10 HiPrep SP FF column on HPLC), which was equilibrated with 60mM lactate, 100 mM NaCl pH 3.5 and eluted with 60mM lactate, 500 mM NaCl pH 5. Fractions under a peak were ran on an 18% SDS-PAGE gel and stained with Coomassie dye. The fractions containing cusF were pooled together and concentrated in a 5000 MWCO concentrator to 4 mL. 4 mL of distilled water was added to drop the salt concentration and the sample was further concentrated to 1 mL. The concentrated sample was then loaded onto a size exclusion column (HiPrep Sephacryl S-100 High resolution column), which was equilibrated with 100 mM phosphate, 100 mM NaCl, pH 7, and eluted with the same buffer. Fractions under a peak were run on an 18 % SDS-PAGE gel and those containing purified CusF were concentrated and stored at -20˚C.

Bis(maleimido)ethane (BMOE) In-vitro Crosslinking

Sample preparation- The samples were CusA M623C (20 uM), cusB M36C (20 uM), and CusF M49C (20 uM). 5 uL of 5 mM Bond-Breaker TCEP solution (neutral pH) was added to 200 uL each of protein samples, and incubated at room temperature for 5 minutes. The TCEP was removed and the proteins buffer-exchanged using a desalting column. CusA was exchanged into

50 mM phosphate, 500 mM NaCl, 100mM sucrose, pH 6.5. CusB and CusF were exchanged into 50 mM phosphate, pH 6.5.

BMOE preparation- 2.2 mg BMOE was dissolved into 0.5 mL of dimethylfluoride (DMF) to according to manufacturer's instruction (13) to obtain a 20 mM solution. 12 uL of 20mM solution was added to 88 uL of DMF to a final concentration of 2.4 mM.

Crosslinking- 12 sterile Eppendorf tubes were used.

Tube 1: 40 uL cusA M623A, 50 uL buffer (50 mM phosphate, 500 mM NaCl, 100 mM sucrose, pH 6.5)

Tube 2: 40 uL cusA M623A, 70 uL buffer (50 mM phosphate, 500 mM NaCl, 100 mM sucrose, pH 6.5)

Tube 3: 40 uL cusB M36C, 50 uL buffer (50 mM phosphate, pH 6.5)

Tube 4: 40 uL cusB M36C, 70 uL buffer (50 mM phosphate, pH 6.5)

Tube 5: 40 uL cusF M49C, 50 uL buffer (50 mM phosphate, pH 6.5)

Tube 6: 40 uL cusF M49C, 70 uL buffer (50 mM phosphate, pH 6.5)

Tube 7: 40 uL cusA M623A, 40 uL cusB M36C, 30 uL buffer (50 mM phosphate, 500 mM NaCl, 100mM sucrose, pH 6.5)

Tube 8: 40 uL cusA M623A, 40 uL usB M36C, 50 uL buffer (50 mM phosphate, 500 mM NaCl, 100mM sucrose, pH 6.5)

Tube 9: 40 uL cusA M623A, 50 uL cusF M49C, 30 uL buffer (50 mM phosphate, 500 mM NaCl, 100mM sucrose, pH 6.5)

Tube 10: 40 uL cusA M623A, 50 uL cusF M49C, 50 uL buffer (50 mM phosphate, 500 mM NaCl, 100mM sucrose, pH 6.5)

Tube 11: 40 uL cusB M36C, 40 uL cusF M49C, 30 uL buffer (50 mM phosphate, pH 6.5)

Tube 12: 40 uL cusB M36C, 40 uL cusF M49C, 50 uL buffer (50 mM phosphate, pH 6.5)

20 uL of 2.4 mM BMOE was added to tubes 1, 3, 5, 7, 9, and 11. All the tubes were incubated at room temperature for an hour. The reaction was quenched with 40 uL of 100 mM DTT for 15 minutes. 37.5 uL of 4X loading dye was added to tubes and each tube was run on 7.5 % SDS-PAGE gels for Western Analysis and silver staining.

