GROWTH STUDIES OF THE COPPER SENSING HISTIDINE KINASE, CUSS

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Statement by Author

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Abstract

The characterization of metal resistance systems in Gram-negative bacteria is an important field of interest with numerous applications. Not only can researchers apply what is learned to eukaryotic systems, but because of the toxic effects that copper can have when it is present in excess amounts in the cell, copper has been found to be an effective antibiotic against bacteria. The *cus* system is one such copper resistance system in *Escherichia coli*. While CusCBA serves as a tripartite copper efflux pump that exports periplasmic Cu(I), the CusRS two component system senses Cu(I) in the periplasm and serves as the signaling system responsible for upregulation of the *cusCFBA* genes. This study set out to explore the role that CusS played in the signaling pathway in the *cus* system. Aerobic growth comparing BW25113 WT and BW25113-*ΔcusS* showed that CusS was not essential for cell survival in copper environments. In the presence of oxygen, the MIC for both strains was found to be 3.0 mM $CuSO_4$ and the growth curves were very similar as well. Under anaerobic conditions CusS was found to play a crucial role in maintaining copper homeostasis. The MIC was found to be 2.0 mM $CuSO_4$ for BW25113-*ΔcusS* compared to the 3.0 mM $CuSO_4$ found in BW25113-WT. A comparison of anaerobic growth in the presence of copper across BW25113 WT, BW25113-*ΔcusS*, BW25113-*ΔcusR*, and BW25113-*ΔcusRS* showed that CusR and CusS are both independently required.

Introduction

The metabolism of essential nutrients in an organism increases in complexity when the benefits of that nutrient can be quickly overshadowed by its lethality. Copper is one such nutrient in prokaryotic and eukaryotic cells that serves as a case in point. Copper is essential for normal cellular function because of its ability to be incorporated as a co-factor in the active sites of proteins that facilitate oxygen transport and electron transfer, as well as play significant roles in respiratory and hydrolytic pathways, in addition to safeguarding against oxidative stress (Quaranta, 2009). A deficiency of copper in cells leads to inactive copper-dependent enzymes, which can ultimately result in disease. However, an excess of copper can prove to be lethal due to its highly reactive redox nature and its production of free radicals. Copper can occupy two distinct redox states: either as the reduced Cu(I) ion, or as the oxidized Cu(II) ion. While intracellular free Cu(I) and Cu(II) ions have both been shown to be toxic at low concentrations, Cu(I) appears to be much more toxic than its oxidized counterpart. Copper toxicity to cells is even more evident in an anaerobic external environment, where copper is most commonly found in the Cu(I) oxidation state (Rensing, 2003). The starkly opposing functions of copper have forced cells of all types to evolve intricate systems dedicated to preserving copper metal homeostasis.

Copper ions within the Gram-negative bacteria *Escherichia coli* give rise to an ever-present obstacle for the organism's survival. The systems that have evolved to maintain balanced copper concentrations must account for the compartmentalization of the cell – where the cytoplasmic (bound by the cytoplasmic membrane) and periplasmic (located between the cytoplasmic and outer membranes) copper concentrations must be regulated. The manner in which copper enters the cell is not well understood, but it is believed that copper ions transverse

the outer membrane through porins, and that Cu(I) is able to cross the cytoplasmic membrane through diffusion (Soncini, 2009). However, it appears that the levels of copper in both the periplasm and the cytoplasm are primarily regulated by two major copper export systems (Outten, 2001).

Genetic analysis of the chromosomal DNA of *E. coli* has identified two systems involved in copper efflux, both of which serve to detoxify the cell by removing excess copper (Outten, 2001). One of these systems is the *cue* system (named for its Cu-efflux functionality), which encodes for the CueR, CopA, and CueO proteins. CueR is a MerR-like transcription factor that upregulates the expression of the *copA* and *cueO* genes upon sensing Cu(I) in the cytoplasm. CopA is a copper exporting P-type ATPase found in the cytoplasmic membrane that serves to detoxify the cytoplasm of Cu(I) by transporting the ion into the periplasm (Rensing, 2003). CopA is a critical protein when it comes to copper regulation because it functions equally well under both aerobic and anaerobic conditions. This efficiency can be attributed to the fact that induction of the *copA* promoter remains relatively constant under both environmental conditions (Yamamoto, 2005). CueO is a periplasmic multi-copper oxidase that couples the oxidation of four Cu(I) ions with the reduction of one molecule of O_2 to generate four Cu(II) ions and two molecules of H_2O. Conversion of the highly reactive Cu(I) species to the more stable Cu(II) species safeguards numerous periplasmic proteins from copper-mediated destruction and decreases the likelihood of diffusion of Cu(I) across the cytoplasmic membrane (Outten, 2001). Thus, CueO helps to reduce the toxicity of copper to the cell, but it is clear that CueO detoxification is dependent upon the presence of molecular oxygen. Therefore, under anaerobic conditions it appears that the *cue* system is fatally flawed. While CopA continues to sequester Cu(I) into the periplasm, CueO cannot oxidize it to its less lethal form.

