Copper homeostasis in *Salmonella enterica*: cloning, expression, and purification of CopA transporter and CueO cuproprotein

A Major Qualifying Project
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ABSTRACT

Salmonella enterica is an important and frequent cause of gastroenteritis and systemic infections, including those of economic relevance. *Salmonella* possesses a highly controlled system of transcription regulators, chaperones, membrane transporters, and target cuproproteins that tightly control the distribution of copper across its compartments. Cu^+ is required as a redox cofactor in the catalytic centers of enzymes, but free Cu^+ is highly reactive and deleterious to cells. Cu^+, along with the oxidative burst, is central in host-pathogen interactions as part of the innate immune response. The goal of this major qualifying project is to contribute to understanding the mechanisms of Cu^+ homeostasis in the cell envelope of the pathogen *Salmonella enterica*. Here, the proteins CopA, an inner membrane Cu^+ efflux transporter, and CueO, a multicopper oxidase, which are essential in Cu homeostasis, were cloned and purified from *Salmonella enterica*. Results indicate that CopA and CueO were successfully cloned and expressed in *E. coli*. Future research should purify these proteins and determine their copper binding kinetics to help understand how Cu^+ is transported and stored in *Salmonella* under conditions of cellular equilibrium.

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INTRODUCTION

Salmonella enterica, referred to as *Salmonella* in this work, includes a zoonotic group of pathogens divided into the *bongori* and *enterica* species (1, 2). *Salmonella* is a large group of gram-negative bacteria comprised of seven subspecies and more than 2,600 serotypes (1, 3). This pathogen causes a variety of clinical ailments in both humans and animals, and ranges from gastroenteritis to more serious systemic infections. Every year there are nearly 94 million cases of enteric *Salmonella* infections and more than 150,000 deaths reported globally (4, 5). People acquire this bacteria through ingestion of contaminated food or water, and in rarer cases by direct contact with infected individuals. Many cases are self-limited and respond well to antimicrobial treatments, but non-typhoid *Salmonella* can cause severe infections and sepsis in more susceptible patients like young children, older adults, or the immune-comprised (4-6).

Copper (Cu) is an essential co-factor for important redox enzymes in cells, yet is also a viable bactericidal agent that pathogens, such as *Salmonella*, must contend with during infection. Heavy metals in small concentrations serve important roles in living organisms, including cellular respiration and as essential co-factors for several key enzymes (1, 3, 7). However, excess free metals in high enough concentrations in cells leads to production of free radicals and associated cellular damage (8). The accumulation of active copper, either in Cu^{+} or Cu^{2+}, inside phagocytic vesicles cause substantial metabolic deterioration in invading bacteria (1). Consequently, the ability to undergo redox reactions as a prosthetic group by donating or accepting one electron during Cu^{+}/Cu^{2+} interconversion makes free copper a potential hazard, which catalyzes the formation of highly reactive oxygen and nitrogen species (3). In sufficiently high-enough concentrations of free copper, copper may also displace other transition metals. For

example, copper can attack and disrupt iron-sulfur clusters, which releases iron that can then cause further oxidative stress and severe cell damage through the Fenton reaction and conversion of hydrogen peroxide, a byproduct of aerobic respiration, into the highly reactive hydroxyl free radical (3, 9). As a consequence, mechanisms to control the intracellular concentrations of metals are necessary for organisms.

The cell envelope of gram-negative bacteria, which is the area of the cell including the inner and outer membranes and the periplasmic space between them, is especially susceptible to $Cu^{2+/+}$ toxicity due to the localization of energy metabolism and nutrient acquisition there (1). Intracellular facultative *Salmonella* species that infect organisms and are of clinical importance have developed distinctive mechanisms through evolution to mitigate $Cu^{2+/+}$ overload in this compartment and its related redox stress. These mechanisms include a system of influx and efflux transporters, chaperones, chelating agents, and regulatory sensors that have a high affinity for copper. As a result, there are low intracellular Cu^{+} requirements, and Cu^{+} moves via chaperone-mediated processes between membrane transporters to prevent the release of free Cu^{+} (10).

Research to elucidate how *Salmonella* manages $Cu^{2+/+}$ stress derives from studies on the *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) (1). *S.* Typhimurium modulates the expression of factors essential for virulence, which enables survival against the surge of $Cu^{2+/+}$ in the *Salmonella* containing vacuole (3). In particular, this pathogen has the profound ability to adapt and survive in harsh conditions, including the epithelial and phagocytic host environments where it is exposed to high $Cu^{2+/+}$ levels and redox stress; thus, the ability to cope with these stresses is determinant for infection, and these bacteria have a versatile genetic arsenal contributing to their virulence. There have been a variety of studies of *Salmonella*

and various *Salmonella* mutants inside macrophages to understand the roles of these various regulators, transporters, and chaperones, and how $Cu^{2+/+}$ handling in the cell envelope contributes to virulence. For example, during infection in host macrophages, research indicates *Salmonella* detects the surge of Cu inside the *Salmonella*-containing vacuole, and mutants with decreased copper resistance have reduced survival compared to the wild-type strain (1, 3, 11, 12).