Disuccinimidyl Suberate (DSS) In-vivo Crosslinking of GR 15 pcusA WT

Overnight of GR 15 *pcusA* was made in Terrific Broth containing amp100. The next day, the overnights were subcultured into 2 flasks each containing Terrific Broth supplemented with amp100 and allowed to shake at 37˚C until the cells reached an OD600 of 1.0. The cells were induced with 20 uL of 10 mg/mL AHT. 500 uL of 1M $CuSO_4$ was added to one flask. The flasks were allowed to shake for 3 hours at 30˚C. Then the cells were spun down at 5000 rpm, 4˚C for 10 minutes. The pellets were washed three times in 300 mL of PBS pH 8.0. Then were resuspended in 200 mL PBS pH 8.0. 100 mL from each flask was taken out as negative control. 1mL of 0.5 M DSS was added to the other two flask and allowed to shake at room temperature for 30 minutes. The reactions were quenched by adding 2 mL of 1 M Tris-HCl pH 7.5 to each flask for 15 minutes at room temperature. The cells were spun down at 16000rpm, 4˚C for 10 minutes. The pellets were frozen at -20˚C. The following day, the cells were purified using CusA purification protocol. Western blot analysis was done using CusA (1:60000) and CusF (1:1000) primary antibodies to detect the presence of crosslinking.

DSS In-vivo Crosslinking of EC950 pcusB WT

EC950 *pcusB* cells were grown and crosslinked just like in GR 15 *pcusA*. Then was purified following CusB purification protocol without running on a monoQ. Western blot analysis was done using CusF (1:1000) and CusB (1:60000) primary antibodies.

Western Blot Analysis

SDS-PAGE gels were run. While the gels were running, transfer membranes were activated by spraying with methanol until translucent. The membranes were soaked in transfer buffer and allowed to shake in room temperature until ready to use. Whatman papers (6 per gel) were soaked in transfer buffer and a sandwich consisting of Whatman, membrane, and gel were made. Transfer from gel to membrane was done using Power PAC 1000 at 150 mA, for 2 hours. The membrane was placed in blocking solution (100mL 5% carnation milk in PBS per membrane), with the protein side up, and refrigerated overnight. The following day, the membranes were placed in 15 mL primary antibody and allowed to shake at 37˚C for 2 hours. Then they were washed with PBS-Tween three times for five minutes each at room temperature. The membranes were placed in secondary antibody (6 uL IgG Anti-rabbit HRP plus 15 mL PBS-Tween for each membrane) and allowed to shake at room temperature for one hour. While the membranes were in secondary antibody, chromogenic solution was made; I tablet of chloronaphthol was dissolved in 1 mL methanol. 200 uL of solution was added to 20 mL of 1X PBS buffer and mixed, and then 20 uL of hydrogen peroxide was added. The membranes were washed twice with 1X PBS-Tween for one minute each, and then with 1X-PBS for one minute each at room temperature. Finally, the membranes were placed in chromogenic solution (20 mL for each membrane) and allowed to shake until bands formed (~15 minutes). The reaction was stopped by washing with de-ionized water.

Silver stain

The gel was placed in fixation solution (50 % methanol, 10 % acetic acid) for 30 minutes while shaking. Then it was washed with distilled water 3 times for 10 minutes each. Next the gel was placed in sensitizing solution (20 mg $Na_2S_2O_3.5H_2O$ in 10 mL water) for 90 seconds. Then it was rinsed 3 times with water for 30 seconds each. Next it was placed in Silver solution (200 mg $AgNO_3$ in 100 mL water) for 30 minutes while shaking. Then it was rinsed 3 times with water for 60 seconds each. Next the gel was placed in developing solution (6 g Na_2CO_3, 50 uL 37 % formaldehyde, 2 mL sensitizer in 100 mL water) for 10 minutes. The reaction was stopped with 6 % acetic acid and washed with water.

Results

Functional Analysis. EC933 cells containing *cusF* derivatives, as well as empty vector were streaked on plates of varying copper concentrations to determine the minimum copper concentration that would inhibit the growth of the strains. Cells containing wild-type CusF and Sil 105 (from Salmonella) genes grew up to 2.75 mM Copper sulfate (table 2). All other strains including the empty vector grew up to 2.5 mM copper sulfate (table 2).

Growth Assay. CusF strains were innoculated into LB media supplemented with amp100, 0.2 % L-arabinose and varying copper concentrations and the Optical density at 600 nm measured to compare the growth of the mutants with wild type and empty vector at different copper concentrations and to determine at which copper concentration does CusF become critical to the cell. At 0 mM and 1 mM CuSO4, there is no significant difference in optical density between the strains (Figure 1 and 2). Figure 3 shows that the double mutant M47I/M49I has a significantly lower OD600 than other strains. Also the wild-type has a larger OD600 than the empty vector with time. At 2.0 mM CuSO4, the OD600 of wild-type is larger than that of the empty vector with time (Figure 4). Figure 5 shows that the OD600 the growth curve of the empty vector and that of the double mutant M47I/M49I are very similar and are significantly lower than those of the wild-type and other mutants. M47C, M47K/H36A, W44M, and W44A, although higher than empty vector in OD600, are significantly lower than the wild-type and other mutants.

