IDENTIFICATION OF *E. COLI* CORE AND UROPATHOGEN-SPECIFIC GENES INVOLVED IN ADAPTATION TO COPPER STRESS

A Dissertation

by

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ABSTRACT

Urinary Tract Infections are a global health concern affecting approximately 7-11 million people in the United States annually. Uropathogenic *Escherichia coli* (UPEC) are the primary etiologic agent of uncomplicated UTIs, accounting for ~80% of infections in otherwise healthy women. With antimicrobial resistance on the rise for most pathogens, including UPEC, understanding mechanisms of bacterial immune evasion is paramount to developing novel therapeutics. We sought to characterize *E. coli* core and uropathogen-specific genes involved in mitigation of copper toxicity, a known host immune effector. To accomplish this, we conducted a genome-wide screen of a commensal *E. coli* mutant library and comparative transcriptomic profiling of UPEC to identify copper-responsive genes. We identified two arms of iron homeostasis, namely ferric-enterobactin uptake and ferrous iron transport, as playing a role in adaptation to copper stress. Here we proposed a model for enterobactin-mediated iron homeostasis as necessary to maintain adequate cellular iron levels to potentially outcompete copper and prevent mismetallation of key metalloenzymes. On the other hand, we identified FeoB, an inner membrane Fe^{2+} permease, to contribute to increased copper accumulation and stress in a commensal strain. However, our UPEC strain did not share this phenotype and may have adapted either FeoB or the genetic regulation of the encoding gene to limit FeoB-mediated copper accumulation. Collectively, our research highlights the complex and dynamic nature of bacterial metal homeostasis and begs for further research into the interplay between iron acquisition and copper toxicity. These studies provide novel insights into *E. coli* core and pathogen-specific copper response. Our findings have the potential for numerous future studies

which will lead to a greater understanding of host-pathogen interactions, providing novel targets for UTI therapeutics and preventatives.

DEDICATION

This dissertation is dedicated to…

God. Ad Maiorem Dei Gloriam

The love of my life, Trae. Every part of my life is greater because of you. I could fill all 200 pages expressing my love and gratitude for you, but for now I hope you know I will spend my whole life trying to love you in the way God calls me to.

Our current children, Rosemary and Augustine, and our future children. You are my motivation to be a better mother, wife, and person every day.

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This work was supervised by my dissertation committee consisting of Professors Sargurunathan Subashchandrabose, L. Garry Adams, and Sara Lawhon of the Department of Veterinary Pathobiology and Professor Keri Norman of the Department of Veterinary Integrative Biosciences.

For this first chapter, all authors played a role in writing, figure development, and literature search and review.

Experiments in Chapter 2 were performed by KCH, AC, SK, AG, and GLD. Data were by KCH, AC and SS. ST contributed key reagents. KCH and SS wrote the manuscript with feedback from all authors.

For Chapter 3, KCH, SK and GLD performed various experiments. Data were analyzed by KCH and SS. KCH and SS wrote the manuscript with feedback from all authors.

All other work completed for this dissertation was completed by KCH independently.

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NOMENCLATURE

E. coli	*Escherichia coli*
UPEC	Uropathogenic *E. coli*
S. enterica	*Salmonella enterica*
UTI	Urinary Tract Infections
Cu^{1+}	Cupric copper
Cu^{2+}	Cuprous copper
Fe^{3+}	Ferric iron
Fe^{2+}	Ferrous iron
Fe-Ent	Ferric-enterobactin
Mbp	Million base pairs
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
AIEC	Attaching and invading *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
PAIs	Pathogenicity-associated islands
LCN-2	Lipocalin-2
DHBA	2,3-dihydroxybenzoic acid
WT	Wild-type
DE	Differential Expression

GSH	Glutathione
ROS	Reactive oxygen species
GSSG	Glutathione disulfide
TGN	*trans*-Golgi network
IFN-γ	Gamma interferon
LPS	Lipopolysaccharide
H	Hour
HL-60	Human myelocyte cell line
IL-1 β	Interleukin 1β
IL-18	Interleukin 18
OHS	Occipital horn syndrome
RNAseq	RNA sequencing
CFU	Colony forming unit
RPM	Rotations per minute
BCS	Bathocuproinedisulfonic acid
OD	Optical density
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
Δcus	*ΔcusRSCFBA*

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION

Escherichia coli (*E. coli*) is a gram negative, ubiquitous, bacilliform bacteria and serves environmental, commensal, and pathogenic roles. It was first identified in 1884 by Theodor Escherich, a German microbiologist and pediatrician.[1,2] Due to its ease-of-use, fast-growing nature, and versatility, *E. coli* has become a model organism in a vast array of scientific fields. Additionally, the original use of *E. coli* in the 1940s, 1950s, and 1960s resulted in a breadth of knowledge about this single species that continues to be expounded upon today.[3,4,5] The combination of *E. coli*'s easy and versatile nature, relevance to human health, and the abundant foundational knowledge developed over the last 100+ years leads to a highly beneficial model organism.

E. coli is a member of the family *Enterobacteriaceae*, alongside other commonly known bacterial pathogens such as *Shigella, Salmonella, and Klebsiella*. It is a facultative anaerobic organism that measures about 1 μm long by 0.35 μm wide. The core genome of *E. coli* spans about 4.5 million base pairs (Mbp), while the pathogenic strains can have over 5.9 Mbp. The pan genome encompasses genes that are found in thousands of *E. coli* genomes and encodes a wide variety of additional features such as hair-like pili, biofilm formation, stress adaptation, and many others that contribute to fitness and virulence in diverse niches and hosts.

Commensal *E. coli* is primarily found in the lower intestine of the mammalian gut. Within this slightly acidic and anoxic niche, *E. coli* forms complex biofilms which allows it to survive in the nutrient-rich mucosal layer.[6,7,19] These biofilms and mechanisms for nutritional

competition allow it to maintain populations resulting in 10^6-10^9 cells per gram of fecal matter.[6,7,8] The *E. coli* strains present here are a small part of a larger gut microbiome of over 100 trillion microorganisms, including bacteria, viruses, and yeast.[9,10] Although some commensal strains will persist through life, antibiotic use and changes in diet or health can have a significant impact on gut microbiome composition. Additionally, strains of *E. coli* have adapted to survive environmental pressures such as variable temperatures, pH, oxygen levels, and nutrient availability and thus can persist outside of a host.[7]

Some *E. coli* pathotypes cause infection and disease within the intestinal tract. These pathotypes include but are not limited to: enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC). Extraintestinal Pathogenic *E. coli* (ExPEC) causes disease in the urinary tract, blood, spinal cord, and brain.[11,12] ExPEC may reside and exhibit commensal-like behavior in the intestinal tract but can use specific fitness and virulence factors to colonize the previously mentioned niches as opportunity arises. Shifting its nutritional needs and evading host immune defenses is paramount to *E. coli* to establish infection and cause disease.

Uropathogenic *E. coli* (UPEC) is the primary causative agent of Urinary Tract Infections (UTI), accounting for about 80% of infections.[13] UTIs generally manifest as either cystitis or pyelonephritis.[13] Cystitis is a lower urinary tract infection confined to the bladder, while pyelonephritis is an infection that has ascended to the kidneys. In some serious cases, urosepsis may occur. Additionally, UTIs can be categorized as uncomplicated and complicated UTIs. Uncomplicated UTIs are commonly defined as an infection in an otherwise healthy, pre-

menopausal, non-pregnant woman with no urogenital abnormalities. Complicated UTIs are defined as infections in men, children, the elderly, or those with previous urogenital health issues or abnormalities or those using urinary catheters. Although these distinctions were originally characterized to assist physicians with treatment plans, the nuances of the categories are ever-changing and being evaluated for their accuracy and helpfulness. Lastly, certain patients can present with asymptomatic bacteriuria, which is defined as having uropathogenic-positive urine cultures without any clinical symptoms. Among these categories and distinctions are differences in epidemiological factors such as incidence, transmission, and etiology.

UPEC transitioning from the intestinal tract to the urinary tract experience increased exposure to host immune response and decreased availability of nutrients. UPEC has acquired numerous horizontally transferred genes and pathogenicity islands (PAIs) to establish infection in the urinary tract. For example, the prototypical strain CFT073 has one of the largest genomes of any sequenced *E. coli* strain and has acquired 13 PAIs.[14,15] Interestingly, there is not a primary virulence or fitness factor determined to be crucial for establishing infection in the urinary tract, but rather a complex, dynamic and redundant use of factors that contribute to the UPEC pathogenesis. Some of the important factors can be divided into broad categories such as nutrient acquisition, motility, toxins, biofilm and bacterial communities, and outer membrane structures and proteins. For an extensive review, see Subashchandrabose and Mobley 2015, Lüthje and Brauner 2014, or Terlizzi, Gribaudo, and Maffei 2017.[16,17,18] Here, I will briefly discuss the most relevant fitness and virulence factors UPEC utilizes in to establish infection in the urinary tract.

Urine, compared to the nutrient-dense gut, is iron-limited, and moderately-oxygenated. Peptides and amino acids are the primary sources of both carbon and nitrogen in urine.[20,21] Transcriptomic and proteomic studies have shown an increase in genes and proteins related to gluconeogenesis, amino acid catabolism, and the TCA cycle in UPEC during growth in urine *ex vivo* and also during clinical UTI in humans and mice.[22,23,24] Nutritional immunity describes the ability of a host to alter trace metal content, either limiting essential metal bioavailability for the use of microorganisms or increasing concentrations to impose metal toxicity, and plays a key role in the innate immune response to UTIs. In the human host, bioavailability of iron is fairly low and is primarily found associated with host proteins such as transferrin, lactoferrin, ferritin and hemoglobin. UPEC has acquired and developed three main mechanisms of acquiring iron, namely, siderophores, ferrous-iron (Fe^{2+}) uptake systems, and hemophores. In 2014 Subashchandrabose et al. utilized RNA-seq to identify fitness genes differentially regulated in UPEC during clinical UTIs.[31] Among these, the Cus and Cue system were reported to be upregulated during infection. Since then, ceruloplasmin, a 151 kDa ferroxidase responsible for carrying approximately 95% of circulating copper in humans and many mammals, has been shown to be mobilized to the urinary tract during infections.[46] This study proposed a model where ceruloplasmin is able to simultaneously impose copper toxicity and limit the bioavailability of iron in the urinary tract in response to infection, encompassing two arms of nutritional immunity with one protein. Hyre et al. concluded that copper is mobilized as part of the host immune response to impede bacterial colonization and limit infection of the urinary tract.

These and other studies suggest iron and copper homeostasis at the host-pathogen interface is composed of highly complex and dynamic systems that are tightly regulated. With the rise of antimicrobial resistance, novel therapeutics are crucial to combating what the World Health Organization describes as a global health threat. Additionally, gaining insight into host-pathogen interactions will help researchers to not only develop new therapeutic strategies and enhance efficacy of current treatments, but also anticipate future bacterial evolutionary adaptations to keep modern medicine one step ahead of dangerous pathogens. We sought to address this need through an exploration of global response to copper stress in *E. coli*. Additionally, we wanted to compare adaptation to copper stress between commensal and pathogenic *E. coli.* This distinction will help to clarify what genes and proteins aid in promoting pathogen fitness during uropathogenesis.

First, we evaluated what is currently known about copper homeostasis in *E. coli* and closely related *Salmonella enterica*, which is discussed further in Chapter 2 and briefly described here.[47] Although our research is focused on *E. coli*, many copper detoxification mechanisms are conserved in *S. enterica*. Importantly, both species are gram negative pathogens of the *Enterobacteriaceae* family. Subsequently, both *S. enterica* and various forms of *E. coli* can cause infection within the human gut.

The primary mechanism of copper toxicity was originally thought to be the generation of reactive oxygen species (ROS) which can lead to destruction of cellular macromolecules.[52,54,55] Copper participates in Haber-Weiss/Fenton-like reactions, generating hydroxyl radicals as byproducts of their redox potential.[53] However, copper has been shown to be more toxic under

anoxic conditions and to even protect from superoxide stress.[56,57] These findings and others have pushed the field towards the potential for metalloenzyme mismetallation as the primary mechanism for copper toxicity. Copper sits at the top of the Irving Williams series because it is thermodynamically favored for the active sites of enzymes compared to other transition metal, forming more stable complexes.[58] This is paradigm is supported through studies such as the ability of copper to mismetallate iron-sulfur clusters within dehydratases involved in branched-chain amino acids.[59] Mismetallation by copper can lead to numerous downstream affects, including generation of ROS. Pathogens have adapted to combat this copper toxicity through the acquisition of copper detoxification systems.

The main mechanisms involved in efflux and detoxification of copper in *E. coli* were described in publications from Rosen and O'Halloran laboratories.[40-43] The two primary systems were named the Cue and Cus system. The Cue system is composed of the P-type ATPase, CopA, and the multicopper oxidase, CueO, which are controlled by the transcriptional regulator, CueR.[41,44,45] The Cus system is composed of a transmembrane efflux system encoded by *cusCBA*, a periplasmic cuprochaperone called CusF, and the corresponding two-component signal transduction gene-regulating system CusRS. These systems and other secondary copper detoxification mechanisms are explored further in the following chapters. Although the primary systems for copper detoxification have been identified and well-characterized, much is still unknown about *E. coli* copper-response and its role in UTIs.

To address this need, we then performed a genome-wide screen of *E. coli* K-12 under copper stress using the KEIO library.[48,49] This library contains 3,985 mutants in the *E. coli* strain

BW25113 background. All mutants have a single, non-essential gene removed and replaced with a kanamycin-resistance cassette. *E. coli*'s core genome is composed of ~4,400 genes, ensuring that the KEIO library has coverage of 91% of the core genome. The deficiencies are primarily in an inability to remove essential genes. This library is well established and has previously been utilized to screen genes involved with biofilm formation and antibiotic tolerance.[50,51] We used it to evaluate the majority of *E. coli*'s core genome for copper-responsive phenotypes. We chose this method, as opposed to a transposon mutagenesis approach, because it allowed us to cover almost the entirety of *E. coli*'s core genome efficiently and reliably. Our findings are discussed further in Chapter 3 but are stated here briefly. We found a total of 60 and 27 copper-sensitive and copper-resistant mutants, respectively. Among these, were multiple members of a siderophore (Enterobactin) iron acquisition system.

Siderophores are secreted, low-molecular mass, high-affinity, ferric (Fe^{3+}) iron-chelating molecules produced by a diverse group of microorganisms. Siderophores are categorized as either catecholate, hydroxamate, or thiazolidine.[25] Enterobactin and salmochelin are closely related catecholate-type siderophores. Salmochelin is a glycosylated variant of Enterobactin. The addition of 1-3 glucose moieties allows salmochelin to go undetected by host immune effector, Lipocalin-2 (LCN-2), whereas enterobactin is rendered ineffective for Fe^{3+} acquisition by LCN-2.[26] Aerobactin is the only identified hydroxamate siderophore synthesized by *E. coli,* however extracellular ferrichrome can be utilized by the cell.[27] Lastly, yersiniabactin is a thiazolidine-type siderophore produced by some UPEC strains. The prototypical strain CFT073, which our lab primarily utilizes, produces enterobactin, salmochelin and aerobactin, but does not have a

functional yersiniabactin system for Fe^{3+} uptake. UPEC can contain all 4 variants, while K-12 strains rely wholly on enterobactin for Fe^{3+}-siderophore acquisition.

Enterobactin is the prototypical siderophore conserved in every characterized *E. coli* strain thus far. The affinity of enterobactin (K_d of 10^{-52} M) is attributed to the three covalently-linked 2,3-dihydroxybenzoic acid (DHBA) catechol groups.[28] Transcription of enterobactin production and uptake genes is regulated by the ferric uptake regulator (Fur), which is derepressed in iron-limited environments.[29] Fur is a transcriptional repressor that responds to intracellular iron levels to maintain homeostasis. When iron bioavailability is sufficient, holo-Fur is able to bind to inverted repeats (Fur boxes) located near the promoter region of Fur-regulated genes, including those of the enterobactin biosynthesis and uptake. The binding of holo-Fur to the Fur boxes represses transcription by sterically inhibiting the binding of RNA polymerase to the promoter of the Fur-regulated gene. When intracellular iron levels are low, there is not enough bioavailable iron to form holo-Fur, resulting in apo-Fur. Apo-Fur has poor affinity for Fur boxes, thus allowing transcription of Fur-regulated genes, in this case enterobactin biosynthesis and ferric-enterobactin uptake.

Synthesis of enterobactin is divided into two steps: (1) the formation of 2,3-dihydroxybenzoate from chorismic acid and (2) the activation of L-serine and trimerization of the DHBA catechol groups (Fig. 1A).[30] The biosynthetic process for the first step is encoded by *entC*, *entB*, and *entA* genes, while the second step is catalyzed by EntE, EntF, and EntD proteins (Fig. 1A). After biosynthesis in the cytosol, apo-enterobactin is exported from the cytosol to the periplasm through the transporter EntS and from the periplasm to the extracellular milieu

through the outer membrane efflux pump, TolC (Fig. 1B). Once in the milieu enterobactin can sequester Fe^{3+} from iron-containing complexes or the rare free iron molecules. Ferric-enterobactin (Fe-Ent) is then imported into the cell through the outer membrane FepA β-barrel protein, powered by the inner membrane TonB-ExbB-ExbD energy transduction complex (Fig. 1B). Alternatively, Fe-Ent could be imported by Fiu, CirA and other TonB-dependent catecholate importers. Once in the periplasm, Fe-Ent is chaperoned by FepB to the inner membrane ABC transporter complex formed by FepD and FepG. The cytoplasmic ATPase FepC powers transport of Fe-Ent from the periplasm through the FepDG channel to the cytosol. Fes is the cytosolic Fe-Ent esterase that releases iron for cellular use by hydrolytic cleavage of the enterobactin backbone (Fig. 1B). While enterobactin has been detected in UPEC-infected UTI patients and transcription of enterobactin-related genes has been reported to be upregulated in UPEC strains from clinical UTI patients, our studies investigate the role of enterobactin in the face of copper stress in *E. coli*, a known antibacterial host effector.[31]

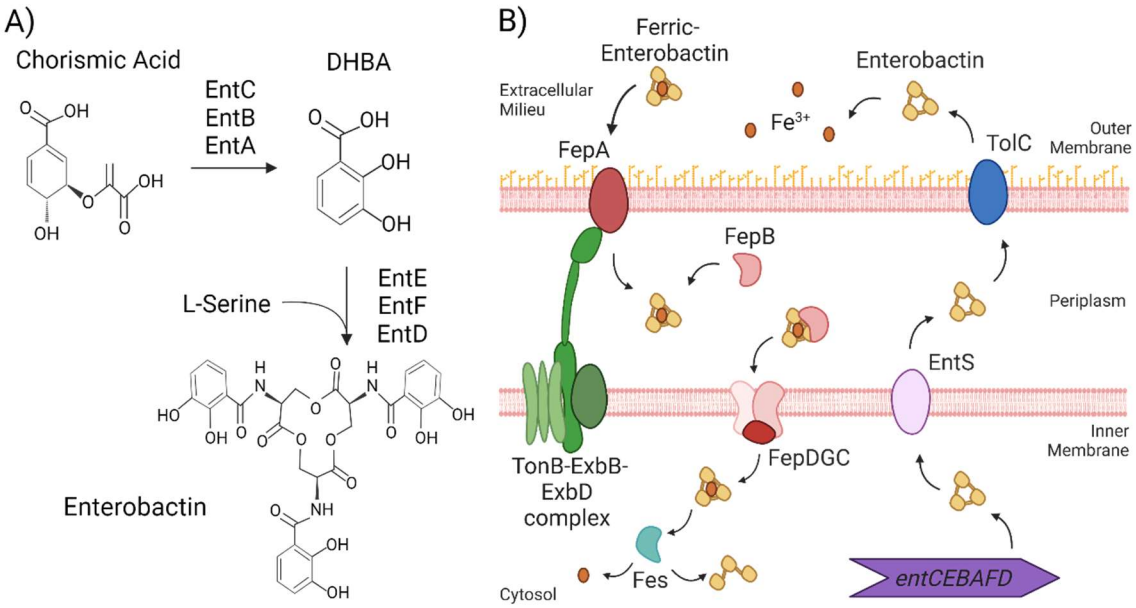

Figure 1. Enterobactin biosynthesis and uptake. A) Simplified schematic of the two-step reaction for enterobactin biosynthesis. EntC, EntB and EntA convert Chorismic acid to 2,3-dihydroxybenzoate (DHBA). EntE, EntF, and EntD convert DHBA to Enterobactin through the activation of L-serine and trimerization of catechol groups. **B)** Model of *E. coli* Fe-Ent uptake. Enterobactin synthesized by the *ent* genes is transported out of the cell by EntS and TolC. Fe-Ent is brought back into the cell through the Fep system which is powered by the TonB-ExbB-ExbD energy transduction complex. Fes allows for utilization of iron and recycling linearized enterobactin

We report that mutants defective in ferric-enterobactin iron acquisition are more sensitive to copper stress than the parental strain. We also demonstrated increased expression of Fe-Ent related genes under copper stress and a dysregulation of iron homeostasis. Taken together, our findings support a role for enterobactin-mediated iron acquisition in mitigating copper stress in *E. coli.*

Next, we wanted to evaluate the global response of pathogenic *E. coli* to copper stress with comparative transcriptomic profiling, which is discussed further in Chapter 4. Briefly, we utilized RNAseq and Differential Expression (DE) of mRNA to analyze RNA samples of UPEC CFT073 exposed to copper compared to controls. We identified 49 and 30 genes to be upregulated or down regulated, respectively, when exposed to physiologically-relevant copper levels. Among these are genes known to play a role in copper homeostasis, such as members of

the *cus* and *cue* system. Notably, two members of the Feo ferrous iron transport system, *feoA* and *feoB*, were found to be upregulated during copper stress in our UPEC strain.

While most research has been focused on ferric iron acquisition, ferrous iron uptake systems are additional machinery possessed by *E. coli* to maintain iron homeostasis. Fe^{3+} is primarily available in aerobic environments, while Fe^{2+} is the primary source of iron in anaerobic or acidic conditions. Soluble Fe^{2+} is primarily present in its free form, as opposed to Fe^{3+}, which primarily exists in complexes. A key difference between Fe^{3+} and Fe^{2+} transport is the ability of Fe^{2+} to traverse the outer membrane of gram-negative bacteria via porins. Therefore, Fe^{2+} transport machinery is responsible for bringing Fe^{2+} from the periplasm to the cytoplasm. There are 4 ferrous iron transporters characterized in *E. coli*: MntH, ZupT, EfeUOB and FeoABC.[32] MntH is a high affinity Mn^{2+} symporter that can also transport $Fe^{2+}$with less affinity.[33] ZupT is a divalent metal transporter with broad substrate specificity, supporting the transport of Zn^{2+}, Co^{2+}, Mn^{2+}, and Fe^{2+}.[34] The EfeUOB system is only functional in certain pathogenic strains, such as EHEC strain O157:H7 and UPEC CFT073.[35] Non-pathogenic K-12 strains contain EfeU as a pseudogene due to a frameshift mutation. Despite this, EfeO and EfeB are still produced by K12 strains. FeoABC is the only transporter specific to Fe^{2+} uptake and conserved in both pathogenic and non-pathogenic strains. For this reason, it is considered the primary bacterial ferrous iron transporter.

FeoABC is encoded by the *feoABC* operon and is regulated by both the Fur transcriptional repressor and FNR transcriptional activator, which is active under anaerobic conditions.[32] FeoA is a cytoplasmic protein composed of 250 amino acids. While the function of

FeoA has not been characterized, studies suggest that it may form a complex with FeoB, although the exact region of interaction is debated.[36,37] FeoB is the inner membrane permease consisting of 773 amino acids and has been shown to be necessary for Fe^{2+} transport, however the exact mechanism of transport is still unknown.[38] FeoC is believed to be an iron-sulfur cluster-dependent transcriptional repressor of the *feo* operon and is only present in γ-proteobacteria. Additionally, some studies suggest a role for FeoC in the protection of FeoB from Lon-mediated proteolysis during anaerobic conditions.[39] Although numerous biochemical and structural studies have been conducted on the Feo system, there is a large gap in the current understanding of the mechanism of ferrous iron transport and how it relates to pathogenesis. Moreover, to our knowledge, no studies have been published examining the interplay of UPEC and K-12 ferrous iron transport and copper toxicity or its role in UTIs.