The second chromosomally encoded copper efflux system of *E. coli* is the *cus* system (named for its Cu-sensing functionality) (Munson, 2000). The *cus* system encodes for the proteins generated from two divergently transcribed operons – the *cusCFBA* and *cusRS* operons. The products of the *cusCBA* genes, the CusCBA proteins, form a CBA-type transport system that removes copper ions from the periplasm of *E. coli*. CusA belongs to a family of resistance nodulation (RND) proteins that is theorized to couple the export of copper cations with the import of protons across the outer membrane. Spanning the cytoplasmic membrane and protruding into the periplasm, CusA is the central protein of the pump involved in copper export in the *cus* system. The channel forming protein CusC spans the outer membrane of the cell and protrudes into the periplasm, providing a path through which copper ions can pass to the extracellular space. CusB is a clamping protein that binds CusA and CusC together and is directly involved in the copper transport system. Together, the CusCBA proteins form a tripartite transporter that is thought to export Cu(I) from the periplasm into the extracellular space. Existing independently from the CusCBA efflux channel, CusF is a small metallochaperone protein present in the periplasm that is able to bind a single copper atom (Rensing, 2003). There are two possible models pertaining to CusF's role in the cell. One model is that CusF serves to bind Cu(I) in the periplasm so that it can be transferred to CusCBA allowing it to be exported from the cell. CusF would thus directly help to eradicate copper from the periplasm. An additional model is that upon binding Cu(I) in the periplasm, CusF interacts with CusA, signaling the presence of periplasmic Cu(I) and activating the CusCBA export system (Kim, 2011). The CusCBA transporter is present in both aerobic and anaerobic environments, but under aerobic conditions, there is considerable overlap in function with the *cue* system. Studies have shown that the *cue* system is sufficient to detoxify the cell of excess copper when O_2 is present,

while the *cus* system assists in copper efflux. However, the situation is quite different under anaerobic conditions (Outten, 2001). Because the CueO protein loses its functionality, an excess of Cu(I) ions builds up in the periplasm, increasing the need for the Cu(I) CusCBA exporter. It has been observed that in the absence of O_2, the promoter for the *cusCFBA* operon is induced at much lower copper concentrations (Yamamoto, 2005). This allows CusCFBA to compensate for the deficiencies caused by the inactive CueO protein.

The second operon of the *cus* system is the *cusRS* operon, which encodes for the proteins CusR and CusS. The CusRS proteins together form a two-component system (TCS) that is believed to upregulate expression of the *cusCFBA* operon in response to an environmental copper stimulus. CusS is a histidine kinase embedded in the cytoplasmic membrane of *E. coli* (Rensing, 2003). Histidine kinases are a family of proteins found as homodimers within the cell and are comprised of a sensory domain in the periplasm and a kinase domain that extends into the cytoplasm. While the kinase domain of histidine kinases is highly conserved across all two-component systems, the sensory domain displays a high degree of sequence variety. This variance reflects the wide variety of stimuli that can be detected (Quaranta, 2009). In the case of CusS, the periplasmic sensing domain is thought to be sensitive for Cu(I) (Rensing, 2003). It is believed that when CusS detects an environmental stimulus in the form of metal ions, the kinase domain undergoes autophosphorylation, where each monomer phosphorylates a conserved histidine in its counterpart. These monomers are then believed to phosphorylate CusR, the response regulator (RR), at a conserved aspartate residue. CusR most likely undergoes a conformational change upon phosphorylation, allowing it to bind the promoter of the *cusCFBA* operon and upregulate the transcription of its gene products. This theoretical signaling pathway is extrapolated from the mechanism employed by canonical histidine kinases (Stock, 2000).

The scope of this project is to determine the role that CusS plays as a sensory protein in copper homeostasis within *Escherichia coli*. Previous experiments have shown that strains deficient in the *cusRS* and *cusR* genes display significant copper sensitivity in anaerobic environments, but lose sensitivity under aerobic environments (Yamamoto, 2005). However, bacterial strains with a deletion in the *cusS* gene have not yet been tested. Thus far, it has been assumed that CusS is strongly associated with the signaling of CusR due to the fact that the genes for both proteins are located on the same operon (Munson, 2000). Homology of CusRS with other two-component systems supports this assumption, but the mechanism for metal-sensing two-component systems has not yet been characterized. Hence, through this project, I will investigate the sensory role of CusS by comparing the results of growth experiments of cells containing deletions in *cusS, cusR*, and *cusRS* with the Wild Type when exposed to different amounts of copper under aerobic and anaerobic conditions. Furthermore, I will attempt to restore the phenotype of each knockout strain by inserting the gene *in trans* and observing if the added gene compensates for the loss of copper resistance. The lethal phenotype of cells will indicate the sensitivity of a given strain to a specific copper concentration. It is hypothesized that *E. coli* genes containing deletions of both *cusR* and *cusS* genes will display the greatest degree of copper sensitivity and that strains containing deletions in either *cusR* or *cusS* will display slightly less or equal degrees of copper sensitivity under anaerobic conditions.

Materials and Methods

Bacterial Strains

All bacterial strains used in this experiment were derivatives of the parent *E. coli* BW25113 strain. The BW25113 Wild Type (BW25113-WT), BW25113-Δ*cusR*, and BW25113-Δ*cusS* strains were obtained from the Keio collection courtesy of Dr. Vahe Bendarian. The BW25113-Δ*cusRS* strain was obtained from Swapna Aravind. All knockout strains used in this experiment were generated using the Datsenko-Wanner method of gene deletion regardless of the source from which they were obtained (Datsenko, 2000). As a byproduct of the method used to generate the gene deletions, all knockout strains have the kanamycin resistance gene inserted into their genomic DNA in replace of the deletion.