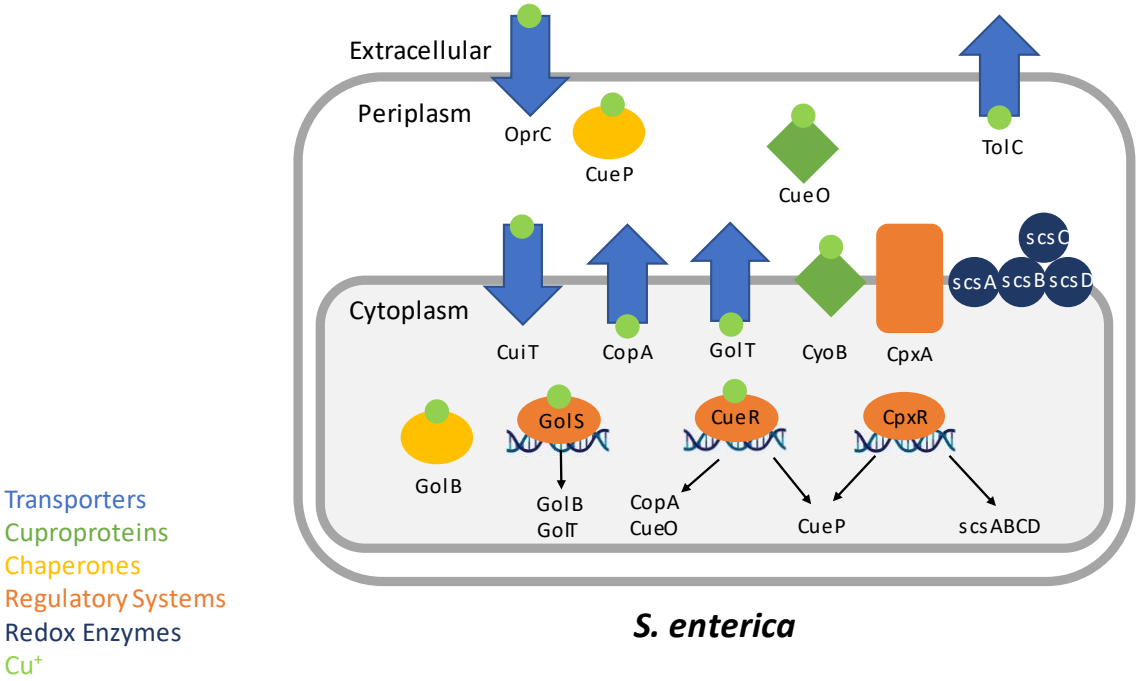

Figure 1. Major elements from the Cu^{+} homeostasis network in gram-negative *S. enterica* (1, 13).

As in other gram-negative bacteria, copper homeostatic systems in *S.* Typhimurium are maintained by transcriptional control by envelope and cytoplasmic Cu sensors, including CueR (1). CueR (Cu Efflux Regulator) is a cytoplasmic sensor and regulator that maintains periplasmic Cu homeostasis under aerobic conditions (3, 14). CueR responds to increasing concentrations of cytoplasmic copper, and drives the expression of associated promoters to maintain intracellular

copper below a toxic threshold (14). CueR is present in many gram-negative bacteria and a member of the MerR family of transcriptional regulators that usually controls the expression of three proteins: a Cu^+ ATPase, CopA; a chaperone, such as CopZ or CueP; and a multi-copper oxidase, CueO (8, 13). CueR directly stimulates the transcription of CopA and CueO where CopA translocate Cu^+ from the cytoplasm to the periplasmic space, where it is converted to the less toxic Cu^{2+} form by CueO (7). However, *S.* Typhimurium lacks the CopZ cytoplasmic chaperone, and instead regulates the expression of the periplasmic chaperone CueP (13). Many of the factors involved in the *Salmonella* copper homeostasis network are shown in Figure 1.

CopA is an inner membrane transporter that is responsible for the movement of Cu^+ to the periplasm. CopA is a P_{1B}-ATPase that couples the hydrolysis of ATP to the efflux of cytoplasmic Cu^+ (8). CueO is a periplasmic multicopper oxidase controlled by the Cu efflux regular, CueR (14). CueO, or the Cu efflux oxidase, is responsible for converting Cu^+ to the less toxic Cu^{2+}, which protects the cell by preventing the reduced species from participating in the Fenton reaction (6). CopA and CueO are regulated in a copper responsive manner by CueR, which increases transcription in response to increasing copper concentration (14). In *S.* Typhimurium, CueO is required for copper tolerance as previous studies demonstrate that deletion of CueO results in enhanced sensitivity to copper and reduced virulence (6). In addition, the *S.* Typhimurium multicopper oxidase has a greater activity toward Cu^+ relative to Fe^{2+}, unlike eukaryotic counterparts with a higher affinity for Fe^{2+}, which would not be inconsistent with the proposed role of CueO in copper oxidation. However, the biochemical basis for the protective role of CueO remains to be established, but it is likely that maintenance of copper in a Cu^{2+} state would reduce the permeability and entry of Cu^+ into the cytoplasm, which prevents the toxic effects of free Cu^+ (6).