In vitro crosslinking. To determine if CusF interacts with CusA and CusB and if CusA and CusB interact at their metal binding sites, strains containing M →C mutants in the metal binding site were crosslinked with BMOE, a sulfhydryl crosslinker. Western blot analysis probing with CusF antibody shows that CusF and CusB interact *in vitro* at their metal binding sites (figure 7). CusA did not cross-link with CusB (figure 6).

In-vivo crosslinking. Previous experiments have shown that CusF and CusB interact *in vitro*. To determine if these two proteins interact *in vivo*, EC950 pcusB cells were induced with AHT and copper, and then crosslinked with DSS. CusB was purified, and checked for the presence on CusF. Western blot analysis probed with CusF anti-body showed that CusF interacts with CusB in-vivo (figure 9). GR 15 *pcusA* cells crosslinked with DSS and induced with AHT and copper showed the interaction of CusF and CusA *in vivo* (figure 8).

Discussion

The Cus system is activated under anaerobic conditions or in extremely high copper concentrations (5). CusS senses copper stress and relays the information to CusR. CusR activates the *cusCFBA* genes that code for the tripartite pump, CBA, and a periplasmic protein, cusF. CusA is an inner membrane protein. CusB is an adapter protein, and CusC is an outer membrane protein. Deletion of *cusA* and *cusB* genes led to complete loss of copper resistance while the deletion of *cusF* and *cusC* genes lead to diminished copper resistance with the effects of *cusF* deletion being the least (7). This suggests that CusA and CusB are absolutely necessary for copper resistance while CusC and CusF are not completely necessary. Three residues coordinate metal in CusF: M47, M49, and H36 (9). The importance of these residues in copper resistance were investigated. The residues were mutated (see Table 1) and a growth assay was done on the different mutants at varying copper concentrations. At 2.5 mM, the double mutant M47I/M49I displayed very similar growth curve as the empty vector and had OD600 readings significantly lower than those of the wild-type and other mutants. This may suggest that the residues M47 and M49 are important for metal coordination. Studies have shown that Cu(I) bound to CusF has a trigonal planar shape coordinated by M47 and M49 at both sides, and H36 at the bottom (9). A deletion of one of these residues may not necessarily lead to loss of activity of the protein as the other two residues might still be able to coordinate copper. However, loss of two residues could lead to loss of the protein's activity because only one residue cannot coordinate copper. This might be an explanation to why the double mutant displayed growth characteristics to empty vector under extreme copper stress. The other double mutant M47K/H36A was less tolerant to copper stress than other mutations although the effects were not as much as that seen in the M47I/M49I strain. An explanation to this phenotype could be that the mutation from methionine to lysine did not lead to complete loss of activity of CusF because the amino group on the lysine might still be able to coordinate copper even though not as efficiently as methionine would. Isoleucine on the other hand has a neutral side chain and so will not coordinate metal. Also, only one of the methionine residues was mutated in M47K/H36A while two were mutated in M47I/M49I.

The minimum inhibitory concentration for wild type CusF was 2.75 mM while that of the mutants was 2.5 mM. The fact that cells are able to grow up to this concentration even when CusF is not fully functional, may support previous studies that suggest that CusF is not a necessary component of the *cus* system in metal resistance. The aim of this project is to understand the function of CusF in the metal efflux as this has not been clearly understood. Studies have shown evidence that it is a metal chaperone (3). Through isothermal titration calorimetry and X-ray absorption spectroscopy, CusF has also been shown to interact and transfer metal to CusB (1). *In vitro* crosslinking of methionine → cysteine mutants in the metal binding site of CusB and CusF with BMOE (spacer arm 11.4 Å) show that the two proteins interact at their metal binding sites (figure 7) and so may be an indication of metal transfer between the two proteins. However, these experiments were done *in vitro* and does not necessarily model what actually happens in the cell. To test if the CusF and CusB interaction occurs in the cell, an *in vivo* crosslinking experiment was done using DSS. DSS is an amine-reactive crosslinker with spacer arm of 11.4 Å (8 atoms). It is membrane permeable and contains N-hydroxysuccinimide (NHS) ester at each end of an 8-carbon spacer arm (3). NHS esters forms stable esters form stable amide bonds when they react with primary esters with the release of N-hydroxysuccinimide leaving group (12). Hence, DSS can capture intracellular interactions of proteins that contain primary amines. This crosslinker gives reliable information on protein-

protein interactions in the cell because it has very small spacer arm. For any two proteins/ amines to be captured by this crosslinker, they need to be very close (8 atoms away) from each other which means that they are undergoing specific interactions with each other. If they are not reacting specifically, the two proteins will not be close enough proximity to be captured by the crosslinker.