Plasmid Constructs

The plasmids used in this experiment were all derivatives of the pBAD24(-) vector (courtesy of Dr. Jon Beckwith, Harvard Medical School). The genes cusR, cusS were amplified using PCR primers from the chromosome of *E. coli* K-12 BW25113 and cloned into the pBAD vector. The resultant plasmids were pBAD24(-), pBAD24-cusR, pBAD24-cusS, and pBAD24-cusRS and they all conferred antibiotic resistance to ampicillin. The plasmids can also be induced for protein expression by adding L-Arabinose to logarithmic phase cultures. The plasmids were created using cloning procedures outlined below.

Cloning

PCR Amplification

The genes of interest were amplified from genomic DNA isolated from *E. coli* BW25113 using the polymerase chain reaction (PCR). PFU Ultra DNA polymerase (Agilent) was the enzyme used to catalyze the amplification of the DNA according to the manufacturer's

directions. The PCR reaction conditions specific to the targeted gene are shown below. After amplification, a 5 μL aliquot of the amplified gene product was run on a 1% agarose gel to very amplification and primer specificity. The remaining PCR product was purified using the Qiagen PCR Purification Kit.

Restriction Digest

The amplified genes and pBAD24(-) plasmid were both independently digested by the restriction endonucleases EcoR1 and Sal1. Digestion began with the Fast Digest EcoR1 restriction endonuclease (Fermentas). Both the plasmid and the amplified genes were digested in an incubator at 37 °C for 30 minutes and the EcoR1 restriction endonuclease was heat inactivated in a hot well plate at 80 °C for 5 minutes. The plasmid and amplified genes were placed on ice for an additional 5 minutes. Then, digestion continued for both the plasmid and the amplified gene using the Fast Digest Sal1 restriction endonuclease (Fermentas). Both the plasmid and amplified genes were digested in an incubator at 37 °C for 1 hour. The Sal1 restriction endonuclease was then heat inactivated in a hot well plate at 65 °C for 10 minutes and the samples were placed on ice for 5 minutes. At this stage, the digested genes were purified using the Qiagen PCR Purification Kit.

Vector Treatment with Alkaline Phosphatase

The plasmid was then treated with alkaline phosphatase (Fermentas). The alkaline phosphatase reaction mixture was placed in an incubator at 37 °C for 20 minutes. The alkaline phosphatase was heat inactivated in a hot well plate at 75 °C for 5 minutes. The plasmid was then placed on ice for 5 minutes. At this stage, the plasmid was purified using a Qiagen PCR Purification Kit.

Ligation

The next step performed was the ligation reaction. First, a 1% agarose gel was run to compare the relative concentrations of the digested gene insert and the digested vector. Five different ligation reactions were set up, never allowing each ligation reaction to exceed 20 μL. Different ratios of insert to vector were made in each reaction, with each ratio being 1:1, 2:1, 5:1, 9:1, and 5:3. T4 DNA ligase (Fermentas) was used to catalyze the ligation reactions. The reaction mixtures were, for the most part, made according to the manufacturer's specifications. The only time the mixture was altered was for the different ratios of insert to vector in the mixture. The ligation reactions were allowed to sit at room temperature for 16 hours.

Transformation and Clone Isolation

The entire volume of each ligation reaction was then transformed into RuCl chemically competent *E. coli* DH5α cells using a heat shock time of 90 seconds. The transformed cells were plated on LB agar plates with 100 μg/mL ampicillin to select for the clones, and allowed to grow overnight for 16 hours in an incubator at 37 °C. Upon detection of colonies after 16 hours of growth, single colonies were isolated and inoculated into 10 mL of fresh LB + Amp100 and allowed to grow for 16 hours in a shaker at 37 °C. The plasmid was isolated from 10 mL overnight cultures using a Qiagen Spin Miniprep Kit and then sent away for sequencing.

PCR Amplification Conditions

cusR

The forward primer used to amplify *cusR* was 5' – AAA AAG AAT TCA TGA AAC TGT TGA TTG TCG AAG ATG – 3' and the reverse primer used was 5' – TTT TTG TCG ACT TAC TGA CCA TCC GGC ACC TCA – 3'. The primers were designed with restriction sites for EcoR1 and Sal1. The *cusR* gene is a total of 693 bp long. The PCR had an initial denaturation

temperature of 95 °C for 5 minutes. Following that was a denaturation temperature of 95 °C for 30 seconds, an annealing temperature of 62 °C for 30 seconds, and an extension temperature of 72 °C for 42 seconds. These three conditions were repeated for a total of 30 cycles. A final extension period of 5 minutes was carried out at 72 °C.