Here we report, in the BW25113 K-12 background, a *feoB*-deficient mutant is more resistant to copper than the wild-type (WT). Surprisingly, a UPEC CFT073 *ΔfeoB* mutant only experienced slight resistance to copper stress. Gene expression suggests a *feoB*-deficient mutant under copper stress experiences decreased copper stress both in the cytoplasm and the periplasm, compared to its BW25113 parent strain. However, in UPEC CFT073, a *feoB*-deficient mutant experiences less cytoplasmic copper stress, but higher periplasmic copper stress. We also report a decrease in cellular copper accumulation in a *feoB*-deficient mutant compared to the BW25113 strain. This suggests FeoB contributes to copper stress, possibly by non-specific transport of copper into the cell during Fe^{2+} transport. It is possible UPEC CFT073 adapted to prevent FeoB-mediated accumulation of copper, allowing for higher fitness in the urinary tract. The difference in copper homeostasis between a non-pathogenic and a pathogenic strain underscores a strain-

specific role for genes that are otherwise highly conserved between commensal and pathogenic strains.

The following chapters discuss known copper homeostasis mechanisms, the results from a genome-wide screen and a transcriptomic analysis of *E. coli* under copper stress.

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2. COPPER HOMEOSTATIC MECHANISMS AND THEIR ROLE IN THE VIRULENCE OF *ESHERICHIA COLI* AND *SALMONELLA ENTERICA*[*]

2.1 Abstract

Copper is an essential micronutrient that also exerts toxic effects at high concentrations. This review summarizes the current state of knowledge on copper handling and homeostasis systems in *Escherichia coli* and *Salmonella enterica*. We describe the mechanisms by which transcriptional regulators, efflux pumps, detoxification enzymes, metallochaperones, and ancillary copper response systems orchestrate cellular response to copper stress. *E. coli* and *S. enterica* are important pathogens of humans and animals. We discuss the critical role of copper during killing of these pathogens by macrophages and in nutritional immunity at the bacterial-pathogen–host interface. In closing, we identify opportunities to advance our understanding of the biological roles of copper in these model enteric bacterial pathogens.

2.2 Introduction

Copper is an indispensable micronutrient required for several biological processes in organisms from all three domains of life.[1] Bioavailability of copper increased around two billion years ago during the Great Oxygenation Event due to the enhanced solubility of Cu^{2+} ions.[2]

[*]Reprinted with permission from "Copper Homeostatic Mechanisms and Their Role in the Virulence of *Escherichia coli* and *Salmonella enterica*" by Amanda Hyre[#], Kaitlin Casanova Hampton[#], and Sargurunathan Subashchandrabose. 2021. EcoSal Plus, Vol. 9, No. 2, Copyright [2021] by American Society for Microbiology. [#]Equal Contribution.

Copper is a transition metal that participates in redox reactions by cycling between cuprous (Cu^{1+}) and cupric (Cu^{2+}) oxidation states. Additionally, copper is at the top of the Irving-Williams series, resulting in the thermodynamically favorable mismetallation of metalloenzymes, particularly iron- and zinc-containing enzymes, with copper. The combination of redox-cycling properties and mismetallation of essential enzymes renders copper toxic when found in excess. *Escherichia coli* and *Salmonella enterica* are Gram-negative bacteria with significant impact on human and animal health globally. These bacteria are exposed to copper in the environment and within mammalian hosts. Toxicity of copper is mitigated in bacterial cells by buffering, efflux, and detoxification systems to limit levels of bioavailable and total copper. Here, we discuss the mechanisms involved in copper homeostasis in *E. coli* and *S. enterica* and the role of copper during interaction between hosts and these bacterial pathogens.

2.3 Bacterial Cuproenzymes

Bioinformatic analysis reveals that 72% of bacteria appear to utilize copper, primarily as a cofactor in enzymes.[3] Around 0.35% of proteins in *E. coli* have been predicted to be copper-containing proteins.[4] The bacterial cuproproteome is relatively small and is largely restricted to aerobic species.[3] Cytochrome oxidases are the predominant group of cuproenzymes, followed by nitrite/nitric oxide/nitrous oxide reductases, copper-zinc superoxide dismutase, multicopper oxidase, NADH dehydrogenase, and amine oxidase in *E. coli* and *S. enterica*.[5] These diverse groups of enzymes are involved in functions such as generation of proton gradient, denitrification, combating superoxide, oxidation of more toxic Cu^{1+} to less toxic Cu^{2+} ions, oxidation of NADH, and utilization of amines as a carbon and nitrogen source. Notably, these

proteins are localized in the inner membrane and/or periplasm, with no known instances of cuproenzymes in the cytoplasm of *E. coli* and *S. enterica*.

2.4 Copper Acquisition

Bacteria with a higher physiological requirement for copper utilize a cuprophore (methanobactin) or a copper specific transporter (CtaA, a P-type ATPase, or CcoA, a major facilitator superfamily protein) to meet the cellular demand for copper.[6-9] Copper-specific import systems have not been identified in *E. coli* and *S. enterica*, suggesting that these bacteria require only relatively low levels of copper. Import via porins and unidentified transporters and uptake of Cu^{2+}-yersiniabactin complexes have been reported as possible means of copper acquisition in *E. coli*.[10-13] Yersiniabactin is a siderophore produced by multiple Gram-negative pathogens, including several uropathogenic *E. coli* strains.[14] There are many gaps in the current understanding of the uptake and trafficking of copper prior to metalation of cuproenzymes in bacteria, and this is the subject of a recent review.[15] An *E. coli* cell is estimated to contain 1.7 x 10^5 copper atoms.[16] Zeptomolar (10^{-21} M) sensitivity of the copper-activated transcription factor CueR, however, suggests that most of this copper is localized outside the cytoplasm in *E. coli* and that copper is buffered to limit its bioavailability in the cytoplasm and periplasm.[17] Bacteria rely on copper export and buffering systems to maintain an infinitesimally low level of bioavailable copper in the cytoplasm. Glutathione (GSH) and other thiols can act as low-affinity buffers for copper in the cytoplasm.[18] *E. coli* and *S. enterica* utilize multiple metallochaperones, discussed below, to minimize levels of bioavailable copper. When copper is not exported or buffered, the periplasmic compartment, delimited by the inner and outer membranes in Gram-

negative bacteria, is the primary site of not only copper utilization but also copper-inflicted damage.

2.5 Copper Toxicity vis Generation of ROS

Historically, copper has been implicated in damaging macromolecules by participating in a Fenton/Haber Weiss reaction to generate hydroxyl radicals.[16,19-23] Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide are produced as metabolic by-products during respiration. During copper toxicity, Cu^{2+} ions are reduced by superoxide to Cu^{1+} and oxygen. Cu^{1+} is then oxidized to regenerate Cu^{2+} during nonenzymatic disproportionation of hydrogen peroxide, resulting in the production of hydroxyl radicals and hydroxide ions. The cyclic nature of these reactions generates a steady stream of the extremely reactive hydroxyl radicals, which then inflict oxidative damage on macromolecules in their vicinity. Since the periplasm is the most oxygenated and copper-replete cellular compartment in Gram-negative bacteria, multiple mechanisms discussed below are utilized to sequester copper and export it to the exterior.

2.6 Mismetallation of Proteins by Copper

Conversely, ROS-induced toxicity contradicts the observations on increased copper toxicity under anaerobic conditions, compared to aerobic environments.[24-26] Disruption of iron-sulfur clusters has emerged as a major molecular target for copper toxicity by imposing conditional auxotrophy of the branched-chain amino acids glutamine and glutamate in *E. coli*.[24,27] Macomber and Imlay published a paradigm-shifting study on the destruction of iron-

sulfur clusters in dehydratases involved in the biosynthesis of branched-chain amino acids (isoleucine, leucine, and valine) by copper.[24] Djoko et al. have reported exacerbation of copper stress in *E. coli* mutants lacking the copper efflux pump CopA and rescue of the copper-induced growth defect by supplementing glutamate and glutamine.[27] Copper induces intracellular scarcity of glutamate in *E. coli* by decreasing the activity of glutamate oxoglutarate aminotransferase, an enzyme containing iron-sulfur clusters.[27] While iron-sulfur clusters are the subject of investigations on copper-mediated mismetallation, it is worth noting that copper has the highest relative ligand affinity compared to other transition metals. The preferential metalation of copper is due to its location in the Irving-Williams series, and proteins containing iron, manganese, cobalt, nickel, and zinc as cofactors are all potential targets of mismetallation by copper.[28] Given the similarities in copper homeostasis and overall biology, it is not unreasonable to speculate that copper disrupts Fe-S clusters in *S. enterica*, a hypothesis that awaits experimental investigation. In addition to overt toxicity, copper also affects multiple facets of cellular biology and metabolism in *E. coli* and *S. enterica*, which is beyond the scope of this review and was recently reviewed by Giachino and Waldron.[29] In summary, copper exerts toxic effects via ROS-dependent and -independent mechanisms.

2.7 Copper Response and Defense Mechanisms

Bacteria mitigate the toxic effects of copper by utilizing copper-binding buffers, transcriptional regulators, efflux pumps, oxidases, and metallochaperones. Early studies in *Enterococcus hirae*, a Gram-positive bacterium, were instrumental in generating a framework for understanding copper homeostasis in bacteria.[30,31] Broadly conserved and lineage-specific copper

homeostasis systems have been identified in numerous bacteria, including pathogens.[5,29,32] Here, we discuss, compare, and contrast the mechanisms utilized by *E. coli* (Fig. 1) and *S. enterica* (Fig. 2) to combat toxicity and ultimately restore the homeostasis of copper in the cytoplasm and periplasm (Table 1).

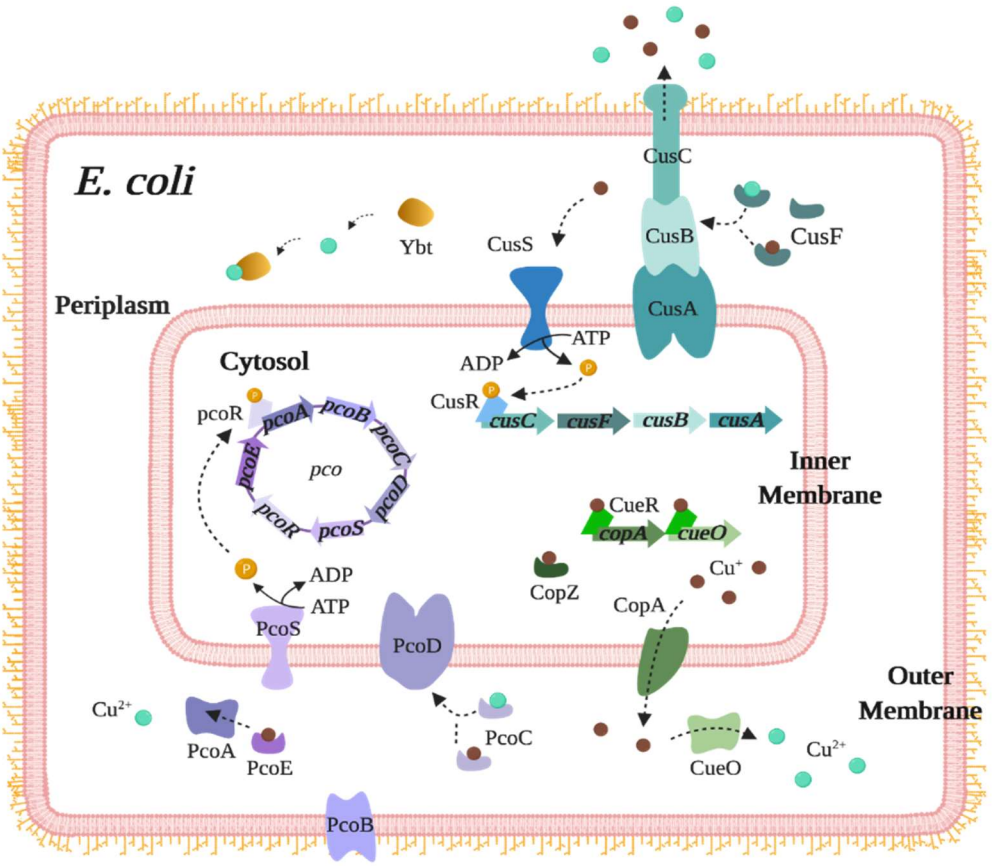

Figure 1. Overview of the Copper Detoxification Systems in *E. coli*. The CueR regulon (green), composed of CopA and CueO, plays a central role in handling excess copper in *E. coli*. Cu^{1+}-bound CueR is the transcriptional activator for *copA* and *cueO* genes. CopA is an inner-membrane ATPase that pumps Cu^{1+} from the cytoplasm into the periplasm. CueO is a periplasmic multicopper oxidase that converts the more toxic Cu^{1+} to the less toxic Cu^{2+}. Another mechanism

for copper detoxification is the Cus system (blue), comprising the transmembrane efflux pump, cusCBA. The periplasmic copper chaperone CusF escorts Cu^{1+} and Cu^{2+} to the CusCBA complex. CusS hydrolyzes ATP upon sensing copper to phosphorylate CusR and activates transcription of the *cusCFBA* operon. The plasmid-borne *pco* operon is found only in selected strains of *E. coli* and is regulated by the PcoSR two-component system. The siderophore yersiniabactin binds to sequester Cu^{2+}, thereby preventing it from reducing to the more toxic Cu^{1+}.

2.7.1 The copper-sensing transcriptional regulator CueR

CueR is a copper-responsive transcriptional regulator belonging to the MerR family.[17,33-35] CueR proteins from *E. coli* and *S. enterica* are highly conserved, with 89.6% identity. The *E. coli* CueR regulon is composed of *copA* and *cueO* genes, encoding a copper-specific inner membrane ATPase and a periplasmic copper-dependent multicopper oxidase, respectively. The CueR regulon in *S. enterica* includes *cueP*, encoding a periplasmic copper-binding protein, in addition to *copA* and *cueO*.[36] *copA* and *cueR* genes are separated by the *ybaS* and *ybaT* genes, which are involved in the glutamate-dependent acid tolerance system in *E. coli*.[27] In *S. enterica*, *copA* and *cueR* are located adjacently in the genome and divergently transcribed.[37] Despite variation in genetic organization, functions of CueR and CopA are essentially identical in these bacteria. CueR exists as a dimer and coordinates one Cu^{1+} ion per monomer in *E. coli*.[17] The dimer binds its target promoter whether bound to copper or not, but only holo-CueR activates the transcription of genes under its control by unwinding the DNA.[38] CueR binds to an inverted-repeat containing consensus sequence (CCTTCCNNNNNNGGA AGG) known as the CueR box

in the promoter region of *copA* and *cueO* genes in *E. coli*.[39] CueR also activates the transcription of *cueO*, *copA*, and *cueP* genes during copper stress in *S. enterica*.[40]

2.7.2 The copper efflux pump CopA

The primary mechanism of defense against cytoplasmic copper in *E. coli* and *S. enterica* is efflux by the inner membrane P1B-type ATPase, CopA, which pumps Cu^{1+} ions from the cytoplasm into the periplasm.[37,41] Specifically, these organisms encode a CopA1-type of ATPase that demonstrates a higher rate of copper efflux from the cytoplasm to the periplasm, compared to CopA2-type pumps. CopA proteins from *E. coli* and *S. enterica* exhibit 92.7% identity. A BLAST search reveals that CopA is highly conserved in multiple biomedically important genera in the family *Enterobacteriaceae*, including *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Shigella*. One of only two known chromosomally encoded soft-metal translocating ATPases in the *E. coli* genome, CopA shares no overlapping specificity with ZntA, which translocate Zn^{2+}, Cd^{2+}, and Pb^{2+}.[41] CopA is also important for delivery of copper ions to the copper-zinc-containing periplasmic superoxide dismutase SodCII in *S. enterica*.[42] Osman et al. demonstrated an accumulation of inactive apo-SodCII in the periplasm of *S. enterica* mutants lacking both CopA and the gold/copper transporter GolT, but not in single mutants, indicating that one of these proteins must be present for delivery of copper to metalate and thereby activate SodCII.[42] CopA is also involved in copper resistance in a closely related enteric bacterium, *Klebsiella pneumoniae*.[43] Once in the periplasm, Cu^{1+} ions could be oxidized to Cu^{2+} ions, sequestered by chaperones, incorporated into cuproenzymes, and/ or exported across the outer membrane.

2.7.3 The copper chaperone CopZ in *E. coli*.

Recent reports reveal that CopZ is an intracellular copper chaperone in *E. coli* that is, surprisingly, encoded by *copA*.[44,45] The full-length *copA* transcript leads to the production of CopZ by a programmed ribosomal frameshifting mechanism, in addition to translation to CopA. A truncated version of the *copA* transcript is also generated in *E. coli* and translated to CopZ. This is a remarkable mechanism of gene optimization where a single gene codes for both an efflux pump and a metallochaperone. Sequence alignment and homology analysis reveals that the nucleotides responsible for ribosomal frameshifting (CCCAAAG) resulting in production of CopZ production in *E. coli* share 100% homology with those in *S. enterica* serovar Typhimurium strain SL1344. While this observation suggests the presence of a conserved mechanism, whether CopZ is produced and utilized as a copper chaperone in *S. enterica* remains to be validated experimentally.

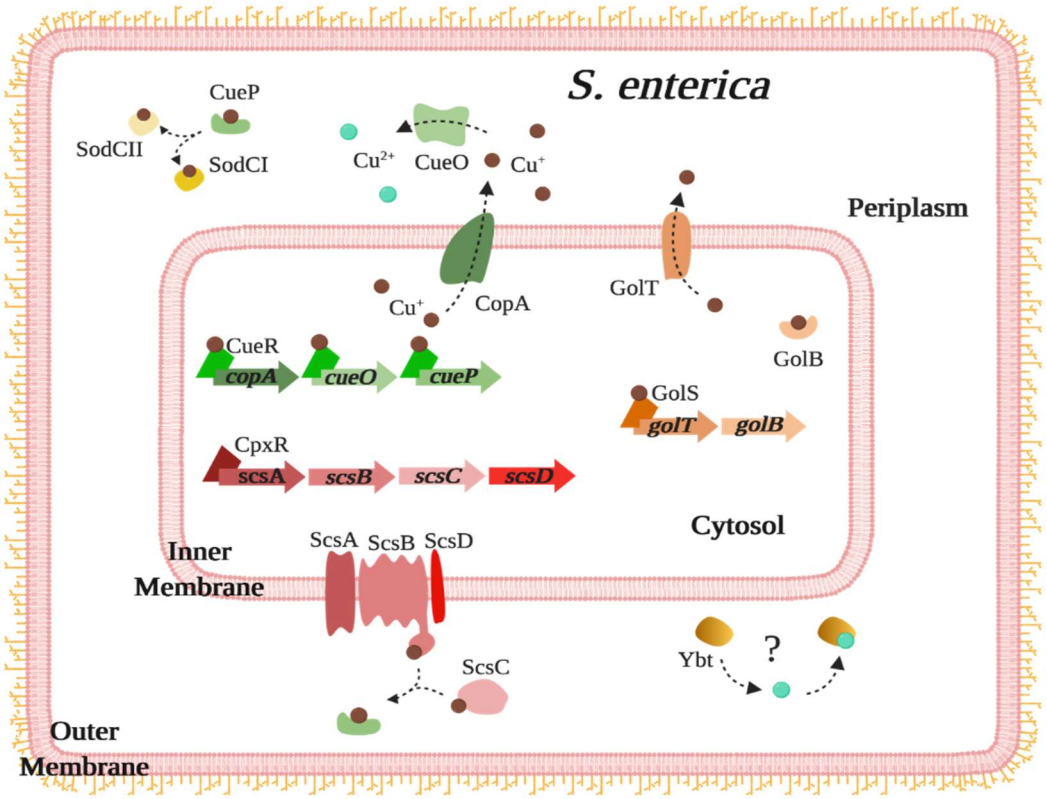

Figure 2. Overview of the Copper Detoxification Systems in *S. enterica.* The CueR system of *S. enterica* is identical to that of *E. coli*, apart from an additional periplasmic copper chaperone, CueP. CueP delivers copper to superoxide dismutases, SodCI and SodCII. The Gol system, transcriptionally regulated by the sensor GolS, includes the GolT inner-membrane Cu^{1+} efflux pump and the cytoplasmic chaperone GolB. ScsB and ScsC proteins from the disulfide reductase system bind and deliver copper to CueP. Some *S. enterica* strains encode Cus and Pco systems, identical to that of *E. coli* (not depicted here; see Fig. 1). The siderophore yersiniabactin is also produced by *S. enterica,* but its role in copper resistance remains to be determined.

2.7.4 The GolST-B system in *S. enterica*

The Gol system is an additional copper efflux system that is encoded in the chromosome of *S. enterica*, but not in *E. coli*.[46] As the name indicates, this system was originally identified for its induction of expression by gold.[46] Cupric GolS, a gold/copper-responsive transcriptional regulator, induces the expression of golST and golB loci.[40] GolS, like CueR, belongs to the MerR family of transcriptional regulators and exhibits a comparable level of affinity for copper.[42,47] Unlike CueR, which senses only copper, GolS responds to the bioavailable levels of both copper and gold in the cytoplasm. The golT gene encodes a CopA-like inner membrane P_{1B}-type ATPase. GolT and CopA restore wild-type levels of copper resistance in mutants lacking both efflux pumps, indicating a redundant role.[48] In the absence of functional CopA, GolT confers copper resistance by efficiently effluxing copper ions from the cytoplasm to the periplasm.[37] Efflux of Cu^{1+} by GolT plays a critical role in metalation of virulence-associated periplasmic superoxide dismutase, SodCII, analogous to the role of CopA in metalating SodCII.[42] The *golB* gene encodes a cytoplasmic gold/copper chaperone with a function reminiscent of CopZ in *E. coli*.

2.7.5 The periplasmic multicopper oxidase CueO

CueO, also sometimes known as CuiD in *S. enterica*, is a periplasmic copper-dependent multicopper oxidase that oxidizes Cu^{1+} to its less toxic Cu^{2+} state, thereby limiting copper-mediated damage to the bacterial cell.[49-51] Apo-CueO is secreted via the twin-arginine translocation pathway, and copper is incorporated into the active site within the periplasm of *E.*

coli.[52] CueO is an exception among proteins transported by the twin-arginine translocation pathway that typically secretes folded holo-proteins. Additionally, CueO oxidizes enterobactin and 2,3-dihydrobenzoic acid (an intermediate in the enterobactin biosynthetic pathway) to preempt reduction of Cu^{2+} ions to more toxic Cu^{1+} ions by these siderophores.[53] This process decreases the efficiency of enterobactin-mediated iron uptake during copper stress, as indicated by overaccumulation of iron in an *E. coli* mutant lacking CueO.[51] CueO plays a significant role in combating periplasmic copper stress in both *E. coli* and *S. enterica* under aerobic conditions.