EC950 *pcusB*, a CusB overexpression strain was grown and induced with AHT and copper overexpress the protein. The cells were then treated with DSS and CusB was purified. A Western blot analysis probed for CusF showed that CusF was crosslinked to CusB (figure 9). This result suggests that CusF interacts with CusB *in vivo* in the presence of metal and supports previous studies of CusF being a metallochaperone that transfers metal CusB (7). CusF does not just interact with CusB *in vivo*. The results shown in figure 8 suggests that the cusF and cusA also interact *in vivo* in the presence of metal. CusF may also transfer metal to CusA. The *in vitro* crosslinking does not show an interaction between the two proteins (figure 7). An explanation could be that the experiment was done in the absence of metal and so the two proteins were not close enough to be captured by the crosslinker. In the future, the experiment will be done in the presence of metal. The results in figure 6 show that the metal binding domain of CusA also does not interact with that of CusB *in vitro*. One explanation could be that the experiment was done in the absence of metal. Another explanation could be that the two proteins do not interact at the metal binding sites and so no transfer of metal occurs between the two proteins. If the latter is the case, it will be good evidence that dismisses the funnel model, where substrate is transferred from CusF to CusB to CusA. Further experiments where the two proteins are crosslinked in the presence of metal and also *in vivo* have to be done to verify that no metal transfer occurs between CusA and CusB.

Current evidence supports the switch model over the funnel model. Firstly, the metal binding domain of CusA is in the periplasmic cleft (7). If CusB were to transfer metal to CusA, its metal binding domain has to be close to the cleft of CusA. However, this is not case (7). Secondly, mutation of methionine residues in the metal binding site of CusB to cysteines displayed residual copper resistance while those of CusA led to copper sensitivity (7). This is an indication that there is more flexibility in the substrate binding and transport domain of CusB than CusA. An explanation to this could be that when bound to metal, CusB undergoes a conformational change that turns the CBA pump into an "on" position to extrude metal out of the cell. So in order to perform its function, CusB just needs to bind Cu(I). If CusB transfers metal to CusA, as the funnel model suggests, there would not have been residual resistance in the CusB mutants. This observation together with the result from the crosslinking experiments support the switch model.

CusF, is a metallochaperone. It transfers metal to CusB, which may induce a conformational change of the protein that would turn on the efflux pump quicker than if CusB had to wait to be bound directly by metal in the periplasm. CusF also transfers metal to CusA. This may probably increase the rate of metal extrusion out of the cell as opposed to periplasmic metal diffusing to CusA. CusF is not absolutely necessary for copper and silver resistance in *E. coli*, however, its presence helps the cell cope better under copper stress. A possible role of CusF in the CusCFBA system is that it is a facilitator that gets metal to the pump to increase its export out of the cell. The absence of CusF may lead to diminished resistance under extreme copper stress but not complete loss of resistance. Its metallochaperone activity facilitates copper export out of the cell by getting copper quicker to CusB and to CusA. This proposed function explains why CusF contributes to copper resistance but is not necessary.

Tables

Table 1- *cusF* derivatives

Background	Relevant genotype	Gene in Trans	Mutation
EC933	ΔcusF ΔcueO	None	
EC933	ΔcusF ΔcueO	*Sil 105*	Wild Type
EC933	ΔcusF ΔcueO	*cusF*	Wild Type
EC933	ΔcusF ΔcueO	*cusF*	W44A
EC933	ΔcusF ΔcueO	*cusF*	W44M
EC933	ΔcusF ΔcueO	*cusF*	M47C
EC933	ΔcusF ΔcueO	*cusF*	M47I
EC933	ΔcusF ΔcueO	*cusF*	M47K
EC933	ΔcusF ΔcueO	*cusF*	M49C
EC933	ΔcusF ΔcueO	*cusF*	M49I
EC933	ΔcusF ΔcueO	*cusF*	M49K
EC933	ΔcusF ΔcueO	*cusF*	H36A
EC933	ΔcusF ΔcueO	*cusF*	M47K/H36A
EC933	ΔcusF ΔcueO	*cusF*	M47I/M49I

Table 2- Minimum inhibitory concentration of $CuSO_4$ for *cusF* mutants.