Another CueR-like sensor, called GolS, is present in most *S. enterica* serovars and controls the expression of GolT and GolB (1, 15). GolT is a CopA-like, P-type ATPase transporter that assists transmembrane Cu efflux at high metal concentrations (1). GolB is a rather small cytoplasmic chaperone that participates in intracellular transport and delivery of various metals with a +1-oxidation state, such as Cu^{+}, Au^{+}, and Ag^{+} (15). In previous studies of *Salmonella*, GolT demonstrated the ability to substitute the copper transporter CopA in a strain lacking CopA, which thereby diminishes the effect of the deletion (7). Further studies are needed to understand the apparent redundancy of these Cu^{+} -ATPases, and the mechanisms of Cu transport among the associated proteins (8). However, the relative importance and interactions of GolT and GolB are beyond the scope of this study on CopA and CueO.

The structure and biochemical characteristics of most of the molecular elements in bacterial and *S. enterica* Cu^{+} distribution have largely been characterized. However, research still needs to be done to understand how all of these integrated systems function jointly, and to develop predictive mathematical models that quantify kinetic parameters such as flux rates Km, and affinities for singular functional elements in the *S. enterica* copper homeostasis network. Studying the roles and transport kinetics of the proteins CopA and CueO requires cloning, expression, and purification. Following various molecular biology and genetic methods to clone the genes of interest, these proteins are overexpressed in competent *E. coli* cells and then isolated through various purification steps. After, these proteins can be characterized and undergo further studies to better understand the functional roles of these genes in copper transport.

METHODS

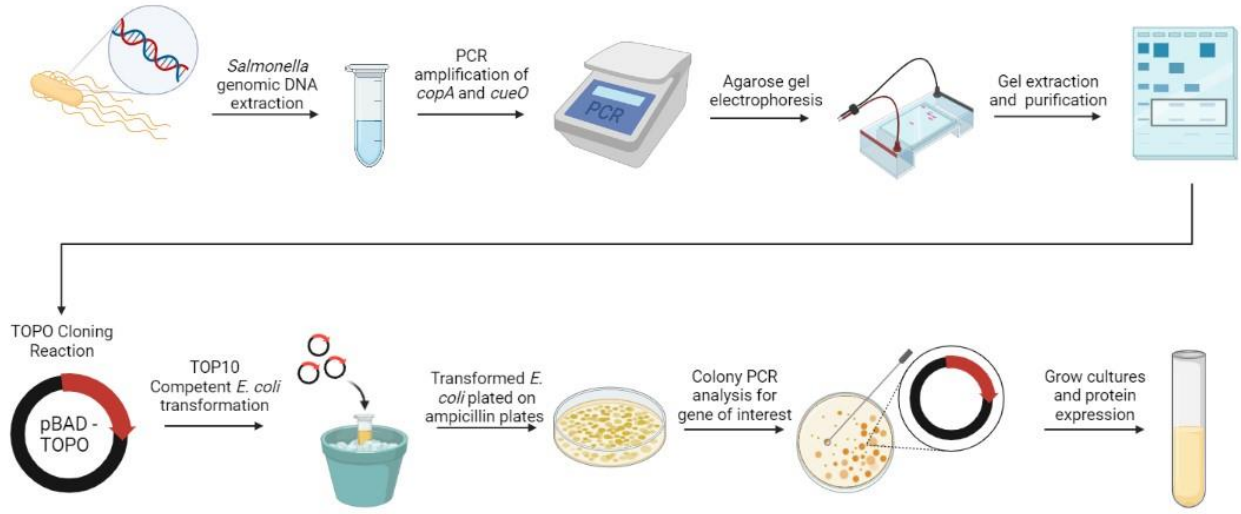

Figure 2. Graphical summary of cloning and expression methodology.

Bacterial strains and growth conditions. The *S. Typhimurium* and *E. coli* strains and plasmids used are listed in Table #. Bacterial strains were grown overnight at 37 °C in Luria broth (LB) with shaking or LB-agar plates. Ampicillin (Amp) was used at 100 μg ml^{-1}. Bacterial stocks were stored at -80°C with 20% glycerol. A final concentration of 2.5% L-arabinose was used when indicated to express CopA and CueO from a pBAD plasmid.