2.7.6 The periplasmic copper metallochaperones CueP and CusF

CueP binds Cu^{1+} ions delivered by CopA and GolT with high affinity (10^{-15} M) and is an integral effector of protection from copper toxicity in *S. enterica*.[36,48,54] In comparison, CusF, found in the periplasm of *E. coli*, has a lower affinity for Cu^{1+} at 1.8×10^{27} M than CueP.[55] It is interesting that CusF from *E. coli*, which uses the CusCBA efflux system to decrease periplasmic copper bioavailability, has a lower affinity for Cu^{1+} than CueP from *S. enterica*, which lacks a known periplasmic copper efflux mechanism. An accumulation of inactive apo-SodCII is observed in an *S. enterica* mutant lacking cueP, indicating a critical role for CueP in the metalation of SodCII.[42] In contrast to CueP, which is regulated by CueR (a cytoplasmic copper sensor), CusF, regulated by CusS (a periplasmic copper sensor), does not appear to play a role in the metalation of SodC in uropathogenic *E. coli* (UPEC).[56] Expression of CueP is coordinately regulated by both CueR, which detects free cytosolic copper, and CpxR/CpxA, which monitors envelope stress.[36] CueP has been proposed as a partial functional equivalent of the Cus transenvelope copper efflux system found in *E. coli*.[26] *E. coli* possesses the periplasmic copper

chaperone CusF, which is discussed below in conjunction with other members of the Cus system.[57]

2.7.7 Periplasmic disulfide reductase ScsC/B pathway in *S. enterica*

The suppressor of copper sensitivity (*scs*) operon was originally identified and characterized for its role in protection from copper toxicity in *S. enterica*.[58-60] ScsA, ScsB, and ScsD are inner membrane proteins, whereas ScsC is a soluble periplasmic protein.[54] Transcription of the *scs* locus is upregulated by copper in a CpxAR-dependent mechanism.[59] ScsA conveys resistance against hydrogen peroxide and does not appear to affect sensitivity to copper.[61] Loss of *scsB* imparts copper sensitivity of a magnitude equivalent to that seen in a mutant lacking *scsBCD* genes, indicating its eminent role in copper resistance among Scs proteins.[59] Binding of Cu^{1+} ions by ScsB and ScsC with high affinity (~10^{-14} M) and delivery to periplasmic copper chaperone CueP are the mechanistic basis of Scs-mediated resistance to copper.[54]

2.7.8 The transenvelope copper efflux system CusCBA and CusF

E. coli minimizes periplasmic copper levels by utilizing the Cus copper efflux complex, which is controlled by a copper-responsive two-component regulatory system.[49,62] The Cus system is comprised of the structural proteins CusCFBA, and the two-component system CusRS encoded by the divergently transcribed cusCFBA and *cusRS* operons.[49,63] The *cus* system contributes to reinstating copper homeostasis under low-oxygen conditions and also during

exposure to near-lethal levels of copper.[64] CusR is responsive to both copper and silver ions, and the Cus system also confers protection from silver toxicity.[65,66] Upon phosphorylation by CusS during copper stress, CusR binds to the inverted repeat AAAATGACAANNTTGTCATTTT, located upstream of *cusCFBA* genes.[39] Transcription of Cus structural genes is also activated by the hydrogen peroxide-responsive two-component regulatory system HprRS, and copper induces the expression of *hprRS* via CusRS.[39,67] The CusCBA structural proteins assemble to form a tripartite, transenvelope copper transporter complex, and CusF is a soluble periplasmic Cu^{1+} chaperone. CusA is an inner membrane proton-substrate antiporter, driven by the proton motive force, containing multiple metal binding sites.[63,64] CusB is a periplasmic adapter protein which links CusA with CusC.[64] CusC is an outer membrane factor possessing no known metal binding sites.[64] Together, these proteins form a channel between the inner and outer membranes, traversing the periplasm.[64] The individual components exist in a disassembled form to minimize the impact on plasticity and dynamic function of the periplasm in the absence of toxic levels of copper or silver ions.[68] CusF delivers Cu^{1+} ions expelled by CopA to the CusCBA complex for export from the cell.[57,63] Recently, the *cus* locus was identified in an integrative conjugative element in antibiotic-resistant strains of *S. enterica*.[69] In summary, CusCBA, aided by CusF, has the unique role of transporting periplasmic copper to the extracellular space.

2.7.9 The plasmid-borne copper resistance system Pco

Plasmid-encoded *pco* operon provides an additional layer of copper tolerance to the chromosomal *cue*, *gol*, and *cus* systems in *E. coli* and *S. enterica*.[70,71] Protein products of the *pco* operon include soluble periplasmic proteins (PcoA, PcoC, and PcoE), an inner membrane copper

pump (PcoD), and an outer membrane copper transporter (PcoB).[72] Djoko et al. demonstrated that PcoA and PcoC interact to oxidize Cu^{1+} ions to Cu^{2+} ions and that they might also interact with PcoB to export copper.[72] PcoC also plays an integral role in supplying copper for proper assembly of PcoA.[72] The CusRS two-component system also induces expression of the plasmid-borne *pcoE* gene in *E. coli*.[62] Other *pco* genes are responsive to the cognate *pco*-specific two-component regulatory system, PcoSR, and are not responsive to activation by CusRS.[62,72] The *pco* locus is typically found across diverse *E. coli* isolates, particularly those originating from swine, due to the use of high levels of copper in animal feed.[73] Recently, *E. coli* harboring the *pco* operon was isolated in Europe from the urine of patients with urinary tract infection.[74] This finding underscores the urgent need for responsible use of trace elements in food animal production to mitigate the spread of metal resistance elements, which are often co-selected with genetic determinants of antibiotic resistance.

2.7.10 Siderophore-mediated protection from copper

Bacteria elaborate high-affinity, iron-chelating molecules known as siderophores to acquire iron.[14] Various siderophores are elaborated by *E. coli* and *S. enterica* strains and may include enterobactin, salmochelin (glycosylated enterobactin), aerobactin, and/or yersiniabactin.[12,75] Enterobactin is produced by both commensal and pathogenic *E. coli* strains, while salmochelin, aerobactin, and/or yersiniabactin are utilized by pathogenic strains.[14] In contrast, *S. enterica* strains produce at least enterobactin and salmochelin, often in conjunction with other siderophores.[12] In addition to its canonical role as a siderophore, yersiniabactin produced by UPEC also binds to copper and other metals, including cobalt, chromium, gallium,

and nickel.[76,77] In the presence of high levels of bioavailable copper in the milieu, yersiniabactin facilitates copper resistance by binding and sequestering Cu^{2+} ions, thereby preventing its reduction to the more toxic Cu^{1+} ions.[78] Oxidized products of enterobactin and 2,3-dihydroxybenzoic acid generated by CueO also act as a sink to sequester copper in *E. coli*.[53] Enterobactin also conveys protection from copper toxicity in *S. enterica*, comparable to its role in *E. coli*.[40] In summary, enterobactin and yersiniabactin confer added protection from copper toxicity in *E. coli* and *S. enterica*.

2.7.11 The sigma E-regulated small RNA MicL and membrane lipoprotein Lpp in *E. coli*

CutC has been proposed to be a cytoplasmic copper-binding protein in *E. coli*, and a mutant lacking cutC is more sensitive to copper than its parental strain.[79] It is now known that copper tolerance previously attributed to CutC is conferred by MicL, a σ^E-dependent small regulatory RNA, encoded within the *cutC* gene.[80] The transcriptional response to maintain homeostasis during membrane stress is governed by σ^E.[81] Independently, loss of σ^E itself also renders *E. coli* more sensitive to the toxic effects of copper.[13] MicL represses the translation of Lpp, an outer membrane lipoprotein, which is also the most abundant protein in *E. coli*.[80] The mutant lacking cutC also lacks micL and produces high levels of Lpp, leading to increased copper sensitivity.[79,80] Enhanced resistance to copper in a mutant lacking *lpp*, compared to the parental strain, further supports the link between Lpp levels, response to copper, and extracytoplasmic stress.[80] In summary, the σ^E -MicL regulatory pathway plays an important role in protection from copper toxicity by decreasing the levels of Lpp in *E. coli*.

Table 1. Effectors of copper homeostasis in *E. coli* and *S. enterica*

Gene	Protein	Function
cueR	CueR	Copper-responsive transcriptional activator
copA	CopA	Inner membrane ATPase for efflux of cytoplasmic copper
copA	CopZ	Cytoplasmic chaperone
cueO/cuiD	CueO/CuiD	Periplasmic Multicopper oxidase
cueP	CueP	Periplasmic binding protein
cusS	CusS	Copper-responsive inner membrane kinase of CusR
cusR	CusR	Transcriptional activator
cusA	CusA	Inner membrane antiporter
cusB	CusB	Periplasmic adapter
cusC	CusC	Outer membrane channel
cusF	CusF	Periplasmic chaperone
golS	GolS	Gold/copper-responsive transcriptional activator
golT	GolT	Inner membrane ATPase for efflux of cytoplasmic copper
golB	GolB	Cytoplasmic chaperone
cutC	MicL (sRNA*)	Post-transcriptional regulator of copper sensitivity

*Small non-coding regulatory RNA

2.7.12 Periplasmic disulfide reductase DsbC in *E. coli*

Disulfide bond formation in the periplasm is critical for structural and functional integrity of many periplasmic and secreted proteins in Gram-negative bacteria. Copper triggers the formation of erroneous disulfide bonds in periplasmic and secreted proteins in *E. coli*.[82] Consequently, disulfide isomerase/reductase DsbC is required to resolve such nonnative disulfide bonds introduced by copper. *E. coli* mutants lacking DsbC exhibit higher sensitivity to copper than the parental wild-type strain, revealing the impact of copper stress on the structure of extracytoplasmic proteins.[82] Whether DsbC interacts directly with copper needs to be tested, in light of the recent findings in *S. enterica* showing that ScsC and ScsB proteins involved in disulfide reduction bind copper.[54]

2.7.13 Glutathione in *E. coli*

Balance between the reduced (GSH) and oxidized (glutathione disulfide [GSSG]) forms of glutathione (L-g-glutamyl-L-cysteine-glycine) plays a critical role in maintaining a reductive milieu in the cytoplasm. Glutathione is involved in mitigating sensitivity to copper in mutants that are defective in copper efflux.[83-85] Failure to produce glutathione, however, has no impact on copper sensitivity in wildtype *E. coli* cells, suggesting a limited role for glutathione in combating copper toxicity.

2.7.14 Functional genomic analysis of copper stress in *E. coli* and *S. enterica*

DNA microarray studies have revealed remarkable similarities in the copper-responsive transcriptional profiles of *E. coli* and *S. enterica*.[39,86,40] As expected, the known copper efflux and detoxification genes *copA*, *cueO*, and *cusCFBA*, regulated by CueR and CusR, are upregulated in *E. coli* during growth in copper-supplemented media. In addition, CpxAR (the key regulatory system for transcription of genes involved in maintaining cell envelope homeostasis), YedVW (renamed HprRS, a two-component system that senses and responds to hydrogen peroxide stress), and SoxS (the master regulator of transcriptional response to superoxide) are also activated during copper stress in *E. coli*. Exposure to high levels of copper also induces the expression of genes encoded in the enterobactin operon, suggesting an increase in cellular demand for iron during copper stress.[86] High CueO activity during copper stress also disrupts enterobactin-dependent uptake of iron in UPEC. A mutant lacking CueO accumulates iron at a higher level than the parental strain and, as a result, exhibits increased virulence in the murine model of urinary tract infection (UTI).[51] Genes under the regulatory control of CpxAR are also highly expressed in copper-intoxicated *S. enterica*.[40] Additionally, copper triggers the expression of the *sitABCD* ferrous iron/ manganese ABC transporter and enterobactin biosynthetic genes. Utilizing a mutant deficient in enterobactin production, Pontel et al. demonstrated a protective role for enterobactin against copper toxicity in *S. enterica*.[40] While *soxS* is induced by copper stress in *S. enterica*, none of the genes from the SoxS regulon were identified as copper-responsive genes. Taken together, these studies highlight the extensive cross talk between regulation of copper homeostasis and other critical cellular processes, such as maintenance of envelope homeostasis, management of iron metabolism, and response to reactive oxygen species.

2.8 Copper at the Host-Pathogen Interface

2.8.1 Overview of pathogenic *E. coli*-mammalian host interaction

Commensal *E. coli* resides in the gut of many mammals, including humans.[87] Pathogenic *E. coli* strains are endowed with virulence and fitness genes that appear to be acquired by an ancestral commensal *E. coli* strain by horizontal gene transfer.[88] These virulence and fitness factors promote successful colonization, induction of infection and inflammation, and subversion of the host immune system. Pathogenic *E. coli* strains can infect the gut or extraintestinal sites, such as the urinary tract, bloodstream, systemic sites, mammary glands, and central nervous system.[88] Intestinal pathogenic *E. coli* strains are further divided into various pathotypes, such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and attaching and invading *E. coli* (AIEC).[87,88] These pathotypes have a unique assortment of virulence factors that induce distinct pathological changes in the host. Intestinal pathogenic *E. coli* organisms are transmitted by the fecal-oral route or as food- or waterborne pathogens. Depending on the pathotype, the clinical symptoms can be self-limiting secretory diarrhea, severe inflammatory diarrhea, dysentery, or potentially fatal hemolytic-uremic syndrome.

Extraintestinal pathogenic *E. coli* strains exhibit a commensal-like lifestyle in the intestine but cause pathological changes at other sites, such as the urinary tract, bloodstream, central nervous system, and mammary glands.[87] Uropathogenic *E. coli* (UPEC) is the

predominant cause of urinary tract infection in humans and is among the most common bacterial infections in humans globally. UPEC is also a major etiological agent of bloodstream infections in humans and is a significant cause of mortality due to urosepsis in the elderly. Studies from various groups have revealed a role for copper during host-UPEC interaction in the urinary tract, and this is discussed below.[78,89] Extraintestinal pathogenic *E. coli* that encodes a K1 type of capsular polysaccharide is a causative agent of human neonatal meningitis. In addition to human infections, *E. coli* is a pathogen of immense importance in veterinary medicine and food animal production.

Regardless of the pathotype, *E. coli* is primarily found as an extracellular pathogen during interaction with mammalian hosts. A notable exception is the intracellular reservoir formation by UPEC in the urothelial cells lining the urinary bladder.[90] The extracellular lifestyle of *E. coli* is an important contrast to that of *S. enterica*, whose intracellular lifestyle is a defining feature of its interaction with mammalian hosts.

2.8.2 A primer for mammalian-host-*S. enterica* interaction

S. enterica is an extremely common foodborne bacterial pathogen.[91] *S. enterica* serovars Typhi and Typhimurium cause typhoid fever and gastroenteritis in humans, respectively.[92] Typhoid fever is a result of systemic dissemination of *Salmonella*. The most common outcomes of salmonellosis in industrialized countries are nontyphoidal gastroenteritis and diarrhea in humans.[91] However, invasive nontyphoidal *Salmonella* strains are emerging as a major public health problem, especially in Africa.[93] *Salmonella* is also an important pathogen impacting the

health of a broad range of animals throughout the world. Both serovars of *S. enterica* invade the enteric epithelium via M cells to reach the lamina propria. Nontyphoidal *S. enterica* organisms are phagocytosed by macrophages and neutrophils, but the phagocytes are killed by the virulence factors elaborated by this pathogen, including those delivered through the type III secretion system. These events culminate in acute enteritis, leading to the clinical presentation of inflammatory diarrhea.[94] On the other hand, upon reaching the lamina propria, typhoidal *S. enterica* undergoes a prolonged replicative phase in macrophages and utilizes this protected intracellular niche to disseminate throughout the body.[95] Following a brief period of gastrointestinal symptoms, typhoid is marked by prolonged fever, reflecting sustained bacteremia. Both serovars of *S. enterica* spend a significant amount of time within the host as an intracellular pathogen. Within host cells, *Salmonella* proliferates within vacuoles known as *Salmonella*-containing vacuoles.[96] By utilizing virulence factors, including type III secretion system-delivered effectors, *Salmonella* modifies the pathogen-containing vacuole to establish a replicative niche.[97,98] *S. enterica* serovar Typhimurium induces a typhoid fever-like systemic infection in mice and has served as an experimental model to understand the pathogenesis and host response during systemic salmonellosis.

2.8.3 A brief introduction to copper homeostasis in mammals

Roles of key proteins involved in copper homeostasis is depicted in Figure 3.[99,100] Copper is absorbed in the small intestine via CTR1 transporter in the enterocytes.[101] Enterocytes utilize ATP7A, a P-type ATPase, to traffic copper to the trans-Golgi network and to export copper into the bloodstream.[102] Mutations in the *Atp7a* gene result in severe systemic copper deficiency,

known as Menkes disease.[103] Hepatocytes also import copper from circulation via Ctr1.[104] ATP7B, another P-type ATPase closely related to ATP7A, is used to mobilize copper to the trans-Golgi network in the hepatocytes to produce ceruloplasmin.[104,105] Ceruloplasmin is the predominant reservoir of circulating copper in mammals.[105,106] Ceruloplasmin is a 132-kDa glycoprotein with six copper atoms that is produced and secreted into circulation by the liver.[105] During copper excess, hepatocytes excrete copper into bile by mobilizing ATP7B to the biliary network.[107] Mutations that disrupt the function of ATP7B result in hepatic copper overload, leading to the hepatolenticular degeneration observed in patients with Wilson's disease.[108] CTR2 is another protein that indirectly affects copper uptake by cells indirectly by modulating the activity of Ctr1.[109] CTR1, CTR2, ATP7A, and ceruloplasmin are expressed in many cell types, including macrophages.

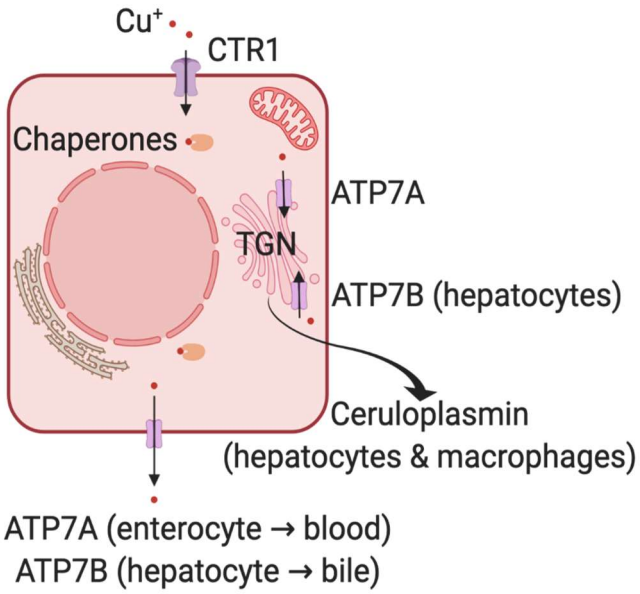

Figure 3. Key Players Involved in Copper Homeostasis in Mammals. Copper (copper^{+}) is imported into the cytoplasm via the CTR1 transporter and is directed to organelles by

metallochaperones. The P-type ATPsaes ATP7A and ATP7B transport copper into the *trans*-Golgi network (TGN). These proteins are also essential for copper absorption in the gut (ATP7A) and biliary excretion of excess copper in the liver (ATP7B). Hepatocytes are the source of circulating ceruloplasmin, and macrophages secrete ceruloplasmin in response to bacterial ligands.

2.8.4 Copper and nutritional immunity

The antimicrobial activity of copper is weaponized by the host immune system during infection. Levels of bioavailable and ceruloplasmin-bound copper increase during the acute-phase reaction triggered by infection and inflammation.[110] Manipulating nutrient availability to curtail pathogen growth during infection is known as nutritional immunity and is an integral part of the innate immune response.[111] Bacterial pathogens, however, have multiple mechanisms to detect and overcome this nutrient depletion strategy, including the well-characterized pathways for acquiring iron by secreting siderophores.[112,113] Mammalian hosts actively limit the availability of iron, zinc, and manganese during infection, with a simultaneous increase in copper availability, suggesting an antimicrobial role for copper at the host-pathogen interface.[113-122] Researchers working on multiple host-pathogen interaction systems have garnered evidence that indicates attempts by the host to poison invading bacteria and fungi through toxic concentrations of copper and zinc.[123-127] Hypoferremia and hypercupremia are synchronously observed during infection, highlighting the opposite effects of these transition metals on pathogen growth *in vivo*.[110,128]

2.8.5 Ceruloplasmin-driven hypercupremia of infection

Ceruloplasmin is a multicopper ferroxidase that oxidizes Fe^{2+} ions to Fe^{3+} ions, which can then bind transferrin. Ferrous iron (Fe^{2+}) is exported by ferroportin, but only ferric iron (Fe^{3+}) binds to transferrin, making multicopper ferroxidases a vital link in iron transport within mammalian hosts.[129] In addition to the ceruloplasmin secreted from the liver, a membrane-anchored form of ceruloplasmin is also found in many tissues.[130] Ceruloplasmin is produced as a major acute-phase reactant during host response to infection and inflammation in humans and nonhuman primates.[110,131,132] In contrast, ceruloplasmin is a minor acute-phase reactant in mice.[133] Thus, hypercupremia of infection is a result of increased hepatic secretion of ceruloplasmin.

2.8.6 Amoebas use copper to kill *E. coli*

The role of copper as a key determinant of the outcome of interaction between bacteria and their protozoal predators serves as an evolutionary framework to understand the antibacterial role of copper during interaction between phagocytes of vertebrate hosts and bacterial pathogens. Bacteria utilize copper efflux pumps to resist digestion within vacuoles when foraged by amoebas. Expression of copper transporters is upregulated in the social amoeba *Dictyostelium discoideum* after engulfment of *E. coli*, resulting in increased levels of intracellular copper.[134] *E. coli* mutants lacking CopA were defective in limiting predation by amoebas, as was evident from their poor survival within amoebas relative to the parental strain.[134] This study highlights the

selective pressure imposed on bacteria to maintain and acquire copper efflux/detoxification systems to protect themselves from protozoa.

2.8.7 Role of copper during microbial infections

Copper is involved in optimal protection from a diverse set of bacterial pathogens, both Gram positive and negative, and fungal pathogens, in addition to *E. coli* and *S. enterica*. A *Pseudomonas aeruginosa* mutant lacking the CueA copper efflux pump is attenuated in colonizing mouse spleens.[135] *Acinetobacter baumannii* mutants lacking a transcriptional regulator of copper efflux (CusR) and a putative efflux pump (CopD) are attenuated during pneumonia in the mouse model.[136] A CopA mutant strain of *Neisseria gonorrhoeae* displays poor survival in human cervical epithelial cells.[137] *Streptococcus pneumoniae* mutants deficient in the CopA copper efflux pump and the CupA copper chaperone are attenuated in a murine model of pneumonia.[138,139] *Cryptococcus neoformans* utilizes copper detoxification machinery to successfully colonize the lungs in a mouse model.[140] In summary, copper is involved in innate protection against a broad range of pathogens.

2.8.8 Antibacterial effect of copper against *E. coli* and *S. enterica* in macrophages

Early insights into the role of copper in antimicrobial function of macrophages were gleaned from studies conducted in copper-depleted rats.[141] Macrophages from copper-depleted rats exhibit decreased capacity for respiratory burst and poor killing of *Candida albicans* compared to controls with adequate copper levels. Wagner et al. utilized X-ray microprobe

technology for precise determination of concentration of selected elements, including copper, inside the phagosomes of mouse macrophages.[142] Copper was found at significantly higher levels inside *Mycobacterium avium*-containing phagosomes from macrophages stimulated with gamma interferon before or after infection relative to unstimulated controls. This direct evidence of copper accumulation within phagosomes was followed by a comprehensive study that shed light on copper-dependent killing of *E. coli* in a murine macrophage-like (RAW264.7) cell line.[143] White et al. demonstrated that RAW 264.7 macrophages stimulated with proinflammatory molecules (gamma interferon [IFN-γ] and lipopolysaccharide [LPS]) import higher levels of copper by using the CTR1 copper transporter.[143] Copper is then trafficked into the phagolysosome by the ATP7A copper transporter. An *E. coli* mutant lacking CopA is killed more effectively by RAW 264.7 cells in 1 to 2 hour (h) postinfection than the parental wild-type strain in an ATP7A-dependent manner.[143]

In the same vein, *S. enterica* mutants lacking both CopA and GolT pumps, but not single mutants, exhibit poor survival within RAW 264.7 cells at 12 and 24 h postinfection.[48] Infection of primary bone marrow-derived murine macrophages with *S. enterica* results in increased abundance in transcripts of copper transporters (*ctr1*, *ctr2*, and *atp7a*), and ceruloplasmin.[125] Chelation of copper promotes survival of *S. enterica* in mouse macrophages. However, copper accumulates in distinct compartments within infected macrophages and does not colocalize with *Salmonella*-containing vacuoles. Copper appears to limit *S. enterica* survival by participating in slower killing mechanisms, such as nitrosative stress, and by limiting iron availability within macrophages, in contrast to its role in faster, oxidative-burst-dependent killing of *E. coli*.[123,143] Ladomersky et al. demonstrated that ATP7A-dependent trafficking of copper plays a role in

killing of *S. enterica* in primary murine macrophages isolated from ATP7A^{LysMcre} and control mice.[144] ATP7A^{LysMcre}-transgenic mice lack ATP7A in cells of myeloid lineage, which includes macrophages and neutrophils. Furthermore, the copper transporters CopA and GolT provide protection from macrophage killing only in wild-type macrophages, indicating an antagonistic use of copper by the host and efflux pumps by the pathogen.[144]

Copper deficiency in humans is associated with reduced neutrophil function and results in significantly compromised bactericidal activity.[126,145] Copper plays a critical role in the differentiation of a human myelocyte cell line (HL-60) into neutrophil-like cells *in vitro*.[146] However, the mechanisms of copper uptake and trafficking to the phagolysosome and its contribution to the bactericidal activity in neutrophils remain to be determined.