Background	Relevant genotype	Gene in Trans	Mutation	MIC mM CuSO4
EC933	ΔcusF ΔcueO	None		2.5
EC933	ΔcusF ΔcueO	*Sil 105*	Wild Type	2.75
EC933	ΔcusF ΔcueO	*cusF*	Wild Type	2.75
EC933	ΔcusF ΔcueO	*cusF*	W44A	2.5
EC933	ΔcusF ΔcueO	*cusF*	W44M	2.5
EC933	ΔcusF ΔcueO	*cusF*	M47I	2.5
EC933	ΔcusF ΔcueO	*cusF*	M47C	2.5
EC933	ΔcusF ΔcueO	*cusF*	M49K	2.5
EC933	ΔcusF ΔcueO	*cusF*	M49C	2.5
EC933	ΔcusF ΔcueO	*cusF*	M49I	2.5
EC933	ΔcusF ΔcueO	*cusF*	H36A	2.5
EC933	ΔcusF ΔcueO	*cusF*	M47K	2.5
EC933	ΔcusF ΔcueO	*cusF*	H36A/M47K	2.5
EC933	ΔcusF ΔcueO	*cusF*	M47I/M49I	2.5

Figures

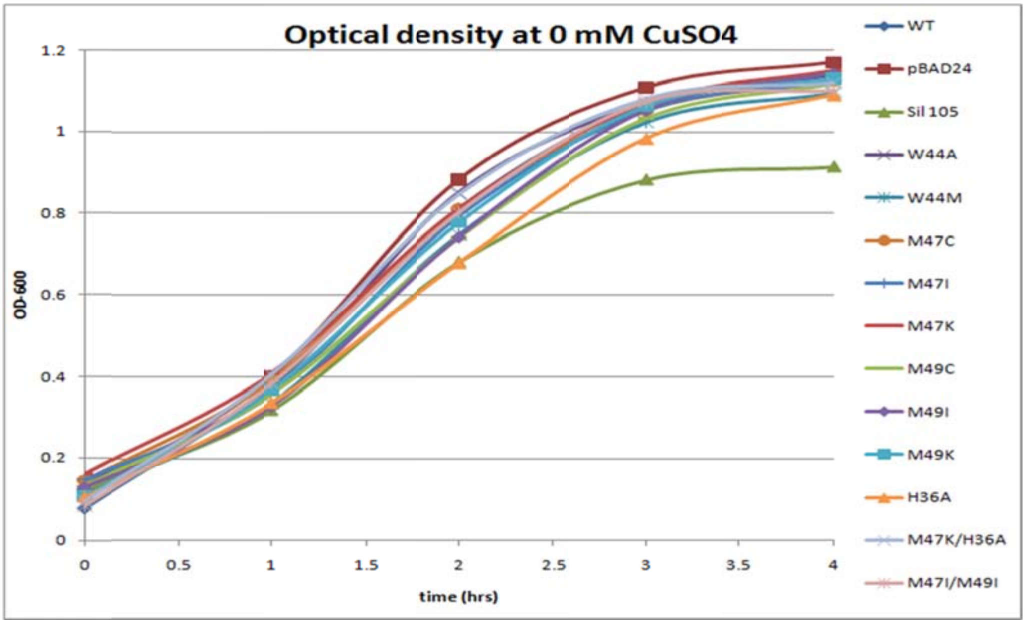

Figure 1

Graph of OD600 versus time after inoculation of cells into LB media supplemented with amp100 and L-arabinose.