Genetic and molecular biology techniques. CopA and CueO proteins were cloned through pBAD TOPO TA expression kit. Genomic DNA was extracted from *Salmonella enterica* S. Typhimurium WT1344 using standard genomic DNA extraction protocols. *S.* Typhimurium WT1344 strain was removed from -80 °C freezer stocks and grown overnight at 37°C in 3 mL overnight LB, and then collected by centrifugation at 7000 x g for 5 minutes. The supernatant

was discarded, the pellet was washed twice with 2.5 mL of TES (10 mM Tris, 25 mM EDTA, 150 mM NaCl, pH 8.0), and then collected as proceeding above. The washed cells were resuspended in 275 uL of a 2 mg/mL lysozyme solution, and the suspension was transferred to a 1.5 mL microcentrifuge tube. After, 300 uL of a 10% Sarkoysl solution (N-Lauroylsarcosine) and 30 uL of 1 mg/mL proteinase K were added. The solution was then incubated for one hour at 55°C. After the incubation, the cells were disrupted by pipetting up and down with a P1000 micropipette. Then, one volume of isopropanol and 1/10 volume of 5M NH_4-Acetate were added, and the solution was mixed by inversion until the was visible as a milky white precipitate. The DNA was collected at 14000 x g for 10 minutes, the supernatant discarded, and then the pellet washed with 500 uL of 80% ethanol. The recovered DNA was dried to remove the ethanol, and then resuspended in 200uL of sterile water. Genomic DNA samples were stored at -20°C. Concentrations of extracted genomic DNA were analyzed using Nanodrop 2000 for DNA purity and contamination.

The *copA* DNA sequence was PCR cloned and amplified from the *Salmonella* chromosome using the primers *copA*-F (5'- ATGTCTCAAACTATCGACCTG - 3') and *copA* -R (5'- CGCTTTCGGTTTAAAACGCAA -3'). The *cueO* DNA sequence was PCR amplified using the primers *cueO* -F (5'- ATGTTACGCCGTGATTTCTTA -3') and *cueO* -R (5'- GACCGTAAATCCTAACATCAT -3'). A list of all primers used in this study are shown in Table 1. Oligonucleotides were synthesized by Azenta Life Sciences. PCR amplicons were produced using Q5 high-fidelity polymerase. Agarose gel electrophoresis was used to verify the quality of the PCR product on a low melt, 0.8% TAE agarose gel with ethidium bromide at 100 volts for 60 minutes. The gel was then read under UV light on an Azure600 gel reader.

Bands were then excised and purified through Nucleospin low-melt gel clean up kit according to the manufacturer's instructions. The eluted DNA was then stored at -20°C until needed for Taq polymerase incubation. PCR products were incubated with Taq polymerase because proof-reading polymerases such as Q5 remove the 3'A-overhangs necessary for TA cloning and generate blunt-ended products. Taq polymerase adds a 3'-adenine overhang to all PCR products, which is necessary for ligation into the PBAD vector. Taq polymerase possesses a non-template dependent terminal transferase activity that adds this single deoxyadenosine to the 3' ends of products. Gel-purified PCR products were incubated with Taq polymerase buffer, dATP, and 0.5 unit of Taq polymerase for 10-15 minutes at 72°C. The PCR product was then used immediately in the TOPO cloning reaction.

Following PCR amplification of *copA* and *cueO*, the PCR products were inserted into the PBAD vector according to manufacturer's instructions. The PBAD vector contains 3' – deoxythymidine residue overhangs that allows PCR inserts efficient ligation. The PBAD vector was incubated in a salt solution with the PCR product. The PBAD vector with the sequence insertion was then transformed into TOP10 competent *E. coli* cells. Cells were plated in LB ampicillin and grown overnight at 37°C for selection.

Ampicillin resistant colonies were selected to verify proper insertion and orientation of the transformed gene through colony PCR. Several TOP10 colonies were selected for CopA and CueO, dissolved in a small volume of distilled sterile water, and then added to a Taq polymerase reaction mix. Taq thermocycling was then conducted with an initial denaturation step at 94°C for

10 minutes. The resultant PCR was then assessed on an 0.8% TAE agarose gel with EtBR at 100 volts for 60 minutes. The results were viewed on an Azure600 imaging system under the UV setting for nucleic acid gels.

After colony PCR, the presence of the entire pBAD and *copA* or *cueO* locus was verified in a colony using DNA Sanger Sequencing at Azenta Life Sciences. Small cultures of the selected colonies were grown overnight, and plasmids were extracted using Zyppy Plasmid mini prep kit according to the manufacturer's instructions. DNA concentrations of the extracted plasmids were determined by Nanodrop 2000 UV-Vis spectrophotometer for nucleic acids. Extracted plasmids were also PCR amplified using Taq polymerase, and visualized on 0.8% TAE agarose gel prior to sequencing tom confirm the presence of the desired region in the plasmid PCR tubes to be sequenced were prepared with the appropriate volumes of extracted plasmid DNA and nuclease-free water for DNA concentrations between 600 and 800 ng per sample. The Sanger sequencing method can only sequence several hundred base pairs from a single primer. To overcome this issue for the longer sequences of *CopA* and *CueO*, sequence specific primers for *CopA* and *CueO* were designed for primer walking via Benchling Software and ordered from Azenta Life Sciences to ensure a primer could sequence a DNA segment every 600-700 base pairs. The primers used to sequence the transformed CopA and CueO plasmid DNA are listed in Table 1 of the Appendix.