cusS

The forward primer used to amplify *cusS* was 5' – TTT TTG AAT TCA TGG TCA GTA AGC CAT TTC AGC G – 3' and the reverse primer used was 5' – AAA AAG TCG ACT TAA GCG GGT AAT GTG ATA ACA AAC – 3'. The primers were designed with restriction sites for EcoR1 and Sal1. The *cusS* gene is a total of 1443 bp long. The PCR had an initial denaturation temperature of 95 °C for 5 minutes. Following that was a denaturation temperature of 95 °C for 30 seconds, an annealing temperature of 66 °C for 30 seconds, and an extension temperature of 72 °C for 1 minute 27 seconds. These three conditions were repeated for a total of 30 cycles. A final extension period of 5 minutes was carried out at 72 °C.

cusRS

The forward primer used to amplify *cusRS* was 5' – AAA AAG AAT TCA TGA AAC TGT TGA TTG TCG AAG ATG – 3' and the reverse primer used was 5' – AAA AAG TCG ACT TAA GCG GGT AAT GTG ATA ACA AAC – 3'. The primers were designed with restriction sites for EcoR1 and Sal1. The *cusRS* gene is a total of 2215 bp long. The PCR had an initial denaturation temperature of 95 °C for 5 minutes. Following that was a denaturation temperature of 95 °C for 30 seconds, an annealing temperature of 63 °C for 30 seconds, and an extension temperature of 72 °C for 2 minute 20 seconds. These three conditions were repeated for a total of 30 cycles. A final extension period of 5 minutes was carried out at 72 °C.

Gene Deletions

E. coli strains W3110 + pKD46 and DH5α + pKD4 were each grown in 5 mL of LB media in a shaker at 37 °C for 16 hours. The plasmids of both strains were extracted using a Qiagen Spin Miniprep Kit. The pKD46 plasmid was transformed into chemically competent BW25113 WT cells using a heat shock time of 60 seconds and plated on LB agar + 100 μg/mL ampicillin for selection. The BW25113 WT strain is the strain in which the *cusRS* knockout will be made. Thus, the L-Arabinose Lambda Red recombinant genes contained in the pKD46 plasmid must be present in the BW25113 cell.

The pKD4 plasmid contains the gene for kanamycin resistance. A PCR was used to amplify the *kan* gene with primers specialized for homologous recombination with the BW25113 *cusRS* gene. The forward primer used was 5' – ATG AAA CTG TTG ATT GTC GAA GAT GAA AAG AAA ACC GGA GAG TGT AGG CTG GAG CTG CTT C– 3' and the reverse primer used was 5' – ACG TTA TTT TTA CAC TGG TTA TAA AAG TTG CCG TTT GCT GAA GGA ATG GGA ATT AGC CAT GGT CCA – 3'. The *kan* gene is approximately 1100 bp long. The PCR had an initial denaturation temperature of 95 °C for 5 minutes. Following that was a denaturation temperature of 95 °C for 30 seconds, an annealing temperature of 69 °C for 30 seconds, and an extension temperature of 72 °C for 1 minute 10 seconds. These three conditions were repeated for a total of 30 cycles. A final extension period of 5 minutes was carried out at 72 °C. After amplification, a 5 μL aliquot of the gene product was run on a 1% agarose gel to very amplification and primer specificity. Then, a restriction digest was performed using the restriction endonuclease DpnI. The DpnI enzyme made by Fermentas was used to carry out this digest, and the reaction mixture was made according to the manufacturer's directions. The digest was performed for 4 hours in an incubator at 37 °C to ensure removal of all DNA

besides the amplified linear *kan* gene fragment. The linear fragment was then purified using a Qiagen PCR Purification Kit.

A colony of the BW25113 + pKD46 strain was isolated and inoculated into 10 mL LB + 100 μg/mL ampicillin and placed in a shaker to grow at 30 °C. The $O.D._{600}$ was measured every half hour until it reached an O.D. of 0.1. 100 μL of 1 M L-Arabinose was added to obtain a final concentration of 10 mM L-Arabinose. The cells continued to grow at 30 °C until they reached an $O.D._{600}$ of 0.4 - 0.5. A Cary 50 Bio Spectrophotometer was used to measure the O.D.'s at a setting of 600.00 nm. At this point, the BW25113 + pKD46 cells were made electrocompetent. The linear fragment of the *kan* gene was then electroporated into the BW25113 + pKD46 cells and 1 mL of SOC medium was added to them. The cells were allowed to shake at 37 °C for 2 hours and then were plated onto LB + agar + 30 μg/mL kanamycin and allowed to grow in an incubator at 37 °C overnight for 16 hours. A colony PCR was then performed to verify that the *kan* gene was inserted into the BW25113 genome (indicating that the *cusRS* genes were deleted) and the genomic DNA was sent away for sequencing (method from Datsenko, 2000).

Aerobic Liquid Media Growth Experiments

A single colony of each cell strain to be included in the experiment was grown overnight in 5 mL of LB medium in a shaker for 16 hours at 37 °C. After 16 hours of growth, 2 mL of each cell strain was inoculated into 200 mL of LB so that the final $O.D._{600}$ was ~0.05. The O.D. was checked every 30 minutes until the cells reached an $O.D._{600}$ of 0.1. The cells were taken out of the shaker for no more than 2 minutes so that a 1 in 20 dilution could be performed by inoculating 500 μL of cells into test tubes containing 10 mL of LB medium. The appropriate amount of $CuCl_2$ was then added to reach the desired copper concentration that the cells would be exposed to. The test tubes were placed back in the shaker at 37 °C to allow the cells to grow in

their copper environment. The $O.D._{600}$ was measured in a Cary 50 Bio Spectrophotometer every hour until the readings became stable or until they decreased.