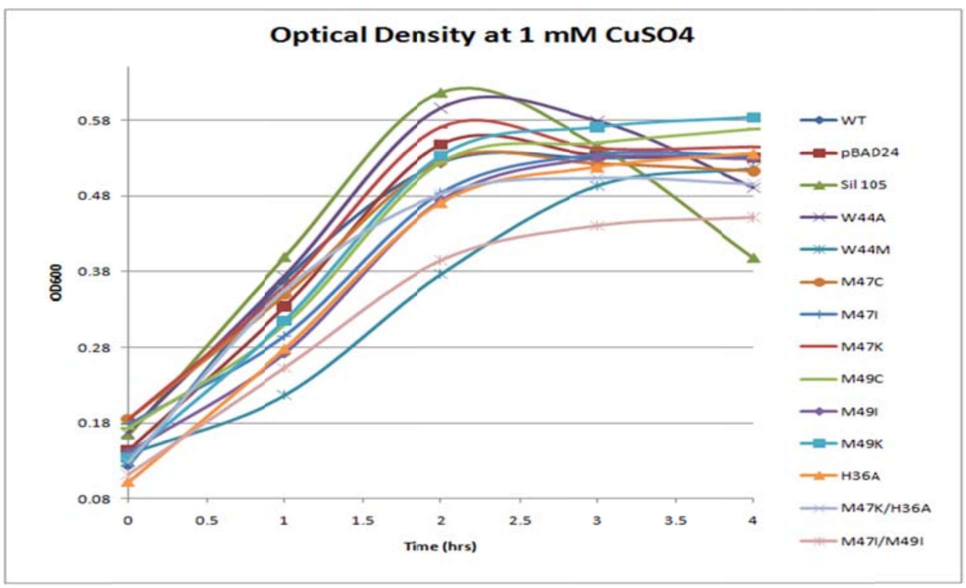

Figure 2

Graph of OD600 versus time after inoculation of cells into LB media supplemented with 1mM Copper Sulphate, amp100 and L-arabinose.

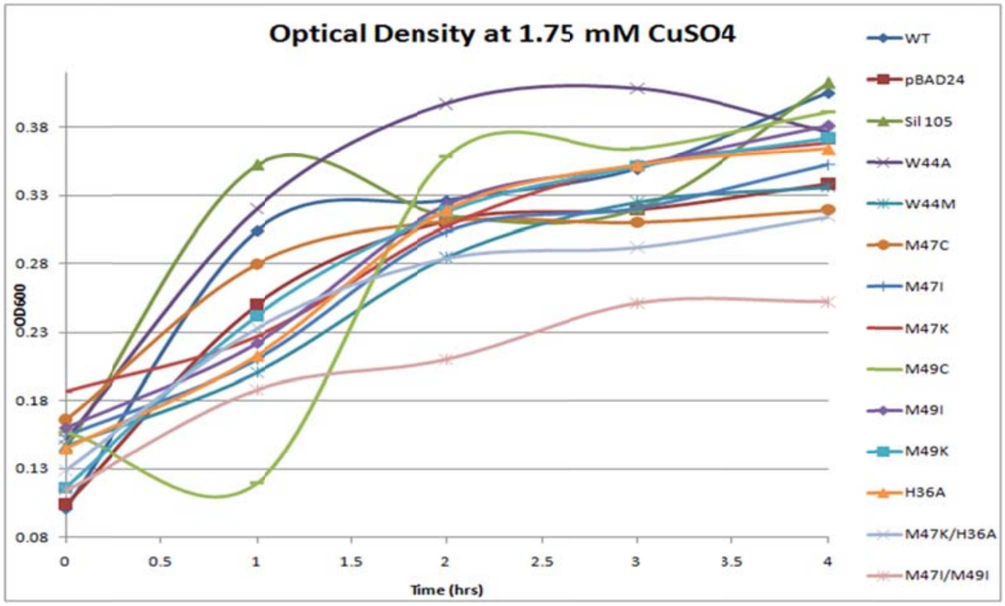

Figure 3

Graph of OD600 versus time after inoculation of cells into LB media supplemented with 1.75 mM Copper Sulphate, amp100 and L-arabinose.