Expression verification by immunostaining assay. A histidine tag immunostaining assay was used for qualitative assessment of CopA and CueO His-tagged protein following arabinose expression. To induce arabinose expression, 5 mL precultures in LB/Amp were grown overnight

and then 250 μL of preculture were added to another 5 mL culture of LB/Amp. The cells were grown until they reached an OD600 of approximately 0.6 after several hours. After, 1 mL of culture was collected and stored in a microcentrifuge tube to serve as the zero-time control. The remaining culture was induced with 0.05% arabinose (2.5% stock) and grown for three hours at 37°C. Following incubation, cells were then centrifuged at 14000 x g for 10 minutes in microcentrifuge tubes. The supernatant was discarded, and the pellet was conserved and then suspended in a small volume (200 uL) of Buffer B. The samples were then sonicated six times for 30 sec each to disrupt the cells. Following sonication, 10 uL of cell homogenate was dispensed on nitrocellulose membrane, and allowed to dry for one hour. The membrane was then incubated with a blocking solution of 1x PBS (phosphate buffered saline) + 5% nonfat dry milk for one hour. The blocking solution was poured off, and then the membrane was incubated with 20 mL of primary antibody: 1:1000 anti-his, rabbit antibody for one hour. The primary antibody was poured off, and then washed three times for five minutes each by 1x PBS + 0.1% Tween. The wash was removed, and then the secondary goat anti-rabbit IgG antibody with HRP at a concentration of 1:2000 was added. After one hour, the secondary antibody was removed, and the solution was washed again three times for five minutes each with 1x PBS. All the washing buffer was poured off, and then two mL each of the Reagent one and Reagent two of Pierce ECL Western Substrate were added for chemiluminescence. After 30 sec, the paper was removed, and then visualized on an Azure600 imaging system with varying exposure times from 10 sec to 40 sec.

Protein quantification by Bradford assay. The concentration of protein samples following arabinose induction and expression were quantified using the Bradford Assay. This assay is

based on the absorbance shift from 465 nm to 595 nm of an acidic solution of Coomassie Brilliant Blue G-250 when the colorant binds proteins. Hydrophobic and ionic interactions stabilize the anionic form of the dye, which causes a visible color change. This assay is useful and replicable because the extinction coefficient of the dye-albumin complex in the standard is constant over a 10-fold concentration range, which enables determination of an unknown protein's concentration within this range (16). Bradford's reagent was made with 50 mg Coomassie Brilliant Blue G-250, 50 mL methanol, 100 mL H_3PO_4 , and 500 mL distilled H_2O, then filtered, and filled to one liter with distilled H_2O. The Bovine serum albumin standard was made at a concentration of 1 mg/mL. Blanks and standards were prepared in triplicate as follows: 0 uL BSA standard + 20 uL H_2O; 2.5 uL BSA standard + 17.5 uL H_2O; 5 uL BSA standard + 15 uL H_2O; 10 uL BSA standard + 10 uL H_2O; and 15 uL BSA standard + 5 uL H_2O. After, 1 mL of Bradford reagent was added to each prepared sample, vortexed, and incubated for 10 minutes at room temperature. Absorbances were then measured at 595 nm, and plotted against concentration as a standard curve. A linear regression was applied to the plot, which produced an equation with x as concentration and y as absorbance. The unknown concentration of samples were then determined by measuring the absorbances of 10 uL and 20 uL of sample, and then substituting these values into the standard curve equation.

RESULTS

PCR cloning and amplification of CopA and CueO using *S*. Typimurium genomic DNA

To study the functional roles of CopA and CueO in *Salmonella* copper homeostasis, the genes for these proteins were cloned using the pBAD TOPO cloning procedure. First, genomic DNA was extracted *Salmonella enterica* S. Typhimurium WT1344, and used as a template to clone the CopA and CueO regions. The high-fidelity Q5 polymerase was used to clone these DNA fragments using specifically designed primers, and the result of the PCR was analyzed on a 0.8% TAE-agarose gel (Figure 3). The bands appeared at the expected locations with *copA* at 2500 bp and *cueO* at 1600 bp. The negative (-) controls are no primers and no genomic template, respectively. The positive (+) control is *cuiT* primers, which have previously been demonstrated to successfully amplify.