Anaerobic Minimum Inhibition Concentration Growth Experiments

$CuSO_4$ LB Agar Plates

The LB Agar plates were made by first adding 1 M $CuSO_4$ to an empty petri plate. The amount of $CuSO_4$ added depended upon the final desired copper concentration of the plate. 20 mL of LB agar was then pipetted into each petri plate and swirled to ensure even copper distribution. The plates were allowed to solidify at room temperature and then stored at 4 °C. Plates were allowed to dry completely before cells were plated on them.

Cell Growth

A single colony was grown overnight in 5 mL of LB medium in a shaker for 16 hours at 37 °C. After 16 hours of growth, a 1 in 500 dilution was performed by inoculating 50 µL of each cell strand into 25 mL of Miller LB medium. These cultures were allowed to grow until they reached an $O.D._{600}$ of 1.0. Cells from each strand were then normalized with respect to one another and plated onto the $CuSO_4$ containing LB agar plates. The plates were then placed into BD GasPak EZ anaerobic containers with 3 oxygen absorbing sachets. These plates were allowed to grow in an incubator at 37 °C for a period of 24 and 48 hours.

Results and Discussion

Although it has been well established through multiple experiments that the *Cus* system does not confer appreciable copper tolerance under aerobic conditions, no study to date has directly tested to see whether or not CusS plays a role in copper homeostasis when oxygen is present. A minimum inhibition concentration (MIC) growth assay was performed where the BW25113 WT and BW25113-*ΔcusS* were grown for 16 hours on LB agar plates containing various concentrations of $CuSO_4$ with no antibiotic. The concentrations of copper ranged increased in 0.5 mM increments from 0.0 mM to 5.0 mM. In this experiment, the MIC is reported as the lowest concentration of copper required to inhibit visible growth of the bacterial cells (Figure 1).

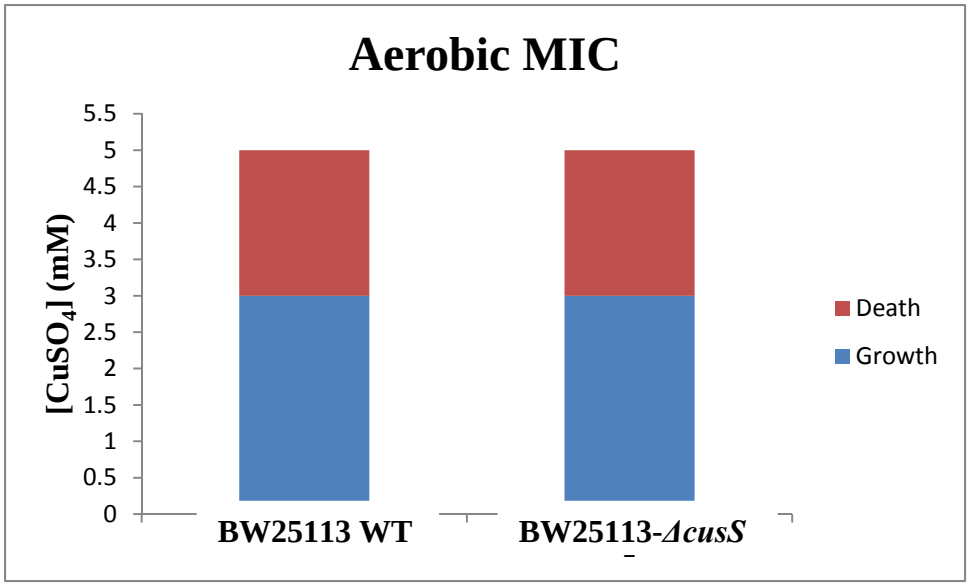

Figure 1
Aerobic MIC data comparing BW25113 WT and BW25113-*ΔcusS.*

The aerobic MIC was determined to be 3.0 mM $CuSO_4$ for both BW25113-WT and BW25113-*ΔcusS*. There were no distinguishing features between either strain. The aerobic MIC data shows that CusS does not affect the phenotype of *E. coli* under aerobic conditions. This

result coincides with the experimental evidence showing that the phenotype for *E. coli* is the same with or without the *cus* system under aerobic conditions. If the CusCBA export pump is not required for cellular viability, then the signaling from CusS that causes upregulation of the *cusCBA* genes would not be essential. This MIC data was used to design a growth experiment for these two strains. This experiment has been repeated three times and the results have been consistent throughout all trials.

A growth experiment comparing the BW25113 WT and BW25113-*ΔCusS* strains was performed aerobically. Both cell strains were grown in LB medium despite the fact that BW25113-*ΔcusS* is resistant to kanamycin in order to keep the growth conditions the same for both cell strains and to avoid having the antibiotic hinder the growth of one strain when the other was not exposed to the antibiotic. Each strain was exposed to different concentrations of copper. The copper concentrations ranged from 0.0 mM to 4.0 mM in 0.5 mM increments. From the previous aerobic MIC data, it was believed that the cells would not have been able to survive in a 4.0 mM $CuSO_4$ copper environment. The 5.0 mM $CuSO_4$ was added as a concentration that should guarantee death of the BW25113 cells. The cells were allowed to grow for a total of 9.5 hours, with time points taken roughly every hour. From this experiment, a set of growth curves was obtained for BW25113 WT and BW25113-*ΔcusS* (Figure 2).