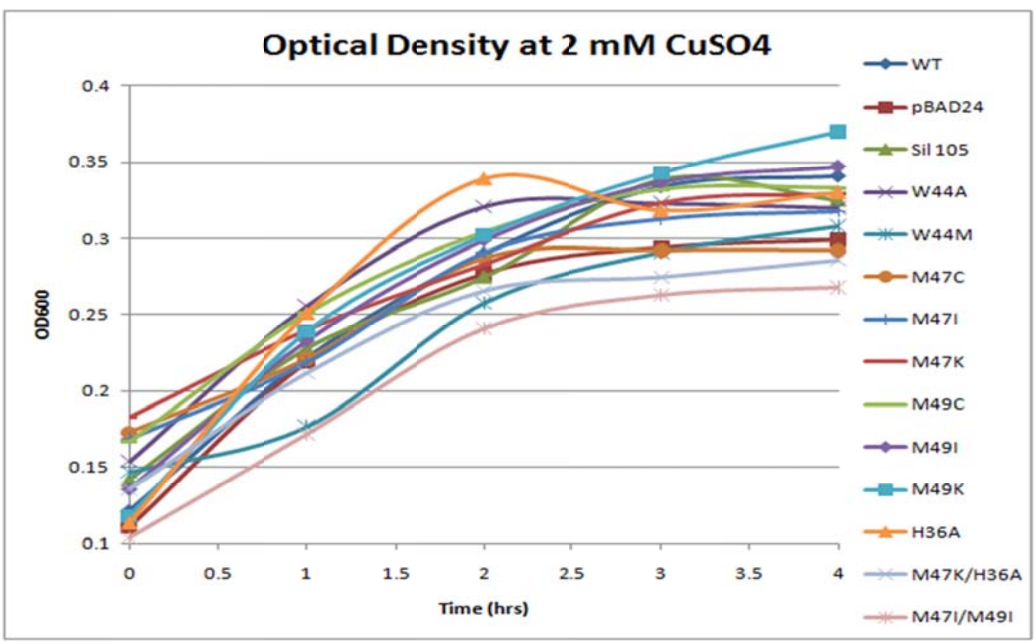

Figure 4

Graph of OD600 versus time after inoculation of cells into LB media supplemented with 2.0 mM Copper Sulphate, amp100 and L-arabinose.

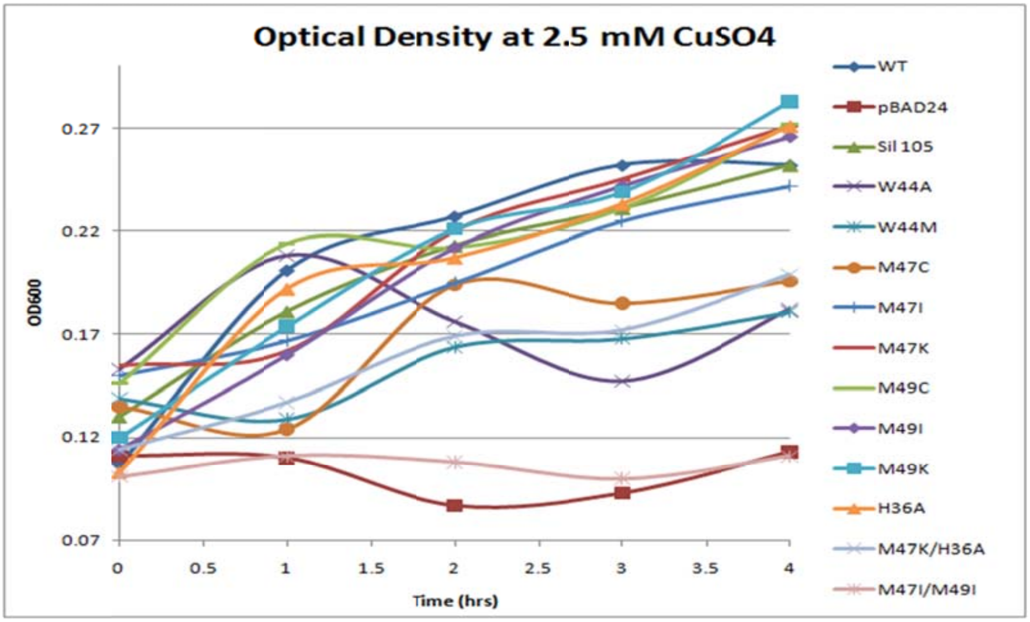

Figure 5

Graph of OD600 versus time after inoculation of cells into LB media supplemented with 2.5 mM Copper Sulphate, amp100 and L-arabinose.