2.8.9 Copper modulates the function of macrophages

Separate from its overt antimicrobial function, copper is also involved in modulating the function of macrophages. Inflammasomes are protein complexes composed of sensor proteins (in the AIM2 or NLR family of proteins) that detect various host- and pathogen-derived signals (Fig. 4).[147] These sensors are coupled with an adaptor molecule and cysteine proteases (caspase 1 or 3). Activation of inflammasomes results in caspases processing key cytokines into active forms (interleukin 1β [IL-1 β] and IL-18) and a unique form of inflammatory cell death (pyroptosis).[147] IL-1 β is a potent proinflammatory cytokine that is active at both local and systemic levels. Intracellular copper activates canonical NLRP3-dependent inflammasome in murine and human macrophages (Fig. 4) by altering redox homeostasis.[148] As a corollary, decreased bioavailability

of copper dampens the outcome of both caspase-1-dependent inflammasome activation and LPS-induced shock in mice.[148] This effect is specific for NLRP3, since copper does not appear to affect the activation of noncanonical NLRP1-, NLRC4-, and AIM-2-dependent inflammasomes.[148]

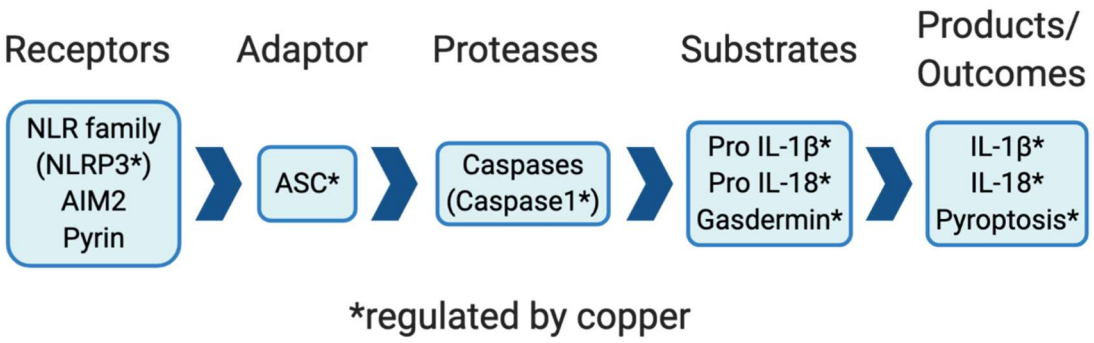

Figure 4. Copper Regulates Inflammasome Activation. Copper is required for signaling via NLRP3 receptor protein to engage the ASC adaptor. This leads to activation of caspase I, which cleaves precursors of substrates to yield active IL-1β and IL-18 (proinflammatory cytokines). Gasdermin triggers an inflammatory form of cell death, known as pyroptosis.

2.8.10 Regulation of mast cell activity by copper

Mast cells are derived from the myeloid lineage of hematopoietic cells and play important roles in hypersensitivity reactions. The involvement of mast cells in the host response to bacterial infections is being increasingly appreciated.[149,150] A key protein released by activated mast cells is tryptase, a serine protease. Tryptase is required for effective control of bacterial growth *in vivo* due to its role in promoting recruitment of neutrophils to the site of infection.[151]

By regulating the expression of tryptase gene, copper exerts an outsized influence on the phenotypes exhibited by mast cells.[152] Mast cells play a key role in the exfoliation of urothelial cells in the urinary bladder during UTI.[153] However, the influence of copper on mast cells in the urinary tract remains to be explored. These emerging roles of copper as a modulator of inflammasome activation in macrophages and mast cell function require further investigation to fully comprehend their scope and impact.

2.8.11 UTI in patients with Menkes disease

The importance of copper in protection against pathogen colonization is illustrated by the increased susceptibility to infection observed in patients with inborn errors in copper metabolism. Menkes disease is an X-linked lethal genetic disease associated with mutations in the *atp7a* gene, resulting in systemic copper deficiency.[103] There has been limited success in restoring copper homeostasis with existing therapeutic approaches, with a promising candidate in the pipeline.[103,154] Historical clinical reports reveal that recurrent and fulminant UTI is a major cause of mortality in patients with Menkes disease.[103,155] Typically, affected individuals die by 3 years of age, but a milder form, occipital horn syndrome (OHS), is often not diagnosed until 5 to 10 years of age.[103] The primary clinical features are related to connective tissue disorder, but these patients are particularly susceptible to UTI, and recurrent UTI is often the first sign which brings the child to the attention of medical professionals.[103] This increased susceptibility to UTI in patients with systemic copper deficiency suggests an important role for copper in limiting bacterial colonization. In a murine model of UTI, a UPEC mutant lacking the Cus copper efflux system is attenuated during infection.[156] In addition to clinical findings, multiple lines of

evidence from experimental studies, discussed below, indicate an important and novel biological role for copper in protection against UTI.

2.8.12 Urinary copper mobilization during infection

A transcriptome sequencing (RNA-seq) study revealed that the *cus* genes were significantly expressed by UPEC during clinical UTI, compared to culture in healthy human urine.[156] Strong induction of expression of *cus* genes, but not *copA* and *cueO*, is not surprising, because UPEC experiences not only copper toxicity but also hypoxia during clinical UTI in patients.[156] Genes encoding CopA and CueO were upregulated ~4-fold in clinical UTI samples but did not meet the threshold for differential expression used in that study.[156] Human urinary copper content, determined by inductively coupled plasma mass spectrometry, is significantly elevated during UTI caused by UPEC, *K. pneumoniae*, and *Proteus mirabilis*.[89,156] Independently, Chaturvedi et al. reported detection of copper-yersiniabactin complexes in UTI urine samples.[78] Ceruloplasmin, the primary carrier of circulating copper, is found at higher levels in urine from UTI patients and is positively correlated with urinary copper content.[89] The reductive milieu of the urine favors formation and maintenance of highly bactericidal Cu^{1+} ions.[89] Urinary copper levels, however, do not increase during UPEC-induced UTI in the mouse model. Interestingly, urine from healthy mice contains inherently high levels of copper, in contrast to that from humans and nonhuman primates.[89] UTI caused by UPEC in the nonhuman primate model (*Chlorocebus aethiops* [vervet monkey]) faithfully emulates clinical features of UTI in humans, including copper mobilization. Furthermore, supplementation and deficiency of copper result in decreased and enhanced UPEC colonization during UTI, respectively.[89,156] An

early (24 to 48 h postinoculation) increase in renal and urinary copper content followed by a decrease (72 h postinoculation) has been reported during systemic infection with *Candida albicans*.[157] It is unlikely that kidneys are copper depleted later during infection because of increased urinary excretion, since serum levels of copper remain elevated during infection, unless abscess formation and associated hemodynamic changes interfere with renal copper uptake during renal colonization by *C. albicans*.[157] However, the effect of systemic bacterial infection on renal and urinary copper content remains to be investigated. Collectively, these findings indicate that copper is a protective innate immune effector mobilized to urine as a conserved host response to UTI caused by Gram-negative bacterial and fungal pathogens.

2.8.13 Yersiniabactin uptake genes are involved in the virulence of Uropathogenic *E. coli*

UPEC strains are more likely to carry yersiniabactin-biosynthetic genes than other pathotypes and commensal strains of *E. coli*.[158,159] Yersiniabactin production is associated with higher copper resistance among UPEC isolates, since it binds and shuttles copper.[78] In addition to sequestering copper, Cu^{2+}-yersiniabactin complexes emulate superoxide dismutase activity to mitigate superoxide stress in UPEC within murine macrophages.[160] Loss of FyuA, the outer membrane receptor for uptake of yersiniabactin-metal complexes, results in attenuation of UPEC in the mouse urinary tract.[161] The genes *ybtP* and *ybtQ* encode ABC transporters localized on the inner membrane that play an essential role in the uptake of yersiniabactin.[162] In a murine model of cystitis, *ybtP* and *ybtQ* genes contribute to the virulence of a prototypical UPEC strain, UTI89.[163] Since the mutants lacking *fyuA*, *ybtP*, and *ybtQ* are competent to produce and utilize

enterobactin and salmochelin as siderophores during infection, decreased virulence could not be attributed to the loss of iron uptake alone. These observations suggest that a non-iron uptake function of yersiniabactin might play a role in attenuation observed *in vivo*. The intracellular copper and iron contents of these mutants and parental wild-type strains should be evaluated to distinguish whether yersiniabactin-mediated protection from copper stress or yersiniabactin-directed acquisition of iron is critical during infection. Taken together, these studies indicate complex siderophore-dependent and -independent roles for yersiniabactin in the virulence of UPEC**.**

2.8.14 Role of host-derived copper in limiting *S. enterica* colonization

The oral gavage route of inoculation more closely emulates the natural course of colonization, infection, and systemic dissemination of *S. enterica* serovar Typhimurium than intraperitoneal inoculation in murine models. Achard et al. reported that CueO plays an important role in the virulence of *S. enterica* (strain SL1344) in a mouse model of intestinal colonization and systemic dissemination following oral gavage.[164] There was an ~100-fold decrease in the recovery of a *cueO* mutant from spleens and livers compared to the parental wild-type strain. A double mutant lacking CopA and GolT exhibited an ~2-fold decrease in fitness during spleen and liver colonization, compared to the parental strain, in a mouse model of systemic salmonellosis (strain SL1344) initiated by intraperitoneal infection.[144] However, oral inoculation of a CopA GolT double mutant (strain SL1344) does not result in a detectable decrease in colonization of spleen and liver.[48] Another study utilizing intraperitoneal route of infection also did not identify a role of CopA, GolT, and CueP in the fitness of *S. enterica* (strain

14028) in various wild-type strains of mice.[165] CopA- and GolT-dependent intramacrophage survival in *S. enterica* exhibited strain-specific differences, with a clear role in strain SL1344 but not in strain 14028. These genes were found to contribute to the fitness of *S. enterica* during systemic infection triggered by a higher-dose inoculation (2×10^4 CFU/mouse), but the loss of fitness was mitigated when a smaller inoculum (5×10^2 CFU/mouse) was utilized. These conflicting pieces of evidence suggest a model system-specific role for copper at the host-*Salmonella* interface. Investigations of the survival of *Salmonella* within cells derived from different lineages in spleen and liver could help reconcile the differences observed in these studies. Key points on the roles of copper at the host-pathogen interface are presented below and summarized in Table 2.

Table 2. Key Points

No.	Observation
1	Effectors of nutritional immunity trigger concurrent hypoferremia and hypercupremia during infection.
2	Copper is co-opted by the innate immune system for protection against several bacterial pathogens.
3	Macrophages import copper via CTR1 during infection, and ATP7A pumps copper into the phagosome to kill to *E. coli* and *S. enterica*.

Table 2 continued

No.	Observation
4	Copper is mobilized to urine during UTI in humans and is recapitulated in a non-human primate model of UTI.
5	Uropathogenic *E. coli* overcome copper toxicity *in vivo* by utilizing Cus efflux system and yerisinia bactin.
6	CueO plays and important role in the virulence of *S. enterica* in a murine model of infection.
7	CopA/GolT in *S. enterica* exhibit bacterial strain and inoculum dose-dependent variation in contribution to fitness during infection.

2.8.15 Copper based therapeutics

Humans have harnessed the antimicrobial action of copper since early civilizations.[115,119,166] Storage of water in copper vessels has been reported to inactivate *E. coli*.[167,168] Copper surfaces function synergistically with common disinfectants and are increasingly being used to minimize microbial contamination in health care settings, and mechanisms of copper intoxication in bacteria exposed to metallic copper are beginning to be unraveled.[166,169-171] There is a keen interest in exploiting the antimicrobial role of copper in the innate immune response through development of novel copper-based therapeutics.[22] This is

primarily driven by the grim outlook on the already high and continuously increasing levels of resistance to antibiotics that pose a threat to global public health. Extended-spectrum β-lactamase-producing bacteria, which are often multidrug resistant, are particularly concerning and lead to increased reliance on carbapenems as an antibiotic of last resort.[172] Copper inhibits metallo-β-lactamase to rescue the sensitivity to carbapenems in resistant *E. coli*.[173] Furthermore, use of copper-pyrithione complexes significantly decreases the concentration of copper required to revive carbapenem sensitivity. Another copper-dependent approach to selectively target β-lactamase-producing *E. coli* also utilizes pyrithione.[174] In this case, pyrithione is released from a complex with cephalosporin only as a product of catalysis by β-lactamase. It shows promising activity against resistant strains in a copper-dependent manner. Copper-dependent killing or growth inhibition has been demonstrated for *Mycobacterium tuberculosis*, *S. aureus*, *N. gonorrhoeae*, and *Mycoplasma* species.[175-178] These reports set the stage for productive inquiry on copper-dependent killing of *E. coli* and *S. enterica*, in addition to other pathogens, and the use of copper as an adjunct or alternative therapeutic to antibiotics.

Concerningly, there is evidence for co-selection of resistance elements against both antibiotics and heavy metals, including copper.[179-181] Bacteria may encounter the selective pressure of copper in a variety of settings, including agriculture, livestock operations, and health care facilities.[182-185] The recent recovery of urinary *E. coli* isolates harboring the *pco* operon is of particular concern, as these isolates often produce extended-spectrum β-lactamases.[74] Understanding the biological and evolutionary basis of co-selection for metal and antibiotic resistance is critical for further exploitation of metal toxicity for therapeutic purposes.

2.9 Outstanding Questions

The essentiality of copper in biological processes and the mechanisms mitigating the toxic effects of excess copper have been subjected to extensive investigation. On the other hand, the process through which bacteria acquire copper remains alarmingly understudied and presents an opportunity to devise new ways to exploit the antimicrobial use of copper by augmenting its import. Although the copper detoxification systems of *E. coli* and *S. enterica* have been extensively studied, the copper response systems of *K. pneumoniae*, other members of *Enterobacteriaceae*, and closely related pathogens such as *Proteus mirabilis* remain ripe for investigation based on insights gleaned from studies on these model enteric bacteria. While the role of copper during UTI is an active area of investigation, the impact of copper during enteric and systemic infection by *E. coli* remains to be investigated. Antimicrobial and growth inhibitory functions of copper have attracted the most attention from researchers. Understanding the effects of copper on expression of virulence factors is yet another critical area that needs to be elucidated. Mechanisms of delivery of host-derived copper to sites of infection also remain to be determined. Ceruloplasmin appears to be the source of copper in urine during UTI.[89] Several questions on the cellular origin of ceruloplasmin, the signals that activate its expression, and its secretion/translocation into the urine remain to be explored. Mounting evidence for copper as an innate immune effector represents an exciting direction for potential research on host-directed therapy against bacterial infections.

In summary, review of copper homeostatic systems and their role at host-pathogen interface in *E. coli* and *S. enterica* has revealed opportunities to advance our understanding of the

biological roles of copper to fully harness its antimicrobial activity. Researchers working on these topics have generated valuable genetic and biochemical tools and animal models that have set the stage for productive inquiry in the years to come.

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3 A GENOME-WIDE SCREEN REVEALS THE INVOLVEMENT OF ENTEROBACTIN-MEDIATED IRON ACQUISITION IN *ESCHERICHIA COLI* SURVIVAL DURING COPPER STRESS*

3.1 Abstract

Copper (copper) is a key transition metal that is involved in many important biological processes in a cell. Copper is also utilized by the immune system to hamper pathogen growth during infection. However, genome-level knowledge on the mechanisms involved in adaptation to copper stress is limited. Here, we report the results of a genome-wide reverse genetic screen for copper-responsive phenotypes in *Escherichia coli*. Our screen has identified novel genes involved in adaptation to copper stress in *E. coli*. We detected multiple genes involved in the biosynthesis and uptake of enterobactin, a siderophore utilized for high-affinity TonB-dependent acquisition of ferrous (Fe^{3+}) iron, as critical players in survival under copper intoxication. We demonstrated the specificity of copper-dependent killing by chelation of copper and by genetic complementation of *tonB*. Notably, TonB is involved in protection from copper in both laboratory and uropathogenic strains of *E. coli*. Copper stress leads to increased expression of the genes involved in iron uptake, indicating that Fur regulon is derepressed during exposure to excess copper. Trace element analyses revealed that iron homeostasis is dysregulated during

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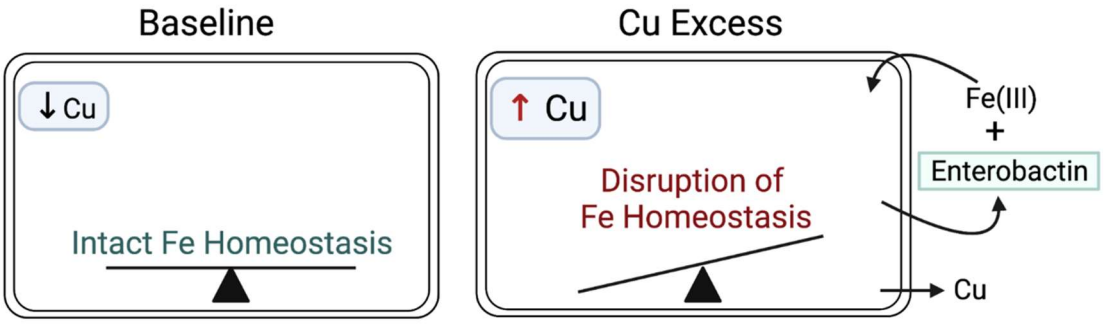
copper stress. Taken together, our data supports a model in which lack of enterobactin-dependent Fe^{3+} uptake leads to exacerbation of copper toxicity and elucidates the intricate connection between the homeostasis of copper and iron in a bacterial cell.

3.2 Graphical Abstract



3.3 Introduction

Copper is a transition metal that is utilized in various Cu-containing proteins in a bacterial cell. Copper homeostasis is a tightly regulated process, since excess copper is toxic due to its potential to mismetallate proteins.[1,2] Copper has higher affinity for noncognate ligands due to its location in the Irving–Williams series and disrupts binding of transition metals such as iron, manganese, and zinc.[3,4] Generation of reactive oxygen species used to be proposed as the mechanism by which copper exerts its toxicity.[5] However, recent studies have challenged this hypothesis by demonstrating that copper can indeed protect *Escherichia coli* from peroxide and superoxide stress.[6,7] Transcriptional regulators CueR and CusR play critical roles in orchestrating

efflux and detoxification of excess copper in *E. coli*.[8] During copper stress, copper-bound CueR activates transcription of *copA* and *cueO* encoding an inner membrane P-type ATPase, and a periplasmic multicopper oxidase, respectively.[9-11] CusR, phosphorylated by CusS during copper stress, induces transcription of *cusCFBA* operon that encodes the CusCBA transenvelope copper efflux system and the CusF periplasmic cuprochaperone.[12] Bioavailable copper in the cytoplasm is maintained at subzeptomolar ($<10^{-21}$ M) levels by the CueR and CusR regulated genes, and sequestration of copper by ligands.[8,13] In addition, copper stress activates other transcriptional regulatory systems including CpxRA and SoxRS, resulting in global changes that mitigate damage and promote survival of *E. coli*.[14,15] While copper efflux and detoxification systems in bacterial pathogens have been well characterized, uptake and trafficking of copper in a bacterial cell remains less clearly defined.[13] Antibacterial activity of copper has been utilized by humans since the metal ages.[4,16] Studies on multiple pathogens have revealed that bacterial copper efflux/detoxification genes are involved in survival and virulence of those pathogens during infection.[16-18] Copper is a key effector in the innate immune response against bacterial pathogens, with a clearly established antimicrobial function in the phagolysosomes of macrophages.[19,20] Unabated increase in resistance to conventional antimicrobial agents in bacterial pathogens has led to a resurgence of interest in the development and use of copper-based antimicrobial agents.[21] Copper is increasingly used in health care settings on high-touch surfaces to mitigate microbial colonization and spread of nosocomial pathogens.[22-24] It is critical to understand how a bacterial cell adapts to copper stress to fully harness the antimicrobial potential of copper, and to preemptively mitigate emergence of resistance. Transcriptional profiling studies have shed light on how gene expression is modulated during copper stress in *E. coli*.[14,15] However, the extent to which many of these copper-responsive genes are involved in

protection against cellular damage induced by copper stress and their precise roles remains poorly understood. To gain a comprehensive and genome-wide understanding of adaptation to copper stress, we utilized the KEIO collection of genetically defined mutants lacking nonessential genes in *E. coli* to screen for copper-responsive (sensitive or resistant) phenotypes.[25] Our findings from this reverse genetic screen revealed that an intact enterobactin biosynthesis and uptake pathway is essential for optimal survival of *E. coli* during copper stress. This study elucidates the central role of TonB in protection against copper intoxication in both laboratory and pathogenic strains of *E. coli*.

3.4 Materials and Methods

3.4.1 Bacterial strains and culture conditions

Strains and plasmids used in this report are listed in Appendix A Table 1. Bacterial strains were cultured in LB broth or agar (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, and agar 15 g/L). Bacterial cultures were incubated at 37°C, and broth cultures were aerated by shaking at 200 RPM, unless noted otherwise. When indicated, spent medium was harvested from cultures in stationary phase by filter-sterilizing culture supernatants. Sterility of spent medium was assessed prior to use by plating on LB agar. Kanamycin (25 mg/mL) or ampicillin (100 mg/mL) was used for selection of mutants and ensure maintenance of plasmids. Chemicals were purchased from Sigma or Fisher Scientific.

3.4.2 Primary screen for copper sensitivity and resistance

KEIO collection was used to start cultures in microtiter plates in LB. Optical densities at 600 nm were recorded in a plate reader (Omega Star, BMG lab tech). A 96-well pin replicator tool was used to stamp the overnight cultures onto LB agar with 0, 3, or 6 mM $CuSO_4$ $5H_2O$. *Escherichia coli* strains BW25113 (parental strain for KEIO collection mutants), CFT073 (wild-type uropathogenic *E. coli*), CFT073 TN32-A2 (copper-resistant mutant), and BW25113 Δ*copA* (copper-sensitive mutant) were also added on each plate as controls, and plates were incubated at 37°C and 24°C to detect the effect of temperature on copper responsiveness. CFT073 TN32-A2 is a transposon mutant in uropathogenic *E. coli* strain CFT073 that has a Tn5 inserted in c4289 encoding a predicted inner membrane protein (YhiM, Appendix A Table 1). This mutant exhibits a high level of copper resistance compared to parental strain CFT073, and this phenotype could be reversed by genetic complementation (manuscript in review, Subash lab). Phenotypic response was determined qualitatively, relative to controls at 24, 48, and 72 h. Strains that exhibited a sensitive or resistant phenotype compared to the wild-type strain were tested in the secondary screen.

3.4.3 Secondary and Fe uptake/metabolism screen

During the secondary screen, overnight cultures were normalized based on optical density prior to inoculation on 3 and 6 mM $CuSO_4$-containing LB agar plates at 37°C, 30°C, and 24°C. Phenotypic response was determined and documented at 24, 48, 72, and 96 h. Strains that exhibited a consistent sensitive or resistant growth compared to wild-type from three

independent experiments were deemed as strains of interest. Thirty-six mutants from the KEIO library containing a deletion in genes related to iron uptake/metabolism (Appendix A Table 5) were screened in triplicate, essentially under the same conditions used in the secondary screen. All further assays for copper-responsive phenotypes were conducted at 37°C with 24 h of incubation, as we did not detect temperature-dependent changes in our screens.

3.4.4 CAS assay for catecholate siderophore production

Level of catecholate siderophore production was determined by CAS assays, as described recently.[26] Briefly, CAS plates were made using the CAS plate preparation protocol found in Himpsl and Mobley 2019.[26] Overnight cultures of strains were then spot plated on CAS agar plates in 5 µL aliquots and incubated at 30℃. Plates were observed after 24 h and the halo around the colonies were assessed for color change and size.

3.4.5 Validation of mutants from KEIO collection and expression of downstream genes in an operon

Oligonucleotide primers used in this study are listed in Appendix A Table 2. Primers flanking genes involved in iron metabolism were designed and used in PCRs to confirm that the mutants utilized from the KEIO collection are indeed lacking the gene and is replaced by the kanamycin cassette. KEIO mutants are designed not to disrupt expression of downstream genes, even when an upstream gene in an operon is replaced by antibiotic resistance cassette. Since many of the mutants of interest in iron metabolic pathways are organized as operons, we

conducted RT-qPCR assays to verify whether the downstream genes were expressed. RNA was extracted from wild-type and mutant strains, reverse transcribed to cDNA, and used in qPCRs to verify the presence of transcripts.

3.4.6 Copper toxicity and chelation assays

Overnight cultures were normalized (OD_{600} = 1.0), diluted 1:100 in LB with or without 3 mM $CuSO_4$, and cultured to stationary phase. When indicated, strains were cultured in spent media from stationary phase cultures of wild-type strain (BW25113). For chelating copper, 6 mM bathocuproinedisulfonic acid disodium salt (BCS) was added. Viable counts were enumerated after 24 h.

3.4.7 Genetic complementation of tonB

Full-length *tonB* and its native promoter were cloned into pUC57 (Genscript) and then subcloned into pGEN_MCS27 at BamHI and EcoRI sites to generate pGEN_*tonB* (Appendix A Table 1). Clones were verified by PCR with primers that bind to the vector (Appendix A Table 2). Empty vector and pGEN_*tonB* were introduced into the wild-type and *ΔtonB* mutant strains by electroporation (Appendix A Table 1).