The growth curves obtained for this experiment were, for the most part, consistent with the expectations for aerobic growth of *E. coli* BW25113. Visual comparison of the WT and *ΔcusS* growth curves show remarkable similarity in growth patterns. It confirms that copper concentrations of 3.0 mM $CuSO_4$ or greater are enough to either completely kill the cell or severely stunt their growth. Additionally, both cell strains that grew in an environment containing 0 to 2.5 mM $CuSO_4$ appear to grow in a tight group with respect to one another,

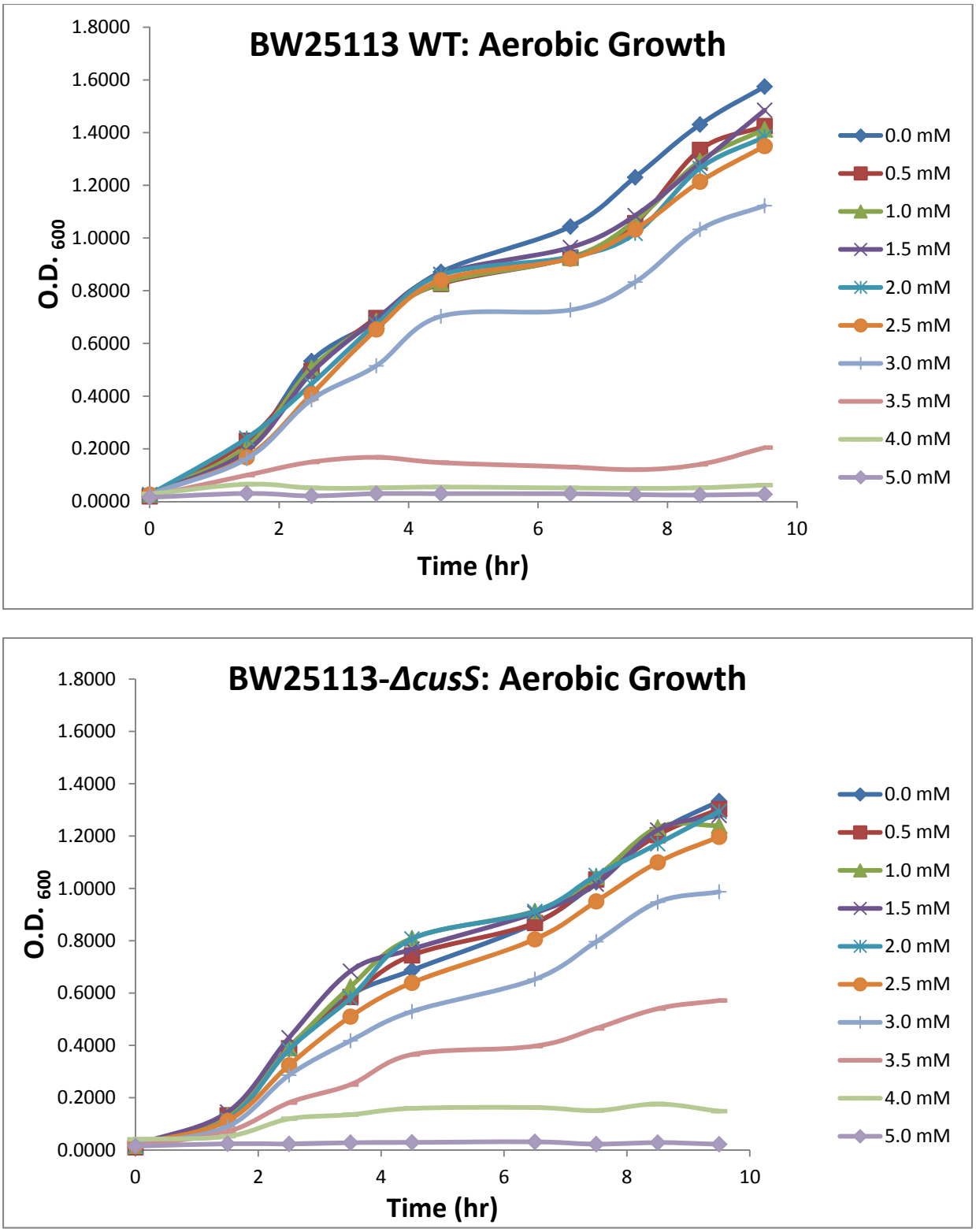

Figure 2
Aerobic growth curves obtained for BW25113 WT (top) and BW25113-*ΔcusS* (bottom) when exposed to various $CuSO_4$ concentrations.

where it is difficult to specifically differentiate between strains growing at different copper concentrations.