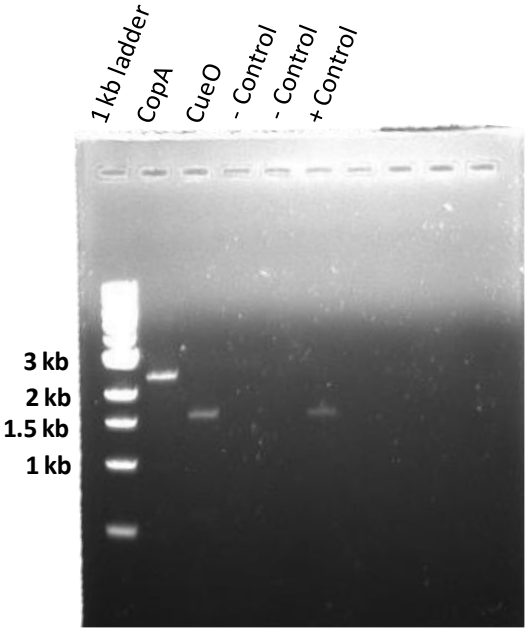

Figure 3: PCR cloning of *copA* and *cueO* genes from *Salmonella enterica* S. Typhimurium WT1344 genomic DNA. Nucleic acids visualized via electrophoresis on 0.8% agarose 1x TAE gel with ethidium bromide. Note the *copA* band at approximately 2500 bp, and the *cueO* band at 1600 bp. Negative (-) controls are no primers and no genomic template, respectively. Positive (+) control is *cuiT* primers, which have previously been demonstrated as successful.

pBAD TOPO cloning into *E. coli* with CopA and CueO DNA fragments

After positive visualization of the genes at the desired bands, the bands were excised from the gel and purified with a gel purification kit. The purified DNA was checked for purity on a Nanodrop spectrophotometer, underwent 3' Adenine overhang addition with Taq polymerase, and cloned into the pBAD vector. The pBAD vectors were transformed into competent TOP10 *E. coli* cells and screened on LB Ampicillin plates. Colonies were then screened using colony PCR, and colonies with the verified gene of interest were made into glycerol stocks. Figure 4 shows the colony PCR of CopA and CueO clones.

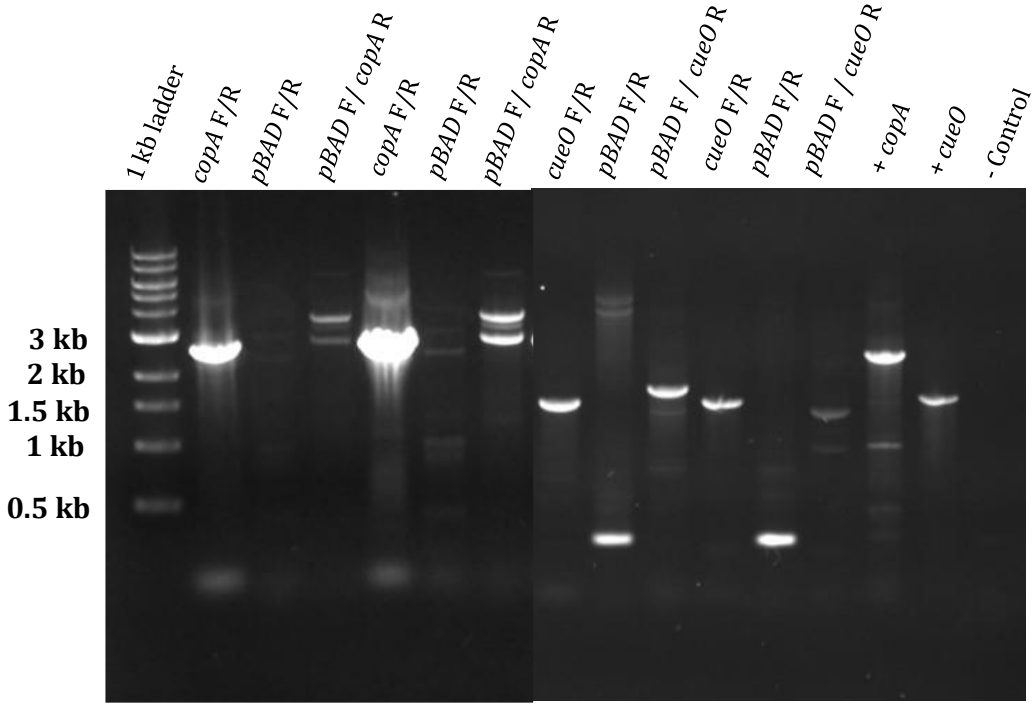

Figure 4. Colony PCR of clones run on 0.8% TAE agarose gel and run at 100 volts for 60 minutes. Each colony underwent primer restriction analysis with primers for the gene of interest, primers for the pBAD vector backbone, and a combination of primers of the gene and pBAD vector backbone to confirm the orientation of the insert. The positive (+) controls use *Salmonella* genomic DNA as a template, and the negative (-) control has no genomic template.