3.4.8 Quantitative PCR

$CuSO_4$ (0.5 mM) was added to cultures of wild-type strain in midlogarithmic phase, and RNA was extracted after 20 min. Transcripts were stabilized with RNAprotect (Qiagen), extracted with RNeasy mini kit (Qiagen). Contaminating DNA was removed by digestion with DNase (Ambion), and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was performed with SYBR Green (Thermo Scientific) and primers (Appendix A Table 2) in a CFX Real-Time system (BioRad Laboratories). *gapA* was used to normalize transcript levels between samples, and relative expression was calculated by using LB without copper as the calibrator.

3.4.9 Trace element analysis

Inductively coupled plasma-mass spectrometry or optical emission spectrometry (ICP-MS or OES) was used to determine transition metal content, as we have recently reported.[7] Briefly, overnight cultures of bacterial strains were resuspended in LB with or without 3 mM $CuSO_4$, incubated for an hour, harvested by centrifugation, and washed thrice with 10 mM HEPES containing 0.5 mM EDTA. Cell pellets were digested with trace element-grade nitric acid and diluted in trace element-grade water. Levels of copper, iron, zinc, and manganese were determined by ICP-MS by an operator blinded to the treatment groups (8800 ICP-MS/MS, Agilent Technologies). Spectral interference was minimized by conducting the analysis in a single quadrupole mode using helium in the collision/reaction cell. Samples containing higher concentrations of copper, iron, and zinc were determined by inductively coupled plasma optical

emission spectrometry (ICP-OES). The sample introduction system in the ICP-OES instrument (5110 SVDV ICP-OES, Agilent Technologies) was composed of a single-pass cyclonic spray chamber and a glass concentric nebulizer. Manufacturer default operating conditions were adopted in all ICP-OES analyses, and atomic/ionic emission signals at 327.395, 238.204, and 213.857 nm were used for copper, iron, and zinc determinations, respectively. Concentration of metals was normalized to the wet weight of *E. coli* pellets.

3.4.10 Statistical Analysis

Experiments were repeated at least three times independently, with two or more technical replicates. Biological and technical replicates were independently assessed and not averaged during statistical analysis. Transcripts from RT-qPCR were analyzed by t-test. Log_{10} CFU/mL and percent killing were analyzed by Mann–Whitney test or ANOVA with Dunnett's test, respectively. The copper chelation assay was analyzed by Kruskal–Wallis test with Dunn's post-test. ICP-MS and ICP-OES were analyzed by ANOVA with Dunnett's test. Mean + SEM is presented for all necessary data sets. Results were analyzed in Prism 7 (GraphPad).

3.5 Results and Discussion

3.5.1 A genome-wide reverse genetic screen for copper-responsive phenotypes in *E. coli*

We utilized the KEIO collection of genetically defined mutants in *E. coli* strain BW25113, derived from K12 lineage, in this study.[25] These mutants were generated by replacement of ORFs encoding nonessential genes with a kanamycin resistance cassette utilizing the lambda Red recombineering technology.[25,28] Mutants (4320) and appropriate controls for wild-type, copper-sensitive, and copper-resistant phenotypes were screened at 3 and 6 mM $CuSO_4$ in LB agar under aerobic conditions (Table 1 and Fig. 1). Based on our pilot assays with wild-type and control strains at various levels of copper, we used 3 and 6 mM copper in our screen for reproducible identification of sensitivity and resistance to copper, respectively (Fig. 1). We utilized a rich medium in this screen to minimize the impact of conditional auxotrophy to select nutrients that develop during induction of copper stress in minimal medium.[1,2] Mutants that exhibited differential growth phenotypes, compared to controls, were verified in a secondary screen, which was conducted in triplicate. We identified a total of 43 and 25 mutants that were more sensitive and more resistant to copper than the parental strain, respectively (Table 1, Fig. 1, Appendix A Tables 3 and 4).

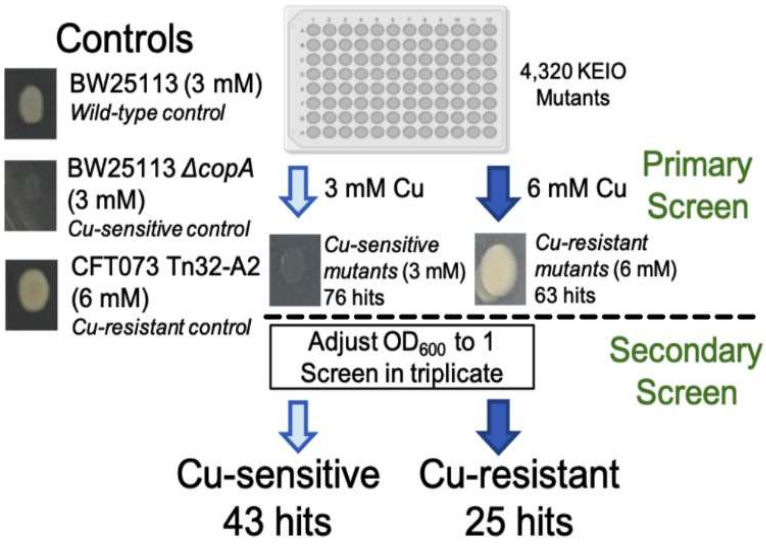


Figure 1. Detection of copper-responsive *E. coli* mutants. Schematic of the reverse genetic screen used to detect *E. coli* mutants from the KEIO library that are more sensitive or more resistant to copper, than the wild-type strain. Representative images of wild-type control strain BW25113 (3 mM copper), copper-sensitive *ΔcopA* mutant (3 mM copper), and copper-resistant CFT073 Tn32-A2 (6 mM copper) mutants growing on copper-supplemented LB agar from the primary screen are depicted here. Hits from the primary screen were validated in the secondary screen.

3.5.2 Previously known and novel copper-responsive genes detected in our screen

Our screen detected genes that were previously known to impart copper-responsive phenotypes in *E. coli*, thereby validating the premise of this study and our findings (Appendix A Tables 3 and 4). Our results revealed that a *lpp* mutant lacking the murein lipoprotein had higher copper resistance (Appendix A Table 4), consistent with the findings of a previous report.[29] We

identified that a mutant lacking the Lon protease was more resistant to copper than the wild-type strain (Appendix A Table 4). Our result supports the recent findings on CueR as a target of the Lon protease, as a *Δlon* mutant would have constitutively high levels of CueR activity leading to a copper-resistant phenotype.[30] Mutants lacking an inner membrane zinc/divalent cation efflux ATPase ZntA—that have potentially higher intracellular accumulation of zinc—were also more resistant to copper (Appendix A Table 4). Surprisingly, a mutant lacking the CusR transcriptional regulator that activates the transcription of *cusCFBA* genes involved in copper efflux under anaerobic conditions was more resistant than the wild-type strain (Appendix A Table 4). This suggests that in the absence of CusR, another transcriptional activator likely induces the expression of the *cusCFBA* operon. Cross-regulation between CusRS and HprRS two component regulatory systems is known and could contribute to the observed copper-resistant phenotype.[15,31] Multiple genes, including *ynaJ*, *yehS*, and *yciU*, whose function has not been characterized yet were also identified as contributors to copper resistance in *E. coli* (Appendix A Table 4). Our screen detected mutants lacking *copA* and *cueO* as copper sensitive (Appendix A Table 3) in line with extensive data on the roles of these genes in copper efflux and detoxification.[17] Mutants lacking the transcriptional regulator CpxR also exhibited increased sensitivity to copper (Appendix A Table 3) in line with previous reports.[32,33] We observed that mutants lacking OmpR and OmpC (an OmpR-regulated porin) are more sensitive to copper, suggesting that the potential for OmpC to act as an importer of copper, as proposed in an earlier study, is less likely.[34] Role of OmpR and OmpC in promoting resistance to copper is aligned with recent observations on the role of these proteins in promoting iron uptake in *E. coli*.[35] We observed that several mutants lacking genes involved in iron acquisition were more sensitive to copper than the parental strain (Appendix A Table 3). A limitation of this screen is that

regulators such as small RNAs that are often found in the intergenic regions will not be detected because they are not represented in the KEIO library. A forward genetic screen will be instrumental in identifying the role of regulators encoded outside of annotated genes in copper-responsive phenotypes. Since our screen was conducted under aerobic conditions, genes involved in adaptation to copper stress under anaerobiosis were not expected to be identified in this study. In summary, our screen has expanded the number of known copper-responsive genes in *E. coli* and represents a resource that lays the foundation for future studies on adaptation to copper stress (Appendix A Table 3 and 4).

Table 1. Summary of screen for copper-responsive phenotypes in *E. coli* KEIO library

Screen	# of Mutants Screened	# of copper-Sensitive Mutants	# of copper-Resistant Mutants
Primary	4320	76 (1.8 %)	63 (1.5 %)
Secondary	139	43	25
Fe homeostasis	36	10	0

3.5.3 Mutants defective in enterobactin biosynthesis and ferric-enterobactin uptake are more sensitive to copper

Enterobactin is biosynthesized by products of the *ent* genes and is imported by the products of the *fep* genes in conjunction with the TonB-ExbB-ExbD complex.[17] Six mutants with defects in uptake of ferric-enterobactin (*tonB*, *exbB*, *exbD*, *fepB*, *fepD*, and *fepG*) exhibited

increased sensitivity to copper in the secondary screen. We screened 36 mutants in the KEIO collection that are involved in enterobactin-mediated Fe^{3+} acquisition pathway, and other known roles in iron uptake/metabolism (Fig. 2A and Appendix A Table 5), for copper-responsive phenotypes. Ten mutants belonging to two functional classes that are defective in enterobactin biosynthesis (*entB*, *entE*, and *entF*), and ferric-enterobactin uptake (*tonB*, *exbB*, *exbD*, *fepB*, *fepC*, *fepD*, and *fepG*), were compromised in growth under copper stress (Fig. 2A, Table 2, and Appendix A Table 5). Notably, none of these 36 mutants exhibited more resistance to copper than the wild-type strain (Table 1 and Fig. 2A). We validated the deletion of specific genes in the mutants from the KEIO collection and assessed the expression of downstream genes in mutants lacking an upstream gene located within an operon by RT-qPCR (Appendix A Table 6 and Appendix A Fig. 1). Next, enterobactin production phenotypes of these mutants were verified on CAS agar and compared to wild-type and fur mutant strains (Fig. 2B). Fur is a repressor of transcription of genes involved in iron uptake, including enterobactin biosynthesis and uptake.[36] As expected, mutants defective in ferric-enterobactin uptake (*tonB*, *fepB*, and *fepG*) produced more enterobactin relative to the parental strain, whereas the *entF* mutant defective in enterobactin biosynthetic pathway did not produce enterobactin (Fig. 2B). Our findings indicate that adaptation to high levels of copper is associated with a disruption in cellular iron homeostasis. Importantly, both enterobactin-deficient and overproducing mutants displayed increased sensitivity to copper, pointing to divergent origins for the copper-sensitivity phenotype. Nichols et al. have also detected ExbD as a contributor of optimal survival and growth of *E. coli* during copper stress.[37] However, loss of other iron acquisition genes identified in our screen exhibited modest copper-dependent decrease in growth in their assay. This difference could be a result of variation in the format of the screens with 1536 versus 96 colony

formats. Additionally, we conducted a screen with 36 mutants lacking various iron uptake and metabolism genes that led to the confirmation and discovery of additional genes in the enterobactin-dependent Fe^{3+}-uptake system in adaptation to copper stress in *E. coli* (Fig. 2A, Table 2, and Appendix A Table 5). Grass et al. had reported that an *E. coli* mutant lacking Fur is conditionally hypersensitive to copper in presence of ascorbate.[38] Our assays were conducted in LB without added ascorbate and is the likely reason that we did not observe copper sensitivity phenotype in the fur mutant.

Table 2. Copper-sensitive mutants that are defective in enterobactin production and uptake.

Gene	Function	Ent-production[a]	Ent-uptake[a]
tonB	TonB subunit complex, TonB	+	-
exbB	TonB subunit complex, ExbB	+	-
exbD	TonB subunit complex, ExbD	+	-
fepB	Ferric enterobactin ABC transporter periplasmic binding protein	+	-
fepC	Ferric enterobactin ABC transporter ATP binding subunit	+	-
fepD	Ferric enterobactin ABC transporter membrane subunit FepD	+	-
fepG	Ferric enterobactin ABC transporter membrane subunit FepG	+	-

Table 2 Continued

Gene	Function	Ent-production[a]	Ent-uptake[a]
entB	Enterobactin synthase component B	-	+
entE	2,3-dihydroxybenzoate-(aryl-carrier protein) ligase	-	+
entF	Apo-serine activating enzyme	-	+

[a]Ent, enterobactin; +, production/uptake competent; -, production/uptake deficient

3.5.4 Transcription of genes involved in enterobactin production and uptake are induced during copper stress

Since mutants defective in production and utilization of enterobactin were more sensitive to copper, we asked whether copper stress induces transcription of the genes involved in enterobactin pathway. Transcription of these genes is repressed by Fur during growth in iron-replete milieu and under anaerobic conditions, and derepression of transcription occurs upon iron starvation.[17,39] Therefore, expression level of these genes could be used as an indicator of cellular bioavailability of iron. Quantitative PCR was performed to assess the abundance of transcripts during copper stress. Expression of *copA* was used as a positive control since it is known to be induced during copper stress (Fig. 2C). Genes involved in the biosynthesis (*entA* and *entF*) and uptake (*tonB*, *fepA*, *fepB*, *fepG*, and *fes*) of enterobactin were significantly upregulated in the wild-type strain during copper stress, compared to growth in LB (Fig. 2C). Our findings suggest that there is increased cellular demand for iron during copper stress leading to derepression of Fur regulon members. Alternatively, elevated copper levels could interfere with transcriptional

repression activity of Fur. As a iron- and zinc-containing metalloprotein, Fur is a potential target for mismetallation by copper. *Escherichia coli* Fur was recently demonstrated to reversibly bind [2Fe-2S] clusters that are less susceptible to mismetallation compared to [4Fe-4S] clusters.[40,41] Our findings on the link between copper stress and derepression of genes in the Fur regulon raise a critical question on whether copper could directly disrupt iron and/or zinc in Fur and requires experimental verification. Recently, zinc excess has been demonstrated to increase cellular demand for iron and a dysregulation of copper homeostasis systems in *E. coli*.[42] Taken in light of the results presented in this report, homeostasis of metals in microbes appears to be heavily interconnected and has major implications for understanding microbial growth and physiology at the host–pathogen interface. Our results support a previous report on copper-dependent transcriptional changes in *E. coli* that had identified upregulation of genes involved in iron uptake, including the enterobactin pathway, upon exposure to copper.[14] Furthermore, Grass et al. have demonstrated elevated production of enterobactin during copper stress in *E. coli*.[38] We and others have reported that the expression of iron-uptake genes is upregulated in uropathogenic *Escherichia coli* (UPEC) during clinical infection in people, which is also recapitulated in experimental animal models of UTI.[43–47] Level of copper is also elevated in urine during UTI caused by uropathogens.[43,48,49] Similar to our findings in *E. coli*, enterobactin is also involved in optimal protection against copper in *Salmonella enterica*.[50] Taken in light of the observations presented in this report, further studies are required to dissect the contribution of iron sequestration mechanisms from copper intoxication within vertebrate hosts on derepression of Fur-regulated genes. Collectively, these findings establish that copper intoxication is intricately linked with changes in iron homeostasis in *E. coli*, a model organism and a pathogen of biomedical significance.

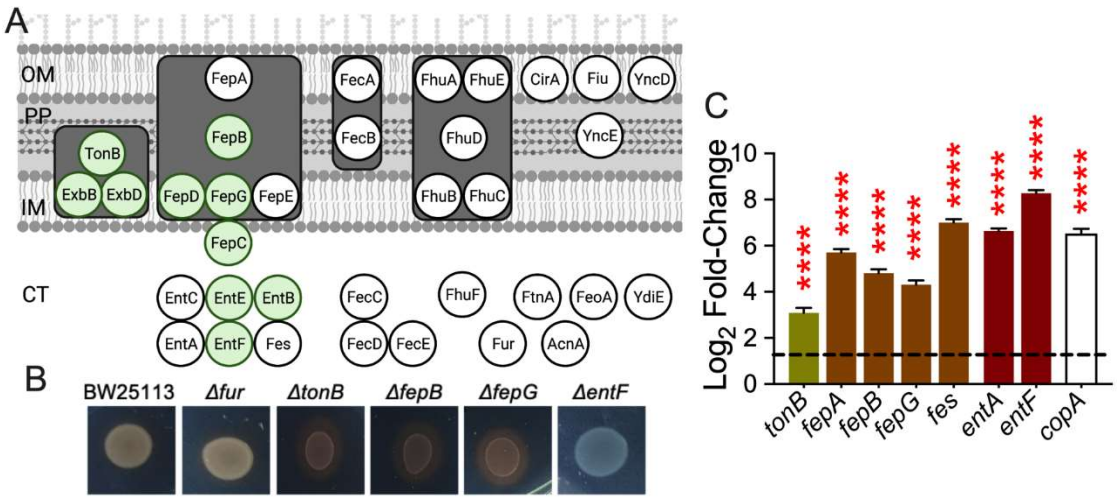


Figure 2. Mutants defective in enterobactin-dependent iron uptake are more sensitive to copper, and transcription of iron uptake genes is induced during copper stress. A) Mutants lacking genes involved in iron metabolism that was tested for sensitivity to copper are depicted here, and clustered based on the pathway and cellular localization. Green circles indicate mutants that display increased sensitivity to copper. OM, outer membrane; PP, periplasm; IM, inner membrane; and CT, cytoplasm. **B)** Production of enterobactin by select mutant, and control strains was visualized on CAS agar. Presence of an orange halo/colony indicates biosynthesis and secretion of enterobactin. A representative image from three replicates is shown here. **C)** Fold-change in the abundance of transcripts corresponding to enterobactin-mediated iron uptake system genes during copper stress was determined. copper-efflux gene *copA* was used as a positive control. All tested transcripts depicted above were $P<0.0001$ by *t*-test in LB + copper, compared to LB alone. N = 3, in triplicate. Mean + SEM is presented here.

3.5.5 Quantitative determination of copper sensitivity

Since bacterial growth was evaluated qualitatively in our screens, we next determined the extent to which copper decreases the viability of the mutants defective in enterobactin production and uptake. Wild-type and mutant strains were cultured overnight in LB with or without 3 mM copper, prior to determining viable counts (Fig. 3A). A mutant lacking *copA* was used as a control to establish the presence of copper stress in these assays (Fig. 3). A mutant lacking Fur was used as a control for excess enterobactin production, compared to wild-type strain (Fig. 3). Quantitative plate counts revealed a significant decrease in viability for all tested mutants, except *Δfur*, during copper stress (Fig. 3A). There was 16% killing for the wild-type strain and 99% killing for the *ΔcopA* mutant in our assays (Fig. 3B). The mutant lacking *fur* exhibited wildtype level of viability during copper stress (Fig. 3A and B). Killing of *ΔfepB*, *ΔfepC*, *ΔfepD*, *ΔfepG*, and *ΔentF* mutants by copper was indistinguishable from that of the *ΔcopA* mutant (Fig. 3B). Other mutants (*ΔtonB*, *ΔexbB*, *ΔexbD*, *ΔentB*, and *ΔentE*) were killed at a significantly higher level than the wild-type strain (Fig. 3B). While we expected increased copper-dependent killing of iron uptake-defective mutants based on our primary and secondary screens, we were surprised that some of these mutants exhibit *ΔcopA* level of sensitivity to copper. The degree of augmentation in sensitivity to copper in these mutants highlights the biological significance of enterobactin pathway in promoting *E. coli* survival during copper intoxication. Our results also suggest that extracellular interaction of enterobactin with copper does not mitigate copper stress because enterobactin overproducing but import defective mutants including *ΔtonB*, *ΔfepB*, and *ΔfepG* (Fig. 2B), exhibit higher level of killing by copper (Fig. 3A and B) than the wild-type strain. The *Δfur* mutant, which overproduces and imports enterobactin, exhibits only wild-type

level of killing (Fig. 3A and B) indicating that excess enterobactin does not augment protection from the toxic effects of copper. Collectively, wild-type level of enterobactin production and import is sufficient to confer optimal protection against copper stress.

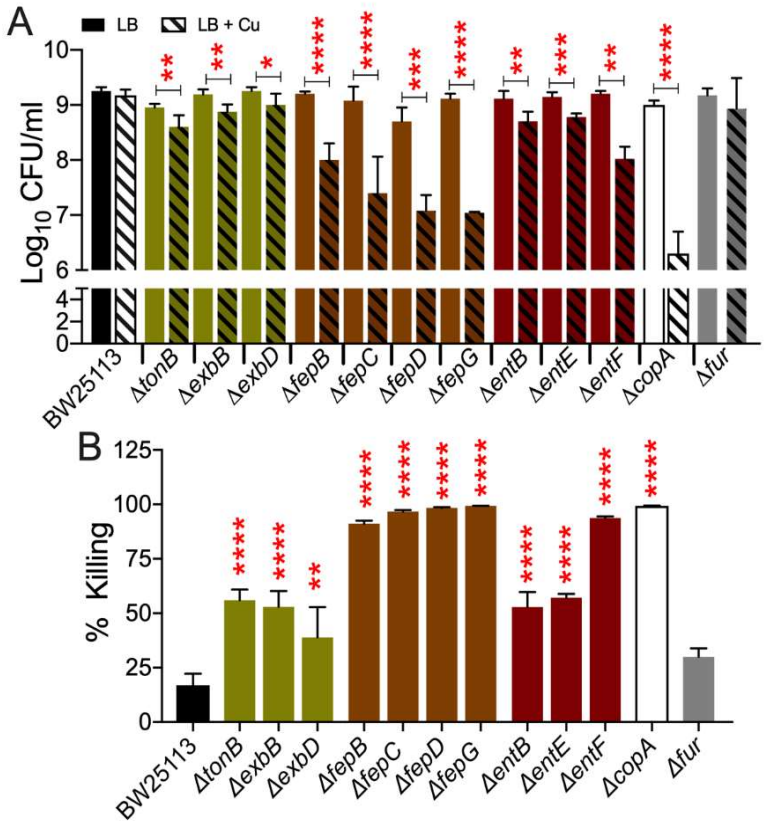


Figure 3. Viability of enterobactin-dependent iron uptake mutants during copper stress. A) Wild-type (BW25113) and mutant strains were grown in LB without (solid bars) or with copper (hatched bars) and viable counts were determined. Bars indicate median and error bars indicate interquartile range. **B)** Percent killing was calculated from viable counts presented in panel **A**) Mean + SEM is presented here. Results from three independent experiments conducted in triplicate

were analyzed by Mann-Whitney test (**A**), or ANOVA with Dunnett's test (**B**). **P*<0.05; ***P*<0.01; ****P*<0.001; and *****P*<0.0001.

3.5.6 Specificity of copper-induced killing in *E. coli*

We addressed whether increased killing during copper stress is specific for copper intoxication by using BCS, a known chelator of Cu^{1+} ions. Our pilot assays indicated that BCS at up to 6 mM levels did not induce a detectible decrease in viable counts of wild-type, *ΔtonB*, *ΔfepG*, *ΔentF*, and *ΔcopA* mutant strains. Quantitative plate counts were performed from LB cultures incubated in the presence of BCS, copper, or BCS + copper (Fig. 4A–E). Supplementation of BCS rescued the growth defect of these mutants that was induced in the presence of copper stress (Fig. 4A, C–E). Previously, yersiniabactin was demonstrated to confer additional protection from copper toxicity in UPEC.[49] Our findings indicate that the enterobactin system, encoded in the core genomes of *E. coli* and many other Gram-negative bacterial pathogens, plays a significant role in protection from copper stress. Collectively, siderophores emerge as key players in cellular defense against the toxic effects of copper in *E. coli*.

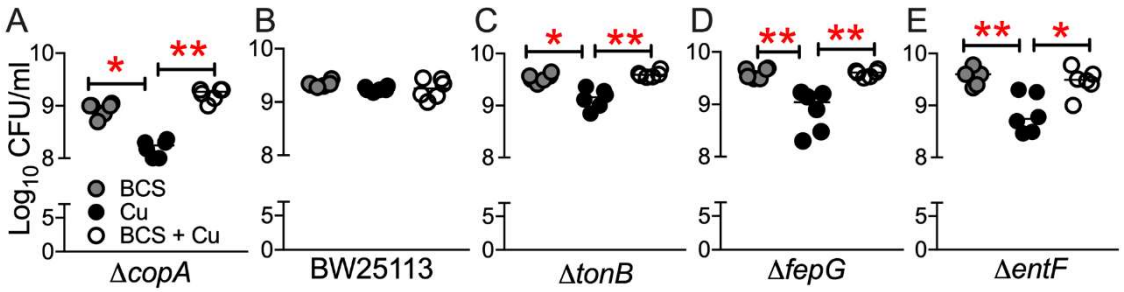


Figure 4. Chelation rescues growth of iron uptake mutants during copper stress. A-E) Indicated mutant or wild-type strain was cultured in LB containing bathocuproine (BCS, 6 mM), copper (3 mM), or BCS + copper to stationary phase, and viable counts were determined. The entire experiment was conducted three times, independently, with each strain/condition tested in duplicate. Bars indicate median. *P<0.05; and **P<0.01 by Kruskal-Wallis test with Dunn's post-test.