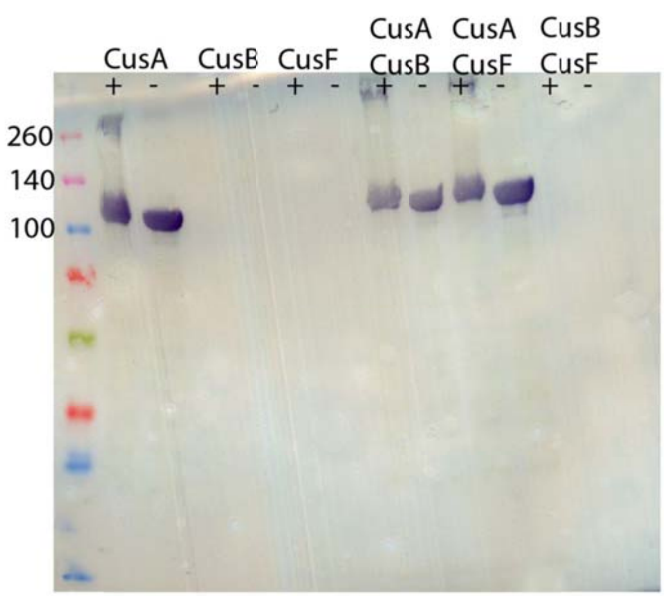

Figure 6

Western blot of BMOE *in vitro* crosslinker probed with CusA primary anti-body (1:60000). Positive sign (+) signifies the presence of crosslinker. Negative sign (-) signifies the absence of crosslinker.

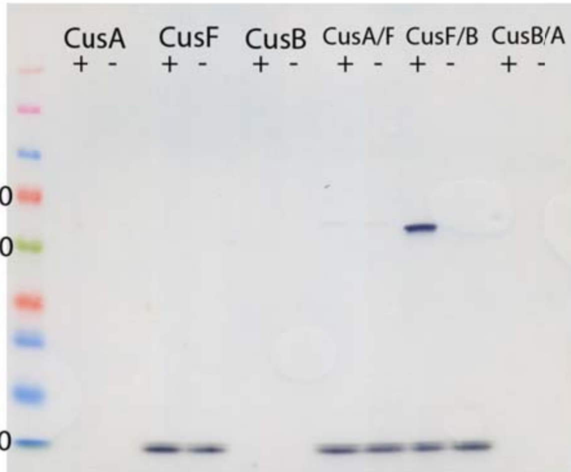

Figure 7

Western blot of BMOE *in vitro* crosslinker probed with CusF primary anti-body (1:5000). Positive sign (+) signifies the presence of crosslinker. Negative sign (-) signifies the absence of crosslinker.

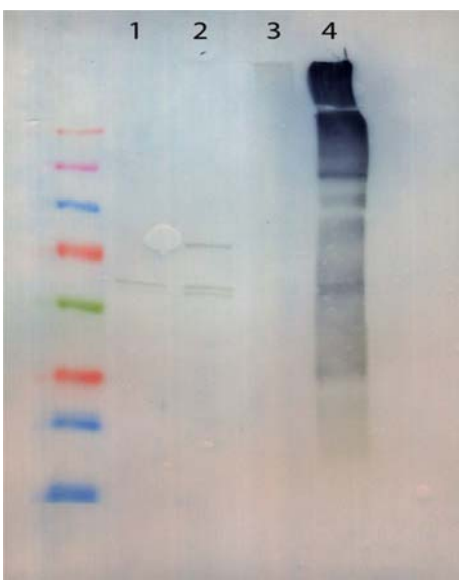

Figure 8

Western blot of DSS *in vivo* Crosslinking of GR 15 *pcusA*. Blot was probed with CusF primary anti-body (1:1000). Lane 1 is from the flask that only had AHT added. Lane 2 had AHT and 1M CuSO4 added. Lane 3 had AHT and DSS added. Lane 4 had AHT, CuSO4, and DSS added.

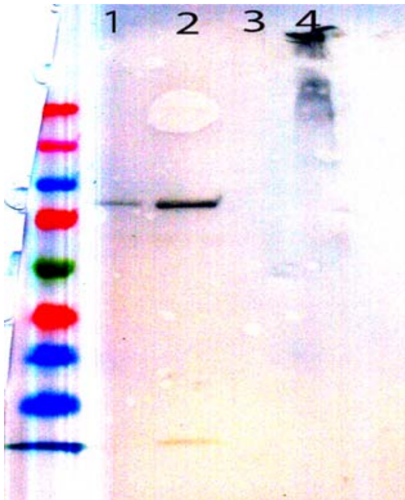

Figure 9

Western blot of DSS *in vivo* Crosslinking of EC950 *pcusB*. Blot was probed with CusF primary anti-body (1:7500). Lane 1 is from the flask that only had AHT added. Lane 2 is had AHT and 1M CuSO4 added. Lane 3 had AHT and DSS added. Lane 4 had AHT, CuSO4, and DSS added.

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