Expression optimization of CopA and CueO

A series of experiments were conducted to optimize the expression of CopA and CueO. The concentrations of arabinose were varied to determine the optimal conditions for expression of each protein. Cultures of CopA and CueO grown in LB Amp were induced with arabinose concentrations ranging from 0.1% to 0.0025%. After three hrs, samples were taken, centrifuged, and then suspended in buffer, and eventually loaded on nitrocellulose membrane. The top row on each membrane contains samples extracted from the supernatant of the resuspended sample. The bottom row, where protein expression is more visible, contains samples from the resuspended pellet.

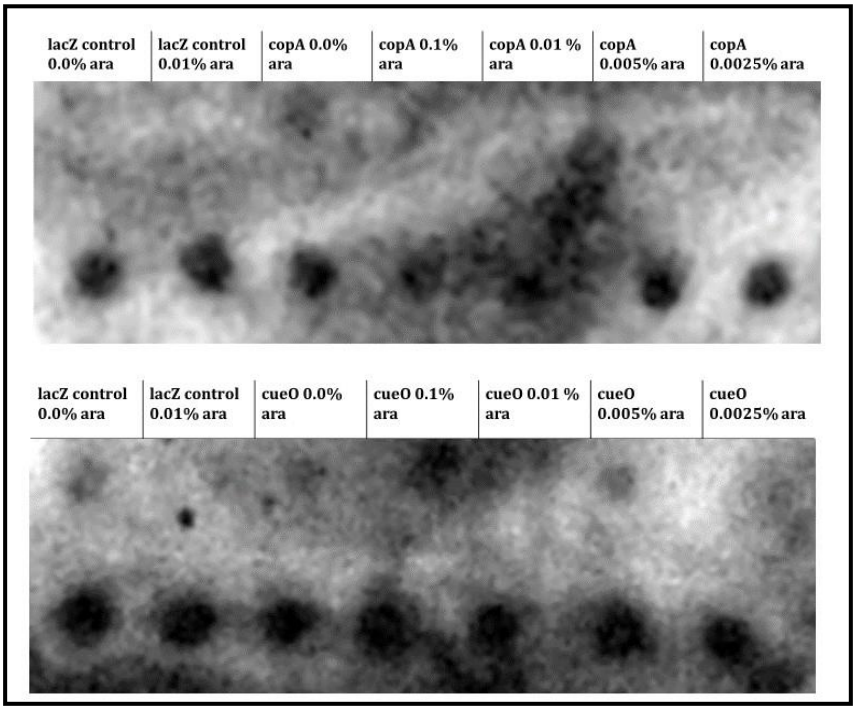

Figure 5. Immunostaining assay of CopA and CueO protein expression on a nitrocellulose membrane. Arabinose concentrations from 0.1% to 0.0025% were used for induction. The top row of each blot is from the supernatant, and the bottom rows are the resuspended pellet.

DISCUSSION

Copper is distributed in the cell compartments of *S. enterica* under conditions of cellular equilibrium. There are various elements and proteins enabling this delicate process, including sensors, influx and efflux transporters, chaperones, and cuproproteins. However, it remains unknown how these copper exchanging partners acquire, transport, and distribute intracellular copper with very high affinities in the femtomolar (10^{-15} M) range. To assess the entire Cu uptake kinetics, the CopA inner membrane efflux transporter and CueO multicopper oxidase from *S.* Typhimurium were cloned from *Salmonella* genomic DNA and expressed in TOP10 *E. coli*. Transgenic colonies were screened on LB ampicillin plates, and orientation of vector insertion in the pBAD-TOPO plasmid were determined via colony PCR analysis. Colony PCR analysis confirmed the direction of the inserts into the pBAD-TOPO plasmid. If the experiments were to be repeated, it would be beneficial and timesaving to use a directional cloning vector, such as the directional pBAD cloning system. Although new primers would be needed to ligate into the directional plasmid, the amount of time and resources needed to screen the clones for the correct gene orientation would be much fewer.

CopA and CueO clones were grown and then expressed using arabinose, and protein expressed detected using a Histidine tag immunostaining assay. Most of the protein expression for both CopA and CueO was visible in the resuspended pellet. CueO was expected to be expressed in the supernatant because it a soluble protein, while CopA is a membrane protein that should be found in the pellet. Additionally, there is visible protein for the 0.0% control and CopA and CueO samples, and this may indicate that these plasmids are constitutively active, which is not inconsistent with knowledge of this plasmid. There is no discernible difference

between the amount of protein expressed between the different concentrations. The experiment needs to be repeated, and it would be helpful to serially dilute each sample extract to view the range of expression.

The next direction in this research would be to grow large cultures of transgenic CopA and CueO *E. coli*, express these proteins, and purify the extracts. Arabinose would induce expression, and proteins would be purified through Ni-NTA affinity chromatography. During these steps, it is essential to conduct Western blot and SDS-PAGE assays to quantify the level of protein expression and verify the correct sizes of proteins. Unlike the immunostaining dot blot, these experiments would likely provide more detailed information about the proteins expressed. Following protein purification, verification of protein size, and quantification of protein concentration in purified extracts, the CopA and CueO proteins would undergo copper binding assays to determine copper binding kinetics.