3.5.7 TonB is required in Uropathogenic *E. coli* to cope with copper stress

Laboratory strains of *E. coli* are reliant on enterobactin as the only siderophore-based Fe^{3+}-uptake system.[51] In contrast, pathogenic strains of *E. coli* produce both enterobactin and other siderophores including salmochelin, yersiniabactin, and/or aerobactin as redundant pathways to acquire Fe^{3+}.[17,52,53] We asked whether TonB, a central player in ferric-siderophore uptake, was also involved in protection from copper toxicity in pathogenic *E. coli*. UPEC strain CFT073 was used because it produces salmochelin and aerobactin, in addition to enterobactin. A *ΔcopA* mutant in the CFT073 genetic background was used as a control in this assay (Fig. 5). Viability of wild-type strain and mutants lacking *tonB* and *copA* were tested in the presence and absence of copper (Fig. 5A). All strains exhibited a statistically significant decrease in viable counts after growth under copper stress (Fig. 5A). The *ΔtonB* mutant was more sensitive to copper stress than the wild-type strain (Fig. 5B), reminiscent of our findings from the laboratory strain BW25113 (Fig. 3B).

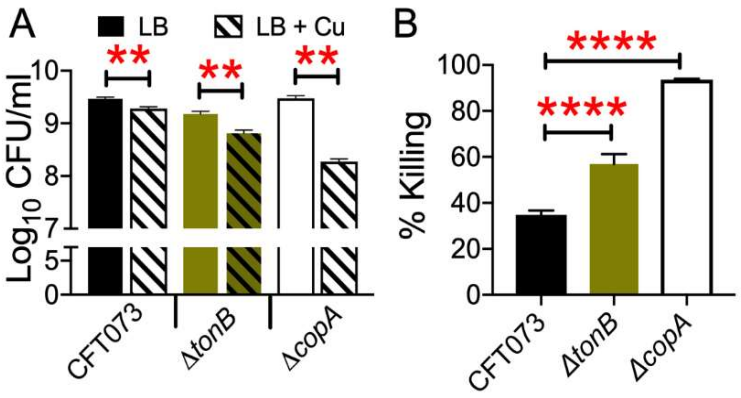


Figure 5. TonB is involved in protecting uropathogenic *E. coli* from copper stress. A) Viable counts of wild-type (CFT073), *ΔtonB,* and *ΔcopA* mutant strains grown in LB without (solid bars) or with 3 mM copper (hatched bars) is presented here. Bars indicate median and error bars indicate interquartile range. **B)** Percent killing was calculated from panel **A**) Mean + SEM is presented here. Results from three independent experiments conducted in triplicate were analyzed by Mann-Whitney test (**A**), or ANOVA with Dunnett's test (**B**). $^{**}P<0.01$; and $^{****}P<0.0001$.

TonB has been reported as a virulence factor in UPEC, since mutants lacking *tonB* and TonB-dependent Fe^{3+}-uptake systems are attenuated in a mouse model of UTI.[53–55] We have reported that endogenous host-derived copper is involved in impeding UPEC growth in the murine urinary tract, since copper-deficient mice are susceptible to higher bacterial burden during UTI than controls.[48] Current observation on the role of TonB in protection from copper intoxication suggests that iron acquisition is not only critical for continued UPEC growth within the host, but also required for optimal survival under copper stress at the host–pathogen interface. Small molecules that specifically inhibit the activity of TonB in UPEC have been described.[56] It will be of interest to test whether these TonB inhibitors modulate the sensitivity of

UPEC to copper, and their impact on modulating UPEC colonization in an experimental model of UTI.

3.5.8 Genetic complementation restores copper resistance in the *ΔtonB* mutant

To establish a causal link between mutants defective in Fe^{3+} uptake and increased copper sensitivity, we took a genetic complementation approach. We selected *ΔtonB* for genetic complementation since many other enterobactin pathway genes are organized as lengthy operons, which makes complementation under the transcriptional control of native promoters a challenging task (Appendix A Fig. 1). Full-length *tonB* and its native promoter was cloned into a low-copy number vector and introduced into the *tonB* strains (Appendix A Table 1). To rule out strain-specific effects, we tested the role of *tonB* in copper resistance in both laboratory (BW25113) and uropathogenic *E. coli* (UPEC CFT073) strains (Fig. 6). Wild-type, mutant, and complemented mutant strains had comparable turbidity (OD_{600}) during growth in LB in the absence of copper stress (Fig. 6A). Reintroduction of *tonB* in trans rescued growth of the *ΔtonB* mutant in the presence of copper in both laboratory (BW25113), and pathogenic (CFT073) strains of *E. coli* (Fig. 6B). To further confirm the specificity of this phenotype, we tested the growth of these strains under iron limitation. Genetic complementation reinstated the ability of *ΔtonB* mutant to grow in the presence of dipyridyl (Fig. 6C).

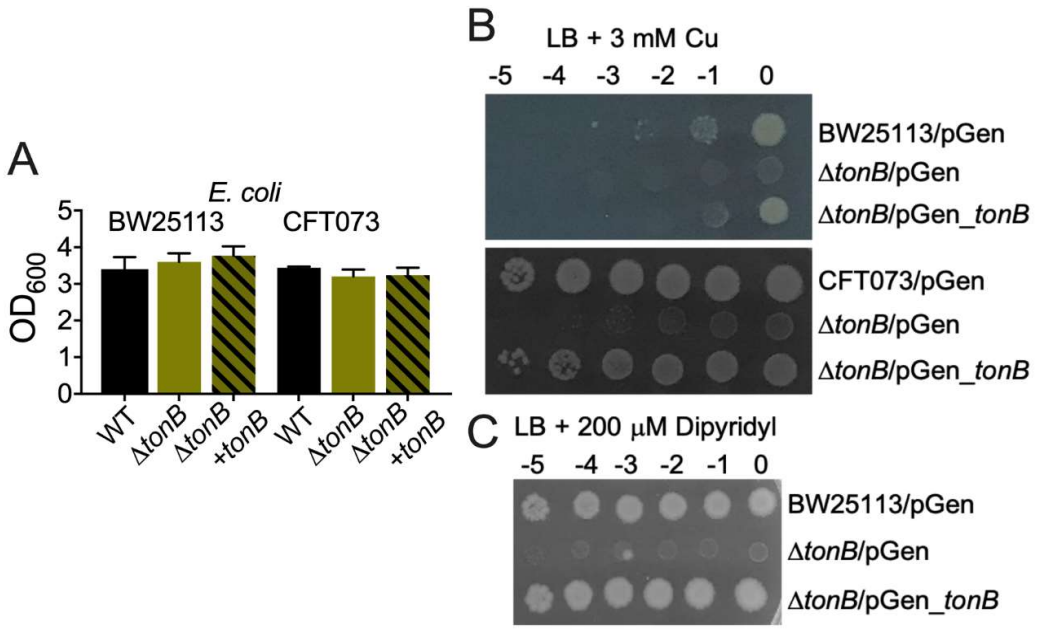


Figure 6. Genetic complementation of the *ΔtonB* mutant. A) Wild-type (BW25113 or CFT073), and *ΔtonB* mutant with empty vector (pGen), and the complemented mutant (pGen_*tonB*) were cultured overnight in LB and optical density was determined at 600 nm. **B)** Serial 10-fold dilutions of overnight cultures, from panel **A**, were plated on LB with 3 mM copper and images were acquired after 24 hours of incubation. The experiments were repeated thrice, and a representative image is depicted. **C**) Complementation of *tonB* reinstates growth of the *ΔtonB* mutant in the presence of dipyridyl, a known iron chelator.

3.5.9 Copper stress is mitigated in *E. coli* during growth in spent culture medium

We utilized culture supernatants in lieu of chemical complementation to further investigate the role of enterobactin biosynthesis and uptake in adaptation to copper stress. The *ΔtonB* and *ΔentF* strains exhibited significantly lower growth in spent medium harvested from

stationary phase cultures of wild-type strain compared to the wild-type strain (Fig. 7). However, the *ΔtonB* strain had significantly lower growth in spent medium supplemented with copper compared to wild-type and *ΔentF* strains (Fig. 7). Interestingly, the WT and *ΔentF* mutant strains, that are competent for importing enterobactin, exhibited comparable growth in spent medium supplemented with copper. These findings on the rescue of growth of an enterobactin biosynthesis defective mutant (*ΔentF*), but not an enterobactin import defective mutant (*ΔtonB*), in the presence of copper in spent medium suggests a role for enterobactin import in adaptation to copper stress. However, this experiment has to be evaluated with pure enterobactin to assess the specificity of our findings.

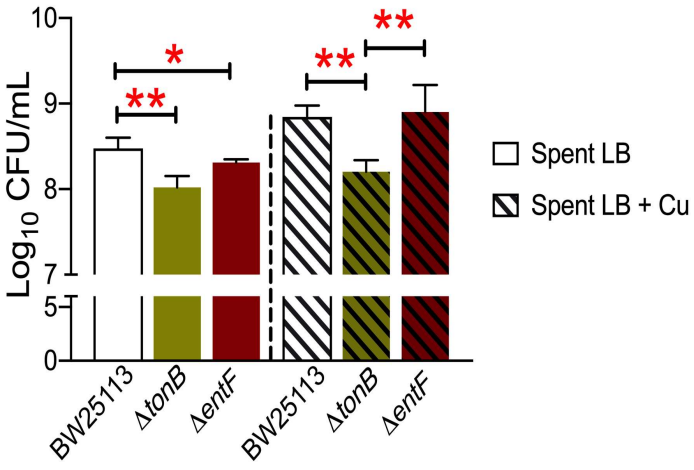


Figure 7. Copper toxicity in spent medium from the wild-type strain. Wild-type (BW25113), *ΔtonB,* and *ΔentF* mutant strains were cultured in spent LB harvested from stationary phase cultures of wild-type strain, that was supplemented with 0 (solid bars) or 3 mM $CuSO_4$ (hatched bars). Viable counts were determined after 24 hours from three biological replicates. Bars indicate

median and error bars indicate interquartile range. *P<0.05; and **P<0.01 by Kruskal-Wallis test with Dunn's post-test.

3.5.10 Cellular transition metal content during copper stress

To test whether these mutants are compromised in their ability to maintain normal level of transition metals, we determined the cellular content of key transition metals (copper, iron, zinc, and manganese) by ICP-MS or OES. There was no detectible difference in the content of these metals in the wild-type, *ΔtonB*, *Δfur*, and *ΔcopA* strains grown in LB (Figs. 8A and C, and Appendix A Fig. 2A and C). Mutants (*Δfur* and *ΔcopA*) were used as controls for dysregulation of iron homeostasis and loss of cytoplasmic copper efflux, respectively (Fig. 8 and Appendix A Fig. 2). As expected, copper content was increased in the wild-type and mutant strains grown in copper-supplemented medium, compared to LB (Figs. 8A and B). Cellular copper content in the *ΔtonB* mutant was indistinguishable from that of the wild-type strain in LB with and without additional copper (Fig. 8A and B). We observed hyperaccumulation of copper in the *Δfur* mutant comparable to that of a known copper-hyperaccumulating *ΔcopA* mutant strain, and significantly higher than the wild-type strain (Fig. 8B). Cellular levels of iron were similar in these strains in the absence of copper stress (Fig. 8C). We noted a significant decrease in cellular iron load in the *ΔtonB* mutant relative to the wild-type strain (Fig. 8D). On the contrary, and as expected, iron content was elevated in the fur mutant (Fig. 8D). Although the *ΔcopA* mutant had higher level of iron than the wild-type strain, this difference was not statistically significant (Fig. 8D). The *ΔcopA* mutant had accumulated significantly higher level of zinc than the parental strain during copper stress (Appendix A Fig. 2B). There were no significant changes in cellular manganese

load in these strains during copper stress (Appendix A Figs. 2C and D). Our ICP-MS and OES analyses suggest that the enhanced sensitivity of the *ΔtonB* mutant is likely due to lower iron content relative to the wild-type strain, although both strains accumulate the same level of cell-associated copper (Fig. 8A and B). Lack of difference in cellular copper content between the wild-type and *ΔtonB* mutant strains during copper stress suggests that enterobactin is unlikely to be involved in importing copper (Fig. 8A), unlike yersiniabactin which is reported to serve as a vehicle for copper import in UPEC.[57] The *Δfur* mutant over-accumulates copper; however, the toxic effects of copper are likely offset by the concurrent increase in iron levels. Overaccumulation of copper, without a concurrent increase in the level of iron in the *ΔcopA* mutant explains increased sensitivity of this mutant to copper that has been long known and utilized as a control in our assays. Changes in cellular iron content, taken in conjunction with increased expression of Fe^{3+}-uptake genes during copper stress reflects a mismatch between total and bioavailable levels of iron and transcriptional repression activity of Fur collectively reflecting disruption of iron homeostasis (Figs. 2 and 8).

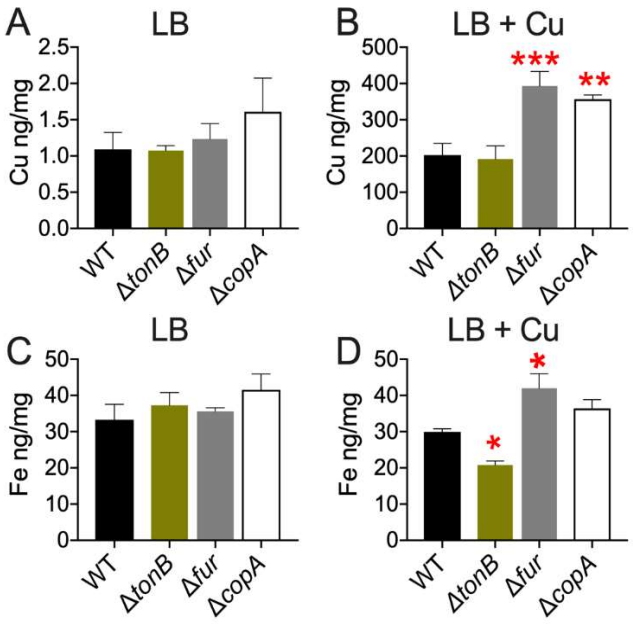


Figure 8. Cellular transition metal content during copper stress. Wild-type (BW25113), *ΔtonB, Δfur,* and *ΔcopA* mutant strains were exposed to 0 or 3 mM copper in LB. Cell pellets were digested and analyzed by ICP-MS or -OES to quantify copper (**A&B**), iron (**C&D**). Mean+SEM of data from three biological replicates is presented here and analyzed by ANOVA with Dunnett's test. **P*<0.05; ***P*<0.01; and ****P*<0.001

Our observations are aligned with the current model on mismetallation of noncognate proteins by copper as a central mechanism of toxicity because maintaining a robust, bioavailable pool of cellular iron is critical to ensure that iron is incorporated into its cognate sites (Fig. 9).

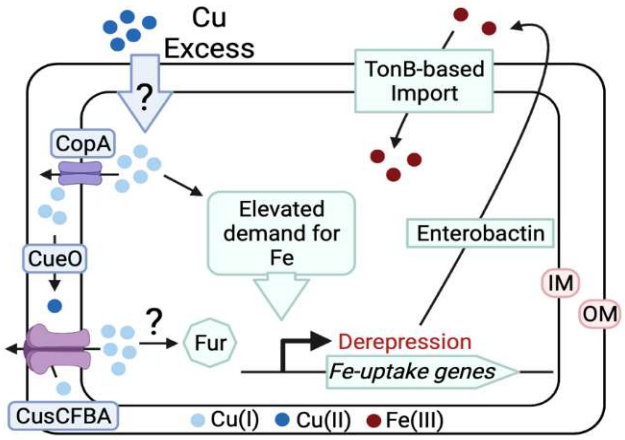


Figure 9. Role of enterobactin in protecting *E. coli* from copper stress. Intracellular copper levels increase during copper stress leading to increased demand for iron associated with disruption of iron homeostasis. Accumulation of copper in the cytosol triggers expression of copper efflux and detoxification systems encoded by the *copA, cueO,* and *cusCFBA* genes. Elevated cellular need for iron results in derepression of Fur-regulated genes that culminates in the production of enterobactin, and uptake of ferric-enterobactin. The ability to ramp up Fe^{3+} import protects wild-type *E. coli* from copper intoxication, and mutants defective in production/uptake of enterobactin are compromised in survival during copper stress. IM, inner membrane; and OM, outer membrane.

3.6 Conclusions and Future Directions

This report provides genome-level insights into the mechanisms involved in adaptation to copper stress in *E. coli*. Here, we report the involvement of enterobactin pathway genes and several other genes in copper-responsive phenotypes in *E. coli*. We provide evidence to support the roles of enterobactin-dependent Fe^{3+}-uptake system genes in promoting *E. coli* survival

during copper stress. Collectively, our data supports a model in which both lack of enterobactin biosynthesis and failure to import enterobactin by TonB-dependent systems result in exacerbation of toxic effects of copper (Fig. 9). Cellular metal content analysis indicates that copper stress is associated with dysregulation of iron homeostasis in *E. coli*. Our findings raise intriguing questions on the potential for direct interaction between Fur, enterobactin, and copper, and the implication of such interactions on metal homeostasis. This report expands our knowledge on the link between homeostasis of transition metals in a bacterial cell and support observations at the host–pathogen interface, including in the urinary tract where concurrent overload of copper and starvation of iron is part of the innate immune response triggered during infection.[48,58]

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4 COMPARATIVE TRANSCRIPTIONAL PROFILING OF UROPATHOGENIC *ESCHERICHIA COLI* UNDER PHYSIOLOGICALLY-RELEVANT COPPER STRESS REVEALS A ROLE FOR FEOB IN COPPER HOMEOSTASIS

4.1 Abstract

Urinary Tract Infections (UTI) are a global health crisis affecting approximately 7-11 million people every year in the United States alone. Uropathogenic *E. coli* (UPEC) are the primary etiologic agent, accounting for 80% of uncomplicated UTIs. Work from our lab and others have shown copper toxicity to be a host innate immune effector during UTIs and copper-detoxification genes to be crucial for UPEC fitness in the urinary tract. We sought to characterize the global response of UPEC to copper stress. To accomplish, this we utilized RNAsequencing (RNAseq) to generate a comparative transcriptional profile of UPEC CFT073 and two copper-detoxification-deficient mutants, CFT073 *ΔcueR* and CFT073 *ΔcusRSCFBA* (*Δcus*), under a physiologically-relevant copper concentration. We report a large data set of transcripts differentially-expressed under copper stress conditions. Notably, we identified numerous iron homeostasis genes to be upregulated during copper toxicity. Among these, were two members of the Feo (ferrous iron uptake) system, *feoA* and *feoB*. We found a *feoB*-deficient mutant to be more resistant to copper stress in a commensal BW25113 background and only slightly more resistant in a UPEC CFT073 background. We report a BW25113 *ΔfeoB* experiences less copper stress than WT, while UPEC CFT073 *ΔfeoB* may be experiencing less cytosolic, but higher periplasmic copper stress. We propose FeoB may be contributing to copper accumulation in the cytosol in a commensal strain, resulting in higher copper toxicity. UPEC CFT073 may be

overcoming this through relying on other mechanisms of iron acquisition and limiting *feoB*-mediated copper accumulation.

4.2 Introduction

Uropathogenic *E. coli* is the primary causative agent of Urinary Tract Infections (UTI), which affects approximately 7-11 million people in the United States every year. These infections were responsible for close to 13 million physician or emergency room visits and approximately 479,000 hospitalizations in 2000 and 2006, respectively, costing approximately $3.5 billion in expenditures in the United States.[1-3] Although UTIs can be self-limiting, they are the second most common infection leading to antibiotic prescription.[4] With antimicrobial resistance being characterized as a global health concern by the World Health Organization (WHO), understanding how pathogens are responding to host immune responses is of immense importance. In an effort to understand UPEC response to clinical infection, Subashchandrabose et al. discovered copper detoxification genes to be significantly upregulated in UPEC from clinical UTI isolates.[5] Our lab has recently reported that ceruloplasmin, a ferroxidase responsible for 90% of blood copper content, is mobilized to the urinary tract during infection, increasing copper toxicity and inducing iron starvation.[6] While the primary UPEC copper detoxification mechanisms have been characterized, much still remains to be known about copper homeostasis and its role in UPEC pathogenesis.[7-12]

Transcriptomics has previously been used in UPEC to determine gene expression during clinical infection, observe the host-pathogen co-transcriptome in a murine macrophage model,

and compare expression profiles in urine compared to lysogeny broth (LB).[5,13,14] While these studies have contributed much to the field, there is still a gap of knowledge on what UPEC's genetic response is to immune responses such as copper. In 2005, a study analyzed the gene expression of *E. coli* K-12 strain MG1655 under minimal, optimal, and high copper concentrations via DNA microarray.[15] However, this was not done using the more sensitive and global RNAsequencing and was in a commensal *E. coli* strain. We sought to assess the global response of UPEC to copper, a known host immune effector. We utilized RNAsequencing to determine differentially-regulated genes when UPEC CFT073 was exposed to physiologically-relevant copper stress. We also chose to investigate how mutants defective in two known copper-detoxification systems namely, CFT073 *ΔcueR*, and CFT073 *ΔcusSRCFBA* (hereafter referred to as *Δcus*), differ in transcription profiles compared to the wild-type strain. We hypothesized that genes that have a role in copper stress response will be differentially-expressed under copper stress conditions as compared to normal growth conditions. We identified 49 and 30 upregulated and downregulated genes, respectively, in the UPEC CFT073 during copper stress. Interestingly, we identified only 6 upregulated genes and no downregulated genes for the CFT073 *ΔcueR* comparison. In the *Δcus* comparison, we identified 45 upregulated and 22 downregulated genes. Among these, were numerous metal and stress related genes. We found two members of the *feoABC* system, specifically *feoA* and *feoB*, to be upregulated under copper stress. Based on our previous work focusing on the interplay of *E. coli* ferric (Fe^{3+}) iron uptake and copper stress mitigation, we chose to explore the role of the ferrous (Fe^{2+}) iron transport system *feoABC* in copper homeostasis.

FeoABC is the most conserved bacterial ferrous iron transporter and is the only known *E. coli* transporter to be specific for ferrous iron.[16] Despite its presence in numerous bacterial strains, much is still unknown about the *feoABC* system. Although the system is represented in multiple bacterial species, not all are comprised as a tripartite *feoABC* operon.[17] In fact, this system is most often found as *feoAB* (54%), and in some cases, only *feoB* (11%) in bacteria. The rarest organization is that of a *feoAB* fusion at just 3% of bacterial Feo systems. The general structure of each member has been identified and is the main focus of a couple publications.[18,19] Of the three Feo proteins, FeoA is the most undetermined in its function; however, it is believed to be a cytoplasmic protein composed of 250 amino acids.[16,17] Orthologous systems suggest it may form a complex with FeoB, although this has not been reported in *E. coli*.[20] FeoB is the largest and most well-known in the Feo system, described as an inner membrane permease consisting of 773 amino acids.[21] Although the mechanism is still unknown, FeoB has been shown to be necessary for ferrous iron transport.[16] Moreover, *E. coli* and *Salmonella feoB*-deficient mutants have shown to have attenuated intestinal colonization in a murine model.[22,23] FeoC is the smallest protein of this system at 78 amino acids and is only present in approximately 13% of bacterial Feo operons. While the function of FeoC is still widely unknown, some structural studies suggest it may serve as a transcriptional regulator of tripartite *feo* operons.[24,25] In *S. enterica*, FeoC has been reported to interact and protect FeoB from FtsH-mediated proteolysis *in vivo*.[26] The differences among bacterial Feo organization and conservation along with the aforementioned data suggest FeoB is the main ferrous iron transporter, while FeoA and FeoC act as accessory proteins.