Although the appearances of the two growth curves look similar, the $O.D._{600}$ measurements are not. As can be seen upon closer inspection of the two curves, the BW25113 WT strain is anywhere from 0.10 to 0.15 O.D. greater than the BW25113-*ΔcusS* strain. This result seems to show that CusS does play a slight role in copper homeostasis even under aerobic environments. Although the *cus* system is not the primary system working under aerobic conditions, it still may be performing a role in copper exportation. The promoters for the *cusRS* and *cusCFBA* operons are not as readily induced when oxygen is present, but the proteins are still found in the cell (Yamamoto, 2005). This growth experiment needs to be repeated in order to verify the result and to optimize the experimental design.

A minimum inhibition concentration (MIC) was also performed anaerobically in order to compare the BW25113 WT and BW25113-*ΔcusS* strains. The cell strains were streaked out on LB agar plates over a range of different $CuSO_4$ concentrations. The copper concentrations varied as they did in the aerobic MIC, increasing in 0.5 mM increments from 0.0 mM to 5.0 mM $CuSO_4$. No antibiotic was used and the cells were subjected to a growth period of 16 hours. The MIC is reported as the lowest concentration of copper required to inhibit visible growth of the bacterial cells (Figure 3). This experiment has been repeated twice and the results have been consistent across both trials.

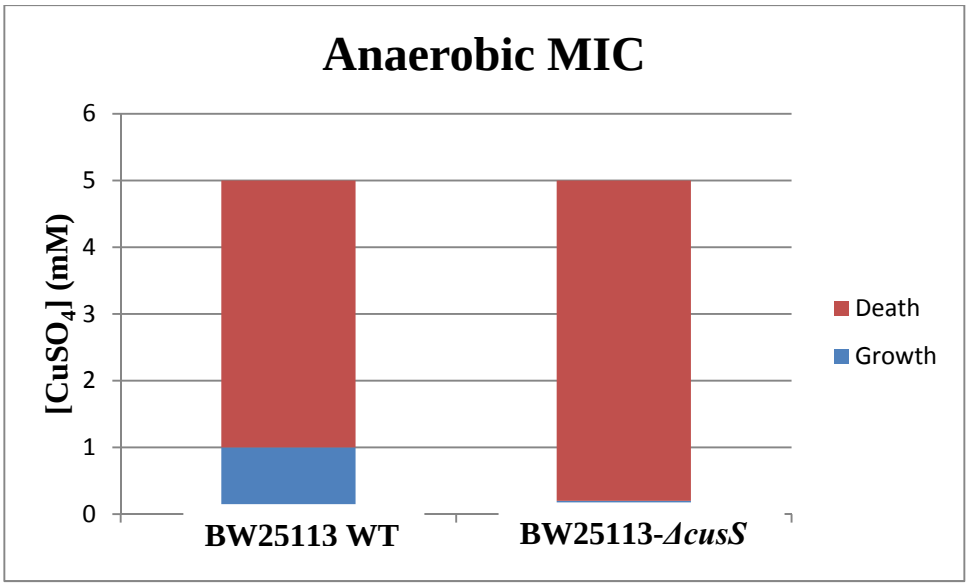

Figure 3
Anaerobic MIC data comparing BW25113 WT and BW25113-*ΔcusS.*

Under anaerobic conditions, the MIC values for BW25113 WT and BW25113-*ΔcusS* are no longer the same. The MIC for the WT was found to be reduced to 1.0 mM and the MIC for *ΔcusS* was reduced to 0.20 mM. The data shows that CusS is an essential protein when it comes to copper homeostasis under anaerobic conditions in *E. coli.* These results are in accordance with what was originally thought about the *cus* system. Under anaerobic conditions, the *cus* system is required for cellular function by serving as a key copper efflux pump in the periplasm. Previous studies have shown that CusR upregulates the *cusCFBA* genes under anaerobic copper stress. However, there was speculation as to whether CusR was phosphorylated by CusS or by another histidine kinase engaging in cross talk signaling. This MIC shows that the periplasmic sensing capabilities of CusS are necessary for the cell to survive. Coupled with the fact that studies have shown that CusR is the only response regulator that CusS phosphorylates, CusS is essential to cause upregulation of the *cusCFBA* genes (Yamamoto, 2005).

An additional minimum inhibitory concentration (MIC) growth assay was performed to compare the phenotypic affects of BW25113 WT, BW25113-*ΔcusS*, BW25113-*ΔcusR*, and BW25113-*ΔcusRS*. The deleted gene was provided *in trans* on the pBAD24 vector to test for restoration of the phenotype of the knockout strains. All cell strains contained a form of the pBAD24 vector to maintain consistency between strains. The addition of the vector also allowed them to be selected for with ampicillin. The cell strains were streaked out on LB agar plates over a range of different $CuSO_4$ concentrations. The copper concentrations varied over a range from 0 to 2.5 μM, at the following concentrations: 0.0 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, and 2.5 mM. The cells were allowed to grow anaerobically for a period of 24 hours and a period of 48 hours. The MIC is reported as the lowest concentration of copper required to inhibit visible growth of the bacterial cells (Figure 4). The data obtained for this experiment is preliminary and still needs to be repeated.