This research will help elucidate Cu uptake kinetics in *S. enterica*, and how organisms such as this transport and distribute transition metals to prevent the harmful release of free Cu^{+}. Future research will ultimately continue to develop predictive mathematical models that quantify kinetic parameters such as flux rates Km, and affinities for singular functional elements in the *S. enterica* copper homeostasis network. The bactericidal role of copper in innate host defenses and pathogen copper detoxification systems may eventually yield novel antimicrobial agents that target this pathway.

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APPENDIX

Table 1.1 List of primers used in this study.

Primer Name	Sequence
CopA forward	5'- ATGTCTCAAACTATCGACCTG - 3'
CopA reverse	5'- CGCTTTCGGTTTAAAACGCAA - 3'
CueO forward	5'- ATGTTACGCCGTGATTTCTTA - 3'
CueO reverse	5'- GACCGTAAATCCTAACATCAT - 3'
pBAD forward	5' - ATGCCATAGCATTTTTATCC - 3'
pBAD reverse	5' - GATTTAATCTGTATCAGG - 3'
CopA sequence 585	5' - TATACCGGTCATGGTCTGGG - 3'
CopA sequence 1148	5' - AAGGCGACAGCGTTCATG - 3'
CopA sequence 1780	5' - AGCGGAAGGTCATCAACTGC - 3'
CueO sequence 597	5' - ACTGGATATTATGACCGCCGC - 3'
CueO sequence 1199	5' - ATGGCAACATGGATCACAGC - 3'

Table 1.2 Q5 polymerase PCR conditions for 25 uL reaction.

Component	25 uL Reaction	Final Concentration
5X Q5 Reaction Buffer	5 uL	1X
10 mM dNTPs	0.5 uL	200 uM
10 uM Forward Primer	1.25 uL	0.5 uM
10 uM Reverse Primer	1.25 uL	0.5 uM
Template DNA	Variable	< 1,000 ng
Q5 High-fidelity DNA polymerase	0.25 uL	0.02U/uL
Nuclease-free Water	To 25 uL	

Table 1.3 Taq polymerase PCR conditions for 25 uL reaction.

Component	25 uL Reaction	Final Concentration
10X Standard Taq Reaction Buffer	2.5 uL	1X
10 mM dNTPs	0.5 uL	200 uM
10 uM Forward Primer	0.5 uL	0.2 uM
10 uM Reverse Primer	0.5 uL	0.2 uM
Template DNA	Variable	< 1,000 ng
Taq DNA polymerase	0.25 uL	0.025U/uL
Nuclease-free Water	To 25 uL	

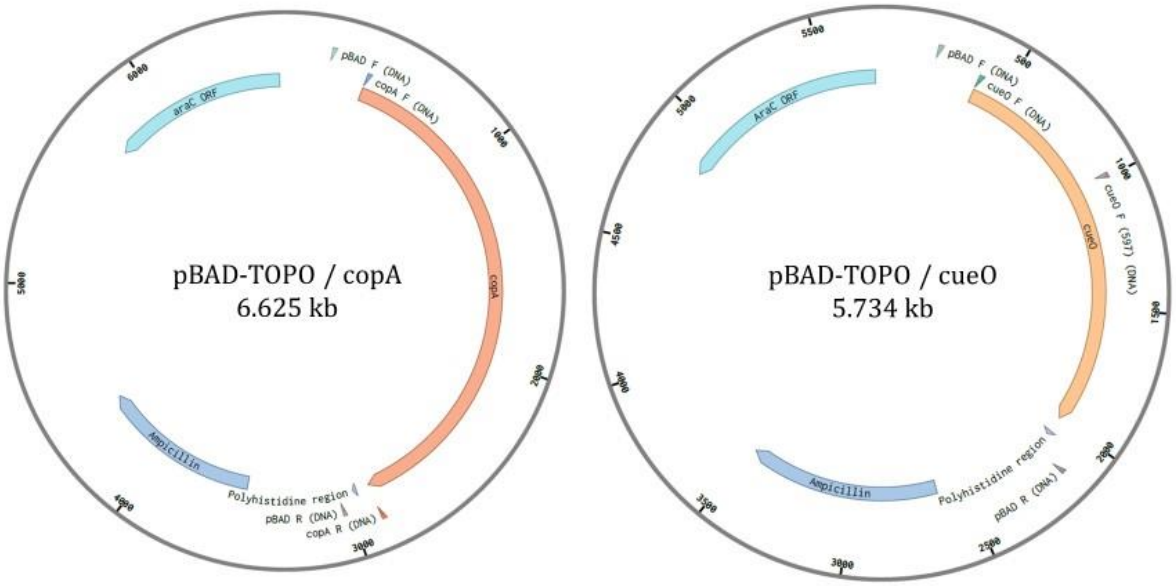

Figure 1.1 Plasmid maps of *copA* and *cueO* in the pBAD-TOPO vector.