Table 1. Known Bacterial Ferrous Iron Transporters

No.	Transport System	Present in BW25113	Present in CFT073
1	FeoABC	Yes	Yes
2	MntH	Yes	Yes
3	ZupT	Yes	Yes
4	YfeABCD	No	No
5	EfeUOB	Yes[a]	Yes
6	SitABC	No	Yes
7	FutABC	No	No

[a]*Non-functional*

Since FeoB is the most characterized and seems to be the main effector of ferrous iron transport, we set out to determine the role of FeoB in copper homeostasis. Additionally, because our previous work revealed a role for a ferric iron import systems in copper stress mitigation was primarily conducted in a K-12 background, we sought to explore the differences between the role of FeoB in commensal (BW25113) and UPEC (CFT073) *E. coli* strains.[27] The Feo system is highly conserved between these strains in both amino acid and nucleotide sequence (Appendix B Table 1). Specifically, BW25113 *feoB* has 97.8% nucleotide sequence similarity and 99.74% amino acid similarity to CFT073 *feoB*. In both the K-12 and UPEC strain, we found *feoB* to be significantly upregulated during copper stress. However, it is worth noting we saw more upregulation in our UPEC strain than in the K-12 strain. Surprisingly, we report a *feoB*-deficient mutant to be more resistant to copper stress in the BW25113 background. Furthermore, we showed genetic complementation of *feoB* restored sensitivity to copper. The CFT073 *ΔfeoB*

showed a slight resistance to copper compared to the wild-type, however this difference was less striking than the K-12 comparison. We also found BW25113 *ΔfeoB* to have less expression of *copA* and *cusC*, two known copper-stress response genes, compared to wild-type. Interestingly, in UPEC CFT073 *ΔfeoB*, we found significantly less *copA* expression, but significantly more *cusC* expression compared to the background strain. Our ICP-MS data corresponds with our copper-resistant phenotype and RT-qPCR results, showing a FeoB-deficient mutant to have less copper accumulation than the BW25113 parent strain. While it appears FeoB leads to higher cellular copper accumulation and increased copper stress in a non-pathogenic strain, the UPEC strain seems to have overcome this problem. The differences depicted between UPEC CFT073 and BW25113 could point to an adaptation of FeoB or genetic regulation of the encoding gene that improves fitness in the urinary tract.

4.3 Materials and Methods

4.3.1 Bacterial strains and culture conditions

Strains and plasmids used in this report are listed in Appendix B Table 2. Bacterial strains were cultured in LB broth or agar (tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, and agar 15 g/L). Bacterial cultures were incubated at 37°C and broth cultures were aerated by shaking at 200 RPM, unless otherwise noted. Chloramphenicol (34 mg/mL) or kanamycin (25 mg/mL) was used for selection of mutants and to ensure maintenance of plasmids. Chemicals were purchased from Sigma or Fisher Scientific.

4.3.2 RNA isolation

$CuSO_4$ (25 μM) was added to cultures of wild-type, *ΔcueR,* and *ΔcusRSCFBA* strains in mid-logarithmic phase, and RNA was extracted after 20 minutes, in accordance with *E. coli* doubling time. Transcripts were stabilized with RNAprotect (Qiagen), extracted with RNeasy mini kit (Qiagen). RNA integrity was assessed using a Qubit Fluorometer (Life Technologies) and TapeStation (Agilent).

4.3.3 RNA-sequencing and analysis

RNA was analyzed with NovaSeq 6000 Illumina sequencing. RNA samples were prepared to maintain directionality. The RNA-seq libraries were checked for quality and adapter content using FastQC v0.11.9, and then mapped to the NCBI *E. coli* RefSeq genome GCF_000007445.2_ASM744v1 using the program HISAT2 version 2.2.1.[49,50] Differential expression between groups was assessed using the program DESeq2 1.30.1 in R.[51] Log2 fold-change between the control and copper stress groups and a false discovery rate (FDR) based on the fold change of the two biological replicates, each with two technical replicates, were calculated to determine significance

4.3.4 Copper-response phenotype assays

Overnight cultures were normalized (OD_{600} = 1.0) and serial diluted to 10^{-7} in 1x PBS. Dilutions were spot plated onto LB with or without 3 mM or 4 mM $CuSO_4$ as indicated. Plates were incubated at 37°C for 24 h. After incubation, plates were observed for growth.

4.3.5 Quantitative PCR

Overnight cultures were subcultured 1:100 in LB. During the mid-logarithmic phase, $CuSO_4$ was added to designated cultures for a final concentration of 500 μM, and RNA was extracted after 20 min, in accordance with *E. coli* doubling time. Transcripts were stabilized with RNAprotect (Qiagen), extracted with RNeasy mini kit (Qiagen). Contaminating DNA was removed by digestion with DNase (Ambion) and cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was performed with SYBR Green (Thermo Scientific) and primers (Appendix B Table 3) in a CFX Real-Time system (BioRad Laboratories). *gapA* was used to normalize transcript levels between samples, and relative expression was calculated by using LB without copper as the control.

4.3.6 Genetic complementation of *feoB*

Plasmids pCA24N and p*feoB* were obtained from the ASKA overexpression library.[28] Plasmids were isolated using a QuickSpin Miniprep Kit (Qiagen), electroporated, and

transformed into the *E. coli* BW25113, BW25113 *ΔfeoB*, CFT073, and CFT073 *ΔfeoB* electrocompetent cells. Clones were selected using LB + chloramphenicol (34 mg/mL).

4.3.7 Trace element analyses

Inductively coupled plasma-mass spectrometry or optical emission spectrometry (ICP-MS or OES) was used to determine transition metal content, as we have recently reported.[27] Briefly, overnight cultures of bacterial strains were subcultured in LB for 2 hours and then incubated for 30 mins with or without 2 mM $CuSO_4$. Strains with plasmids were supplemented with 0.1 mM IPTG to induce plasmid expression. Pellets were harvested by centrifugation and washed thrice with 10 mM HEPES containing 0.5 mM EDTA. Cell pellets were digested with trace element-grade nitric acid and diluted in trace element-grade water. Levels of copper, iron, zinc, and manganese were determined by ICP-MS by an operator blinded to the treatment groups (8800 ICP-MS/MS, Agilent Technologies). Spectral interference was minimized by conducting the analysis in a single quadrupole mode using helium in the collision/reaction cell. Samples containing higher concentrations of copper, iron, and zinc were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The sample introduction system in the ICP-OES instrument (5110 SVDV ICP-OES, Agilent Technologies) was composed of a single-pass cyclonic spray chamber and a glass concentric nebulizer. Manufacturer default operating conditions were adopted in all ICP-OES analyses, and atomic/ionic emission signals at 327.395, 238.204, and 213.857 nm were used for copper, iron, and zinc determinations, respectively. Concentration of metals was normalized to the wet weight of *E. coli* pellets.

4.4 Results and Discussion

4.4.1 RNAsequencing reveals differentially-expressed of uropathogenic *E. coli* genes during physiologically-relevant copper exposure

Our goal for this study was to determine the global changes in UPEC gene expression under physiologically-relevant copper stress when compared to normal growth conditions.[29] We also sought to explore the effect of known transcriptional regulators of copper-detoxification genes on UPEC transcription under these conditions.[30] To accomplish this task, UPEC CFT073, CFT073 *ΔcueR,* and CFT073 *ΔcusRSCFBA* (here after, referred to as *Δcus)* strains were grown in LB and then either exposed to 25 μM $CuSO_4$ or no copper control during the mid-logarithmic phase (Fig. 1). Changes in transcript levels between strains and conditions were then determined by DESeq2 package in R. UPEC CFT073 served as our wild-type (WT) UPEC strain, while CFT073 *ΔcueR* and CFT073 *Δcus* are the two copper-dependent transcriptional regulator mutants. CFT073 *ΔcueR* lacks the transcriptional regulator CueR that activates transcription of *copA* and *cueO* genes when bound to copper. CFT073 *Δcus* is deficient in the entire Cus copper efflux system and its cognate two component regulatory system. These two systems are discussed in detail in chapter two.[30] We hypothesized that genes differentially-expressed under physiologically-relevant copper concentrations will play a role in UPEC mitigation of copper toxicity. We compared the transcriptome of each strain's copper-exposed condition back to their no copper control condition. The UPEC CFT073 comparison had 49 and 30 upregulated and downregulated genes, respectively (Appendix B Table 4). Interestingly, the CFT073 *ΔcueR* comparison only had 6 significantly upregulated genes and 0 downregulated genes (Appendix B

Table 5). The CFT073 *Δcus* comparison was more similar to the wild-type strain with 45 upregulated genes and 22 downregulated genes (Appendix B Table 6).

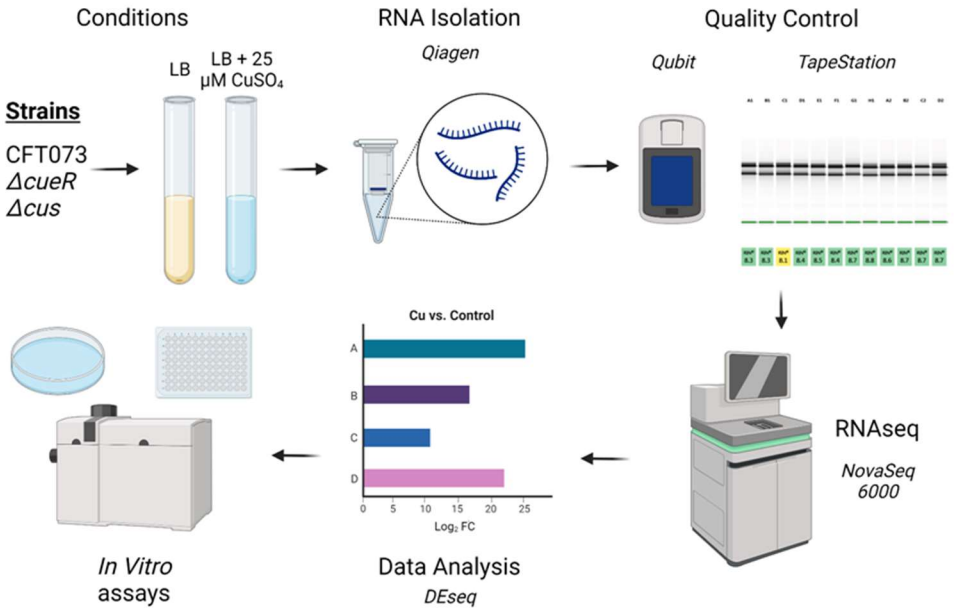

Figure 1. Overview of RNAseq protocol. Strains CFT073, CFT073 *ΔcueR,* and CFT073 *ΔcusRSCFBA* (*Δcus*) were incubated in LB to mid-logarithmic phase and then either exposed to 25 μM $CuSO_4$ or kept a no copper control. RNA was extracted using Qiagen kit. Samples were tested for quality control with Qubit Fluorometer and TapeStation. RNAseq was conducted using Illumina NovaSeq6000 to maintain directionality. Data Analysis was completed using the DEseq2 package in R. *In vitro* assays were then used to assess the genes reported in the RNAseq datasets for a copper-responsive phenotype and further analyses.

4.4.2 Expression of known copper-responsive genes detected in uropathogenic *E. coli* by RNA sequencing

In all 3 comparisons, our results were validated through the expected upregulation of known *E. coli* copper detoxification systems. In the wild-type comparison, we saw both members of the Cus and CueR system among the most upregulated genes, confirming that the cells are experiencing copper stress at this physiologic concentration of copper (Fig. 2a). In the *ΔcueR* comparison, only members of the Cus system were upregulated (Fig. 2c). This is expected as the transcriptional regulator CueR is not present in the mutant to induce the expression of *cueO* and *copA*. As expected, in the *Δcus* comparison, only members of the CueR system were identified (Fig. 2d). As the *Δcus* mutant is missing all members of this system, our results are validated through these internal markers.

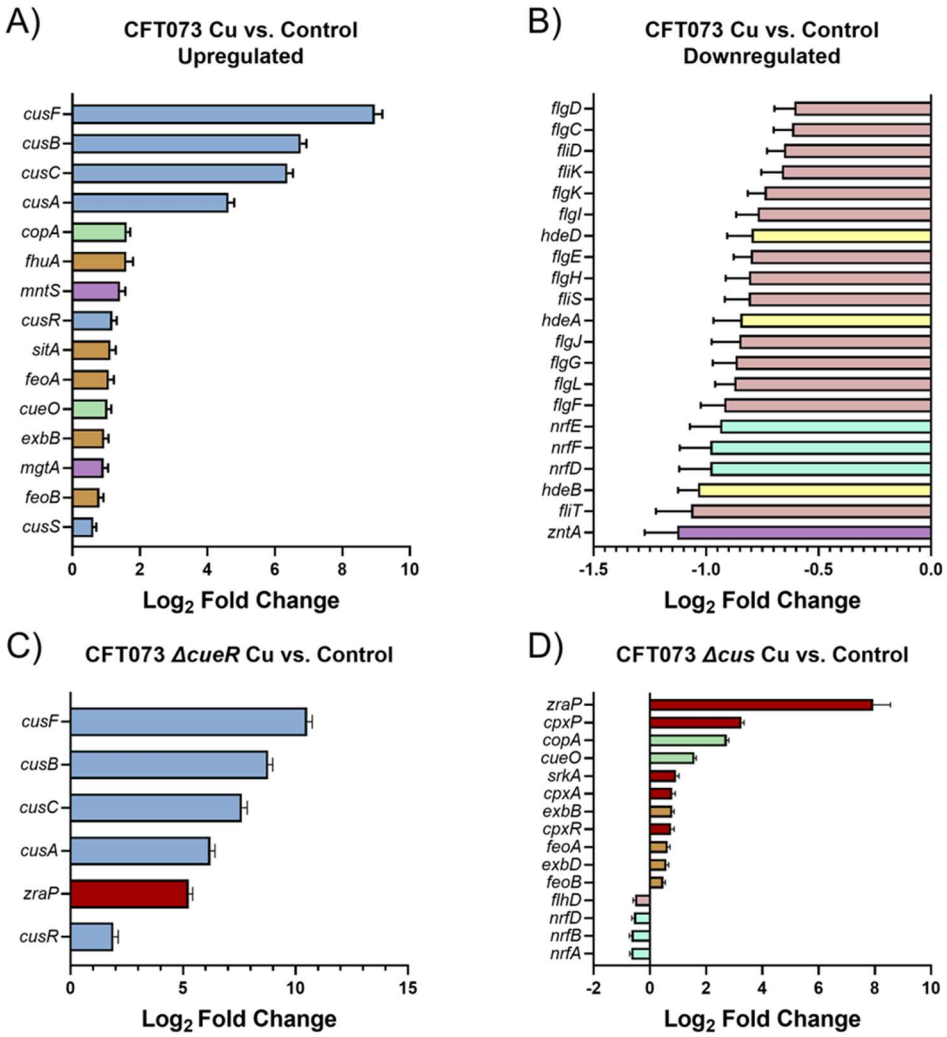

Figure 2. Summary of key genes detected in RNAseq of CFT073, CFT073 *ΔcueR*, and CFT073 *Δcus* under copper stress. Strains were exposed to 0 or 25 μM $CuSO_4$ during mid-logarithmic phase and RNA was extracted and RNAseq was completed. Log_2 fold change is presented here for **A)** Key upregulated genes in the CFT073 copper vs. control comparison; **B)** Key genes downregulated in the CFT073 copper vs. control comparison; **C)** All genes differentially-expressed in the *ΔcueR* comparison; **D)** Key genes in the *Δcus* comparison. See Appendix B Tables 4-6 for full data sets. Dark blue: cus system. Green: cue system. Orange: Iron

homeostasis. Purple: Metal-related. Pink: Flagellar-related. Yellow: Acid stress. Light blue: nrf operon. Red: Envelope stress response. Mean + SEM from 2 independent experiments, in duplicate, are presented here and were analyzed for significance with the DEseq2 package in R.

4.4.3 Iron uptake genes are upregulated in uropathogenic *E. coli* during copper stress

Of interest, genes related to iron homeostasis, namely *fhuA, tonB, sitA, feoA,* and *feoB,* were upregulated under copper stress in the wild-type comparison (Fig. 2a). This corroborates our recent study showing the involvement of the bacterial ferric iron chelating system, Enterobactin, in mitigation of copper stress.[27] We reported mutants deficient in ferric-enterobactin biosynthesis or uptake are more sensitive to copper toxicity in both K-12 BW25113 and UPEC CFT073. We presented a model where copper stress results in an increased demand for cellular iron. FhuA is the outer membrane transporter for ferrichrome, a hydroxamate-class siderophore.[31] TonB is the largest part of the TonB-ExbB-ExbD energy transduction complex, which provides energy for ferric-siderophore import systems, such as ferrichrome, enterobactin and other siderophores.[32] SitA is a metal-type ABC Mn^{2+} and Fe^{2+} transporter.[33] Although originally characterized as a Fe^{2+} transporter, it appears to have a higher affinity for Mn^{2+}. Interestingly, two genes of the Feo system, *feoA* and *feoB,* were upregulated under copper stress. The Feo system is the earliest and most conserved bacterial ferrous iron transport system and is regulated by both the Fur transcriptional repressor and Fnr transcriptional activator.[16] Our transcriptome data supports our previously proposed model of increased iron demand during copper stress with the upregulation of multiple iron acquisition genes.

4.4.4 Expression of other metal transport genes in uropathogenic *E. coli* during copper stress

Within the wild-type comparison, other metal and stress related genes were also differentially-expressed. Both *mntS* and *mgtA* were significantly upregulated under copper stress conditions (Fig. 2a). MgtA has not been well characterized in *E. coli*, but has been extensively studied in *Salmonella spp.* as a P-type ATPase Mg^{2+} importer.[34] MntS is a small protein which either directly or indirectly modulates intracellular Mn^{2+} concentrations.[35] ZntA is a P-type ATPase Zn^{2+} exporter and was significantly decreased in the WT comparison under copper stress (Fig. 2a).[36] The current mechanism for copper toxicity appears to be due to the ability of copper to mismetallate the active sites of key metalloenzymes. This mismetallation leads to the inactivation of key bacterial enzymes, resulting in numerous downstream effects. The decreased expression of *zntA* and increase of *mntS* and *mgtA* expression suggests an increased demand for Zn^{2+}, Mn^{2+}, and Mg^{2+} under copper stress, validating this mechanism of copper toxicity.

4.4.5 Other genes that are differentially expressed during copper stress in UPEC

Many genes were differentially-expressed that do not have a known role in UPEC remediation of copper toxicity. Multiple flagellar biosynthesis genes were significantly downregulated, including multiple members from regions I and III as described by Iino et al (Fig. 2b).[37] Some of these flagellar related genes were also reported to be in a K-12 DNA microarray study assessing copper-responsive genes.[15] Here, they suggested the response is due to an influence of copper on OmpR-EnvZ and H-NS transcriptional regulation on the flagellar

biosynthetic operon. Although neither of these systems were represented in our data, the possibility of their influence cannot be negated. Additionally, all three genes that encode the *hdeABD* periplasmic acid stress system were significantly downregulated in WT comparison (Fig. 2b).[38] Interestingly, hdeABD expression is also dependent on H-NS regulation.[39] More research needs to be conducted to understand the potential role these systems play in mitigation of copper toxicity.

4.4.6 Role of CueR in regulating expression of copper-responsive genes

In the *ΔcueR* comparison, the only non-*cus* related gene differentially-expressed was *zraP*, which modulates the Zra envelope stress response pathway (Fig. 2c).[40] Purified ZraP has been shown to bind multiple divalent metal ions, including Cu^{2+}.[41] Although *zraP* has been shown to play a role in defense against antimicrobials, acidic pH, osmotic shock, and bile salts, to our knowledge, no studies have assessed its role in copper homeostasis.[40]

4.4.7 Role of CusRS in regulating expression of copper-responsive genes

Similar to the *ΔcueR* comparison, *zraP* was also upregulated in the *Δcus* comparison, suggesting neither system plays a role in directly nor indirectly interacting with ZraP. We also saw increased *cpxP, cpxR,* and *cpxA* expression (Fig. 2d).[42,43] This system is responsible for envelope stress response, confirming previous studies suggesting copper stress leads to disrupted membrane homeostasis. Interestingly, *feoA* and *feoB* were upregulated in the wild-type and *Δcus* comparison, but not the *ΔcueR* (Fig. 2c-d). Further studies are needed to confirm if CueR plays a

direct or indirect role in the regulation of the Feo system. Other iron homeostasis genes, *ftnB,* *exbB* and *exbD*, also were upregulated in this comparison, supporting our previously mentioned copper-induced dysregulation of iron homeostasis model (Fig. 2d). The SrkA protein is an understudied stress response kinase and was significantly upregulated in our *Δcus* comparison (Fig. 2d).[46] Although not yet reported, it is possible that the SrkA is activated under copper toxicity. Multiple members of the Nrf cytochrome c-552 nitrite reductase system were significantly downregulated (Fig. 2d).[44,45] This system has no known role in copper homeostasis, but its role in metabolism may prove to be a hindrance to overcome copper stress.

4.4.8 Uropathogenic *E. coli* CFT073 and K-12 BW25113 strains have increased *feoB* expression under copper stress

Due to our previous work in exploring the intersection of iron homeostasis and copper toxicity, we decided to assess the role of the Feo system in copper stress response. Specifically, we chose *feoB* as our gene of interest as it is the largest and most characterized within this system. Since our previous proposed model was primarily established in a K-12 strain, we also wanted to explore the potential role in both a commensal and uropathogenic strain.[27] We exposed UPEC CFT073 and K-12 BW25113 to copper during the mid-logarithmic phase and then extracted RNA. We then sought to confirm our RNAseq findings through RT-qPCR. We confirmed that *feoB* is significantly upregulated under copper stress conditions in both the UPEC CFT073 strain and a K-12 lab strain (Fig. 3a-b). It is worth noting that *feoB* expression was upregulated ~2x higher in UPEC CFT073 than in BW25113. Our results confirm our RNAseq findings and suggest a role for *feoB* in both UPEC and K-12 copper homeostasis.

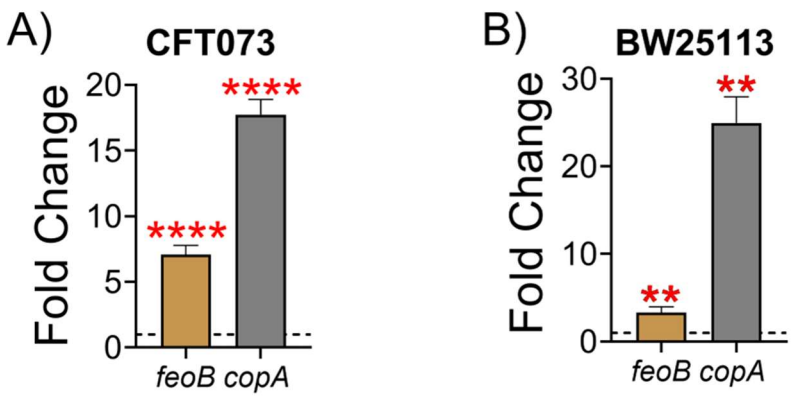

Figure 3. Expression of feoB is induced under copper stress in a commensal and UPEC strain. Strains were exposed to 0 or 0.5 mM $CuSO_4$ during mid-logarithmic phase and then processed for RT-qPCR. Fold change in abundance of transcripts shows significant induction of *feoB* under copper stress in **A)** UPEC CFT073 and **B)** BW25113. Here, *copA* is the copper-responsive control. Mean + SEM from 3 biological replicates, in triplicate, is presented here and were analyzed by t-test. **P<0.01, ****P<0.0001.

4.4.9 A FeoB-deficient mutant is more resistant to copper stress and genetic complementation restores wild-type level of copper-sensitivity

We sought to characterize the growth phenotype of a FeoB-deficient mutant in both UPEC and K12 background. We constructed a UPEC CFT073 *ΔfeoB* mutant using lambda red recombineering as described in Datsenko and Wanner.[47] For the K-12 BW25113 mutant, we utilized the *ΔfeoB* strain from the KEIO collection, a genetically-defined mutant library of non-essential genes.[48] Both mutants were selected through their individual antibiotic resistance

cassettes. Additionally, we confirmed both mutations by PCR and gel electrophoresis. We also determined that neither mutant has a growth deficit or improvement in LB compared to their parent strain (Fig. 4a-b).