The minimum inhibitory concentration growth data shows that the WT strain was able to survive in copper concentrations of 1.5 mM or less, but growth was inhibited in all greater concentrations for both the 24 hour anaerobic growth sample and the 48 hour anaerobic growth sample. A comparison of the MIC data after 24 hours of growth shows that the MIC's for *E. coli* containing deletions in *cusR* or *cusS* are the same, but the MIC for a deletion in *cusRS* is significantly less. The MIC data after 48 hours of growth showed that all knockout strains had the same MIC. As expected, 48 hours of growth showed significantly less cell survival than at 24 hours. As was originally predicted, the *ΔcusRS* strain showed the greatest sensitivity to copper. This was due to the fact that deletion of the entire two-component system is expected to completely halt upregulation of the *cusCFBA* genes. What was not expected was that the *ΔcusS*

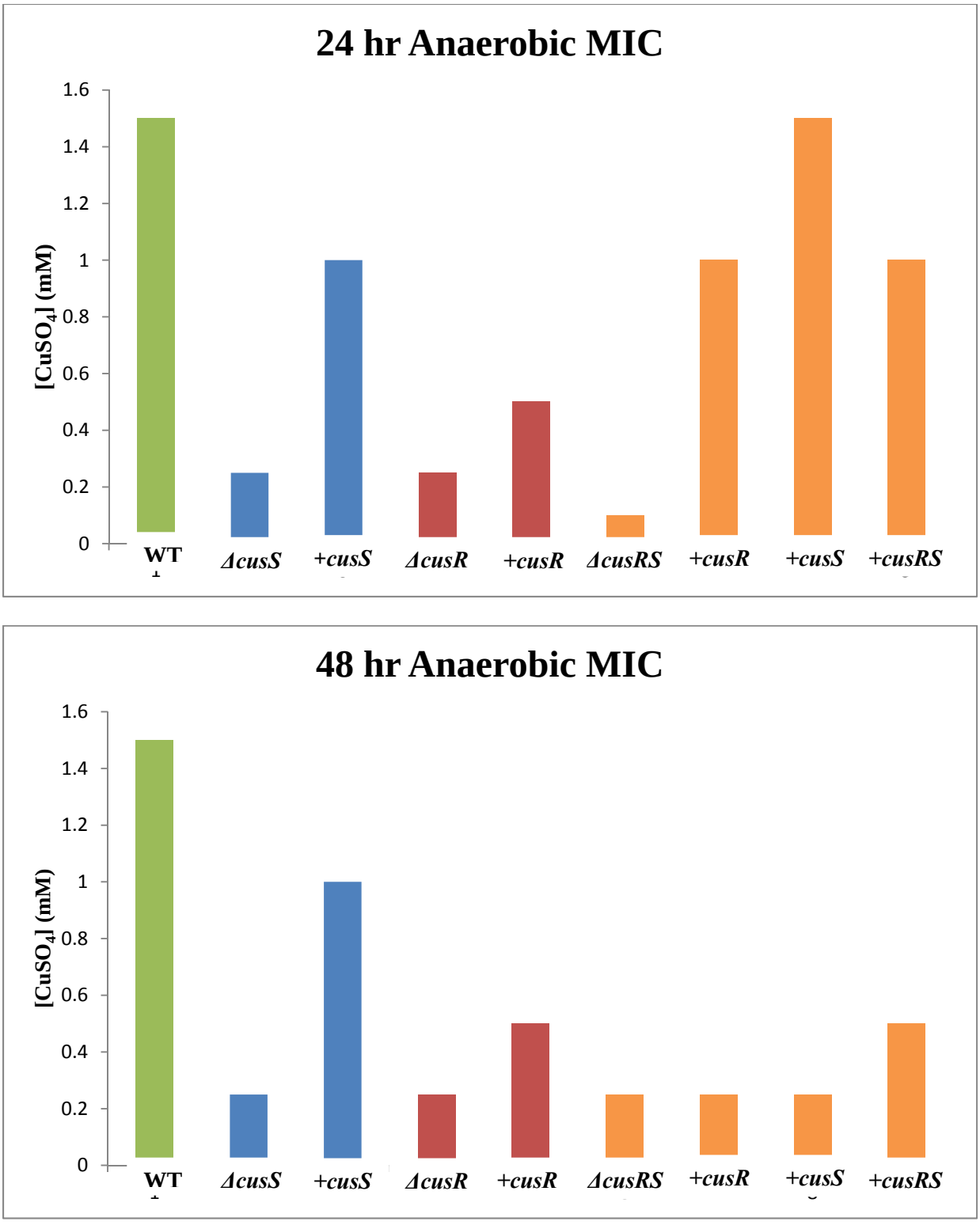

Figure 4
Anaerobic MIC data comparing BW25113 WT (green), BW25113-*ΔcusS* (blue), BW25113-*ΔcusR* (red), and BW25113-*ΔcusRS* (orange) and their recovered phenotypes. Cells were grown for 24 hours (top) and 48 hours (bottom). The plot shows the range of cell growth of each strain.

strain would have the same MIC as the *ΔcusR* strain. This proves that the *cusS* gene is just as essential as the *cusR* gene under anaerobic conditions.

When the deleted gene for each knockout strain was presented *in trans* the phenotype was significantly recovered, but not to the extent of the WT. Recovery to the WT phenotype would have been ideal because resupplying the gene of interest should restore functionality to the knock out strain. I may have been unsuccessful in completely recovering the phenotype due to the expression levels of the plasmid. The expression levels may have differed from those in the *E. coli* genome.

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