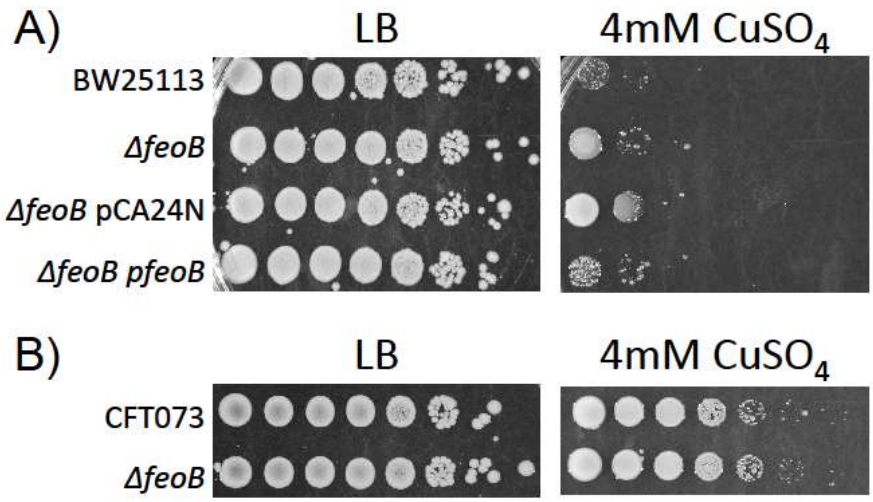

Figure 4. A *feoB*-deficient mutant is more resistant to copper stress in BW25113 than UPEC CFT073. Cultures of **A)** BW25113 *ΔfeoB*, BW25113 *ΔfeoB* pCA24N, and BW25113 *ΔfeoB pfeoB* or **B)** CFT073 and CFT073 *ΔfeoB* were grown overnight, normalized for growth to an OD_{600} of 1.0, serial diluted, and spot plated onto LB or LB + 4 mM $CuSO_4$. After overnight incubation, plates were qualitatively assessed for mutant copper-response phenotype compared to the parental strains

To assess the growth phenotype under copper stress, we grew both FeoB-deficient strains alongside their parent strains on LB and LB containing 4 mM $CuSO_4$. Surprisingly, we found BW25113 *ΔfeoB* to have qualitatively more growth than the wild-type strain (Fig. 4a). Even

more interesting, the CFT073 *ΔfeoB* exhibited only a mild increase in copper resistance compared to its parental strain (Fig. 4b). This could be due to the redundancy in Fe^{2+} and Fe^{3+} iron transporter systems in UPEC CFT073 that is not found in BW25113. Additionally, the reduction in *feoB* expression in the K-12 strain compared to the UPEC strain, could point to FeoB contributing to copper stress in BW25113, but not CFT073. This could represent a strain-specific adaptation that allows UPEC CFT073 to overcome copper toxicity more effectively than a commensal *E. coli* strain.

To determine if this phenotype is Fe^{2+} iron- or feoB-specific, we tested several Fe^{2+} iron transporters from the KEIO collection. No other Fe^{2+} iron transporter showed differential growth compared to the WT BW25113 strain. Results are summarized in Appendix B Table 7. We also wanted to check if the other genes of the Feo operon are involved in modulating growth during copper stress. Neither a BW25113 *ΔfeoA* or *ΔfeoC* strain exhibited differential growth compared to their parent strain. Collectively, this data suggests the resistant phenotype is specific to the FeoB inner membrane permease and not associated with ferrous iron transport via other transporters or other members of the *feo* locus.

To confirm the specificity of resistance to FeoB, we complemented the BW25113 *ΔfeoB* with a *feoB* overexpression plasmid from the ASKA library.[28] Genetic complementation restored copper sensitivity of the *ΔfeoB* comparable to the wild-type strain (Fig. 4a). Notably, introduction of the empty vector plasmid, pCA24N, in the FeoB-deficient mutant (BW25113 *ΔfeoB* pCA24N) strain exhibited a resistant growth phenotype comparable to BW25113 *ΔfeoB*. However, the complemented mutant strain (BW25113 *ΔfeoB* p*feoB*) was more sensitive than the

empty vector control. Together, this data confirms the specificity of the copper resistant phenotype to the FeoB, inner membrane permease.

4.4.10 A *ΔfeoB* mutant has decreased expression of genes related to copper detoxification

We next sought to determine how FeoB-deficient UPEC and K-12 strains continue to thrive in the presence of copper stress. We exposed UPEC CFT073 *ΔfeoB* and BW25113 *ΔfeoB* to copper, extracted RNA and conducted RT-qPCR. We found that a FeoB-deficient mutant in both UPEC and K-12 background have decreased expression of *copA* compared to their wild-type strain (Fig. 5a-b). This suggests that in the absence of FeoB *E. coli* cells experience decreased cytoplasmic copper stress, relative to the parental strain. The K-12 *ΔfeoB* strain also exhibited decreased *cusC* expression, suggesting overall less copper stress compared to the parent strain (Fig. 5b). Interestingly, the UPEC *ΔfeoB* strain had increased *cusC* expression compared to its parent strain (Fig. 5a). Collectively, this data suggests that a UPEC strain may experience lower cytoplasmic copper stress, but higher periplasmic copper stress. However, expression of *cusC* is under highly complex regulation and is affected by other transcription factors.

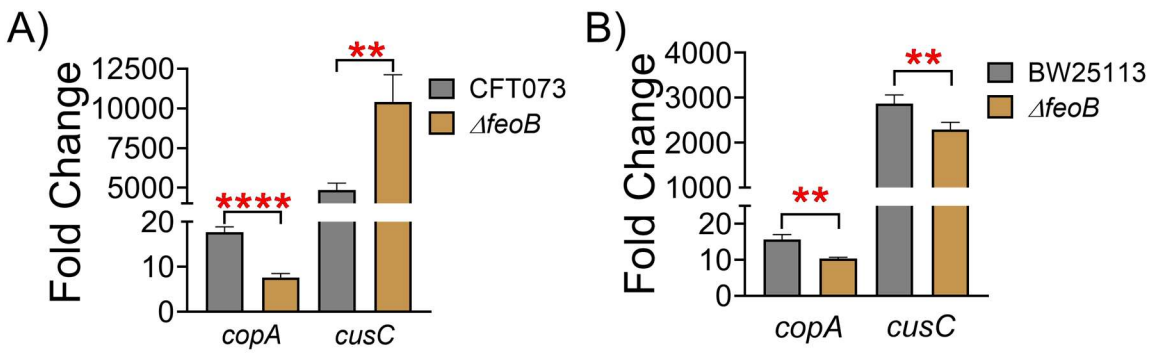

Figure 5. A *feoB*-deficient mutant has reduced expression of *copA* and *cusC* in BW25113 background, but reduced *copA* and increased *cusC* in UPEC CFT073 background under copper stress. Strains were exposed to 0 or 0.5 mM $CuSO_4$ during mid-logarithmic phase and then processed for RT-qPCR. Fold change in transcript abundance *copA* and *cusC* under copper stress in **A)** UPEC CFT073 and **B)** BW25113 was determined. Mean + SEM from 3 biological replicates, in triplicate, is presented here and were analyzed by t-test. **P<0.01, ***P<0.001.

4.4.11 A FeoB-deficient mutant accumulates less copper and iron during copper stress

We wanted to further investigate the mechanism by which the FeoB-deficient mutant is more resistant to copper stress than its parental wild-type strain. We exposed BW25113 and BW25113 *ΔfeoB* to 2 mM copper during the mid-logarithmic phase and analyzed samples for trace metal content with ICP-MS. As expected, the *ΔfeoB* mutant has less iron accumulation than the wild-type strain under both normal and copper stress conditions (Fig. 6c-d). Of interest, we report that BW25113 *ΔfeoB* accumulates less copper under copper stress conditions than the parental strain (Fig. 6b). This corresponds with our RT-qPCR data showing a FeoB-deficient

mutant to have decreased expression of known copper-detoxification genes (Fig. 5b). Together, this data suggests BW25113 *ΔfeoB* experiences less copper stress than wild-type, corroborating copper resistant growth phenotype and decreased expression of copper efflux gene *copA*. Although not much is currently known about non-ferrous cargo that could be imported via FeoB, our data suggests that FeoB might act as a conduit for periplasmic copper to enter the cytosol.

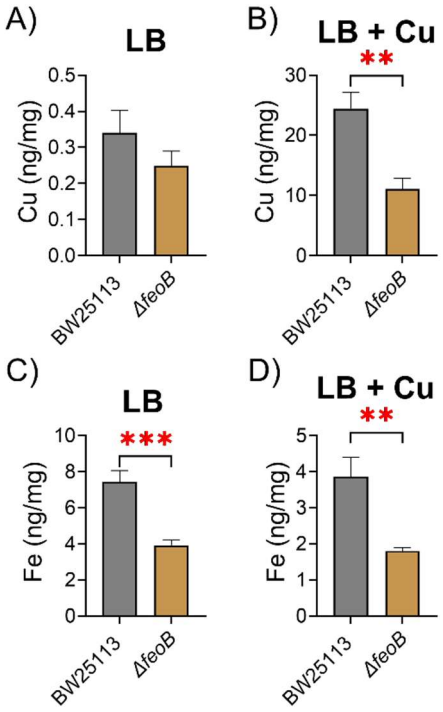

Figure 6. Cellular transition metal content shows decreased copper accumulation in a *feoB*-deficient mutant under copper stress. BW25113 and *ΔfeoB* mutant were exposed to 0 or 2mM $CuSO_4$ in LB. Bacterial cell pellets were digested and analyzed by inductively coupled plasma-mass spectrometry or optical emission spectrometry (ICP-MS or OES) to quantify cellular copper (**A** and **B**) and iron (**C** and **D**). Mean + SEM from 3 biological replicates, completed in triplicate, is presented here, and analyzed by t-test. **P<0.01, ***P<0.001.

4.5 Conclusions and Future Directions

This study establishes the transcriptional landscape of UPEC CFT073 under physiologic copper-stress. This data set has the potential for many follow-up studies on the numerous differentially-expressed genes reported. Additionally, repeating the RNAseq experiment in the BW25113 background to compare it to the current data set from the pathogenic strain would unravel commensal *versus* pathogen differences in expression of copper-responsive genes. Ultimately, such studies will lead to a better understanding of pathogenesis. Importantly, this study has identified a difference in the role of FeoB in copper homeostasis among *E. coli* strains. We report that a BW25113 *ΔfeoB* is more resistant to copper stress. This is seen both in the growth phenotype under copper stress and in the decreased expression of copper detoxification genes. Our ICP-MS data confirms this reduction in copper stress through the decreased cellular copper concentration in a FeoB-deficient mutant. Interestingly, we report that UPEC CFT073 does not share this strong copper resistant phenotype. It is possible that UPEC CFT073 pathogenesis includes an adapted FeoB that does not allow for cytoplasmic copper accumulation while still efficiently transporting Fe^{2+} iron. Our previous model proposes an increased need for uptake of Fe^{2+} iron during copper stress. Here our data shows increased *feoB* expression in UPEC CFT073 compared to BW25113. Adapting the *feoB* transporter to limit copper accumulation would be advantageous for UPEC to mitigating copper toxicity while still increasing iron transport. However, the DNA and amino acid sequence percent identity is highly conserved between BW25113 and UPEC CFT073, which limits the extent of niche adaptation. Yet, we cannot rule out specific mutations or moiety changes that could contribute to the higher

copper accumulation and stress in BW25113. We are currently working towards exploring FeoB-dependent changes in copper and iron accumulation in UPEC CFT073. A more plausible explanation for this divergence in phenotype is the redundancy of UPEC CFT073 Fe^{3+} and Fe^{2+} acquisition systems. Commensal strain BW25113 only has one ferric-siderophore system, enterobactin, while UPEC CFT073 has 2 additional functional siderophore systems, aerobactin and salmochelin. Additionally, UPEC CFT073 also utilizes more ferrous iron transporters (Table 1). The divergence in phenotypes may be due to UPEC CFT073 utilizing other systems and not relying as heavily on *feoB* for iron acquisition. This would limit the amount of *feoB*-mediated copper accumulation while still allowing iron uptake for cellular use, such as in the active sites of metalloenzymes. To our knowledge, no studies have been conducted to explore whether FeoB (BW25113 or UPEC CFT073) directly interacts with copper. Additionally, no studies have been published exploring if FeoB has any direct role in UTI pathogenesis *in vivo*. These follow-up studies are paramount to understanding the differences we report in BW25113 and UPEC CFT073 FeoB's involvement in copper homeostasis.

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5 CONCLUSIONS AND FUTURE DIRECTIONS

We sought to determine the difference between *E. coli*'s core and uropathogen-specific genome response to copper stress. We explored this through independent but complementary global analyses of a K-12 and UPEC strain. In both studies, we found members of iron homeostasis systems to play a crucial role in the mitigation of copper stress. Notably, mutants defective in ferric-enterobactin uptake are more sensitive to copper stress, while a mutant defective in ferrous iron transport is more resistant to copper stress.

Through our screen of the KEIO collection under copper toxicity, we were able to identify *E. coli* mutants that are copper-responsive at genome-wide scale. We found 60 and 27 mutants that are sensitive and resistant to copper, respectively. Although we chose to explore a specific class of mutants identified, our data set provides ample opportunity for future studies. It would be of interest to follow up on other mutants from the data set, including those of unknown function and those that are already known to play a role in metal or stress response. Additionally, this screen was highly discriminatory and only explored mutants with the most extreme phenotypes. Although it would be an extensive project, serial diluting the mutants and then comparing to wild-type growth would likely result in more hits. We are hopeful that our results will not only lead to future research in our lab, but also be utilized by the scientific community.

After our initial screens, we noticed a number of mutants deficient in iron acquisition, which led to an iron metabolism screen. We demonstrated all members of the Fe-Ent system, whether biosynthetic or uptake deficient, showed copper-specific sensitivity and increased gene

expression under copper stress. Surprisingly, some of these mutants showed *ΔcopA*-level sensitivity, highlighting the importance of iron homeostasis in overcoming copper stress. These studies have led to important questions such as if copper directly interacts with enterobactin or its transcriptional repressor, Fur.

Our data confirms the role of *tonB* and, moreover, Fe^{3+} acquisition in both commensal and UPEC copper homeostasis. It would be of interest to explore if recently identified *tonB* inhibitors could modulate copper sensitivity in UPEC and how this may affect pathogenesis in an *in vivo* UTI model. Our data suggests a model where dysregulation of iron homeostasis exacerbates copper stress. This supports the current paradigm shift towards mismetallation of key metalloenzymes as the primary mechanism for copper toxicity. Our research also supports current evidence for host innate immune response involving copper toxicity and iron starvation in the urinary tract. Collectively, our studies expand the current knowledge and illuminate the importance of highly interconnected metal homeostasis in bacterial survival and pathogenesis.

While this study has shown a role for enterobactin-mediated Fe^{3+} acquisition in the mitigation of *E. coli* copper stress, further research is needed to elaborate on the mechanism of action. Due to involvement of Fur regulation, it would be of interest to explore Fur's direct involvement in copper toxicity. Additionally, evaluating the other siderophores found in *E. coli,* such as aerobactin, for a role in evasion of copper stress is also of interest. Investigating the intersection of ferric iron uptake and copper toxicity among other common uropathogens, such as *Klebsiella spp.* and *Proteus spp.*, is an additional area of future research that has translational applications.

Here we also reported a global gene expression profile of UPEC CFT073 under physiologically-relevant copper stress. The potential and future projects of this data set is innumerable and is a novel contribution to the field. As expected, members of known copper-detoxification systems were detected in our RNAseq data, serving as internal validators. Numerous metal and stress related genes were reported and are of interest for future projects. Additionally, many genes with an unknown role in copper homeostasis were identified and would be of interest to explore. As we are interested in highlighting the difference between commensal and pathogenic *E. coli* response to copper stress, it would be interesting to repeat this RNAseq with commensal strain BW25113. Additionally, comparing our results from a UPEC strain of *E. coli* to another pathogenic *E. coli*, such as EHEC or EPEC, would provide insight on copper-responsive genes important to uropathogenesis.

We chose to investigate the role of the largest and most characterized member of the Feo system, FeoB. Surprisingly, we found a *ΔfeoB* mutant to be more resistant to copper stress compared to the wild-type BW25113, whereas a UPEC CFT073 *ΔfeoB* showed only slight resistance compared to its parent strain. We determined that BW25113 *ΔfeoB* has decreased expression of known copper detoxification genes, *copA* and *cusC* compared to wild-type. This suggests a commensal FeoB-deficient mutant experiences overall less copper stress. Interestingly, a CFT073 *ΔfeoB* has decreased expression of *copA,* but increased *cusC* expression. This could possibly be explained through the highly nuanced and complex nature of gene expression. For example, *cusC* gene expression has been shown to be cross-regulated by another transcriptional regulator, *hprR*. However, another explanation could be a genetic difference in

feoB between commensal and uropathogenic *E. coli* that would allow less cytoplasmic copper stress and more periplasmic copper stress. We report that a FeoB-deficient strain, when compared to the BW25113 strain, has less accumulation of cellular copper under copper stress conditions. Together, our data suggests FeoB leads to increased copper stress in commensal *E. coli*, while our UPEC strain has adapted away from this phenomenon. It would be a niche advantage if UPEC's adapted to limit FeoB-mediated copper accumulation while still efficiently importing Fe^{2+} in order to combat nutritional immunity and host innate immune effectors.

Future research should include further exploring the discrepancies between the role of FeoB in BW25113 and CFT073 copper homeostasis. We are currently working to identify trace metal concentrations in the CFT073 and CFT073 *ΔfeoB* strains. To our knowledge, no studies have been conducted in either background to determine if FeoB physically interacts with copper directly, which would also be a beneficial pursuit. Most studies assessing *feoB's* role in pathogenesis and virulence have been conducted in gut infection models, where mutants deficient in *feo*-mediated Fe^{2+} uptake have shown to be attenuated. However, assessing FeoB's role in pathogenesis in an *in vivo* UTI model would provide novel and exciting insights to this understudied system. Additionally, one limitation of an RNAseq is genetic expression does not always correlate with phenotype. This can be due to numerous factors including, but not limited to, post-translational modification and genetic redundancy. One way to overcome these limitations is through the integration of multi-omic approaches. Assessing and comparing the transcriptomic, proteomic, and metabolomic profiles of *E. coli,* commensal and UPEC, under copper stress would help to overcome some of these limitations and is of interest for follow-up

studies. These would provide the most robust analysis of *E. coli* cu-toxicity response and provide greater insights into which genetic elements contribute to pathogenicity.

Despite our exciting findings and the breadth of knowledge that will come from future studies, the screen of the KEIO collection also came with some limitations. One limitation of our study, as a reverse genetic screen, is regulators encoded outside of coding regions, such as small RNAs, will not be detected. A forward genetic screen would be necessary to identify copper-responsive genetic elements not included in the KEIO library. Additionally, this screen was at set conditions, including an aerobic and nutrient-rich media. To identify copper-responsive mutants in anaerobic or minimal media conditions additional screens would need to be conducted. Overall, our screen encompassed most of the *E. coli* genome under baseline conditions. While this provides a foundation for numerous future projects, it would be of interest to fill the gaps delineated here.

Together, these two projects highlight the role of two different mechanisms of iron acquisition: Fe^{3+} siderophore uptake and Fe^{2+} uptake via an inner membrane permease. Both these systems are subject to regulation by the Fur transcriptional repressor. This begs for further studies into the role of Fur in *E. coli* copper stress response. These could include looking for potential Fur and copper-containing protein interactions through bio-layer interferometry (BLI). Additionally, it would be of interest to study comparative "omic" profiles of fur-deficient, Fur-competent, and Fur-overexpressed pathogenic and commensal strains under copper stress. The knowledge gained from these studies would have a multitude of benefits, including foundational data sets, novel insights into pathogenesis, and translational applications.

Our work presented here highlights two main themes. Firstly, iron and copper homeostasis are intricately linked in both commensal and pathogenic *E. coli.* Secondly, the differences observed between commensal and pathogenic bacteria can lead to insights on niche-specific adaptations that promote pathogen fitness leading to increased pathogenicity. Through these studies, we were also able to contribute two large data sets focused on the global response of *E.coli* to copper stress, a known host immune effector. The data sets generated in the studies described here will be a resource for future studies for the scientific community. The insights derived from our research presented here and future studies have the potential to lead to novel targets for therapeutic approaches to combat UTI.

APPENDIX A

SUPPLEMENTARY INFORMATION FOR CHAPTER 3: A GENOME-WIDE SCREEN REVEALS THE INVOLVEMENT OF ENTEROBACTIN-MEDIATED IRON ACQUISITION IN *E. COLI* SURVIVAL DURING COPPER STRESS

Table 1. Bacterial strains and plasmids used in this study

Strain/Plasmid	Description[a]	Source
E. coli		
BW25113	Wild-type strain	KEIO library (1)
BW25113 *ΔtonB*	*ΔtonB::npt*	KEIO library (1)
BW25113 *ΔexbB*	*ΔexbB::npt*	KEIO library (1)
BW25113 *ΔexbD*	*ΔexbD::npt*	KEIO library (1)
BW25113 *ΔfepB*	*ΔfepB::npt*	KEIO library (1)
BW25113 *ΔfepC*	*ΔfepC::npt*	KEIO library (1)
BW25113 *ΔfepD*	*ΔfepD::npt*	KEIO library (1)
BW25113 *ΔfepG*	*ΔfepG::npt*	KEIO library (1)
BW25113 *ΔentB*	*ΔentB::npt*	KEIO library (1)
BW25113 *ΔentE*	*ΔentE::npt*	KEIO library (1)
BW25113 *ΔentF*	*ΔentF::npt*	KEIO library (1)
BW25113 *ΔcopA*	*ΔcopA::npt*	KEIO library (1)
BW25113 *Δfur*	*Δfur::npt*	KEIO library (1)
BW25113/pGEN	Empty vector control	This study

BW25113 *ΔtonB*/pGEN	Empty vector control	This study
BW25113 *ΔtonB*/pGEN_*tonB*	*tonB* complementation	This study
Uropathogenic *E. coli*		
CFT073	Wild-type strain	(2)
CFT073 *ΔtonB*	*ΔtonB::npt*	(3)
CFT073 *ΔcopA*	*ΔcopA::npt*	(4)
CFT073/pGEN	Empty vector control	This study
CFT073 *ΔtonB*/pGEN	Empty vector control	This study
CFT073 *ΔtonB*/pGEN_*tonB*	*tonB* complementation	This study
CFT073 TN32-A2	*c4289::Tn5,* copper resistant	Subash Lab[b]
Plasmids		
pGEN	Low-copy number vector	(5)
pGEN_*tonB*	*tonB* complementation	This study

[a]*npt*, neomycin phosphortransferase; and *cat*, chloramphenicol acetyl transferase

[b]manuscript in review

Table 2. Oligonucleotide primers used in this study

Primer ID[a]	Sequence
Purpose: Verification of KEIO library mutants	
p469 *tonB* F	5'GCGTTTTTCGAGGCTATCAG3'
p470 *tonB* R	5'AAGTATGTCGCGGTTGATCC3'
p475 *fepA* F-Out	5'TTAACGCCGTCACACCATAA3'
p476 *fepA* R-In	5'GCGATACAGACGGTTGGTTT3'
p477 *fepB* F	5'AGGGTTAATGTTCGCACCAG3'
p478 *fepB* R	5'GCGGTTTTGGTTTGTTGATT3'
p485 *fepG* F	5'GATTGCCTTTATTGGCCTGA3'
p486 *fepG* R	5'CAGATTTTCCGCAACGGTAT3'
p487 *fes* F	5'TAATTAATGTCCGCGCTTCC3'
p488 *fes* R	5'TCGACCAGCAAATGGTGATA3'
p489 *entA* F	5'GAGCATTTGATGTCGCTGAA3'
p490 *entA* R	5'CCATTGTGTTATCGCTGGTG3'
p497 *entF* F-Out	5'AATTTTGGGCGCAAAGTATG3'
p498 *entF* R-In	5'CGAATCAACCTCTCCGGTTA3'
p499 *fepA* F-In	5'AACGACCTTCACCTGGTACG3'
p500 *fepA* R-Out	5'TCCGGCTAAATGCTCTGTTT3'
p501 *entF* F-In	5'TGAAGGCAACTACGCTGATG3'
p502 *entF* R-Out	5'TTCCAGCAAGCTAACCGACT3'
p503 *copA* F-Out	5'CATTTTGTCCGCCGTTAAGT3'

p504 *copA* R-In	5’TATCAGGCCGATAACCAACC3’
p505 *copA* F-In	5’TGCAAAGTGAAGGACGTCAG3’
p506 *copA* R-Out	5’ACAAGAAAACCGACGACACC3’
Purpose: RT-qPCR	
p554 *tonB* F	5’GTGGCGGGTCTGCTCTATAC3’
p555 *tonB* R	5’TCGAGATCAGCAGGCGTAAC3’
p556 *fepA* F	5’AATCCGCAAAAACCCGGTTG3’
p557 *fepA* R	5’GGTTATTCCCACGCTGACCA3’
p558 *fepB* F	5’CTACCGCAACGCCCTTCTAT3’
p559 *fepB* R	5’GCCACGGCTGTCAGTAATCT3’
p560 *fepG* F	5’TGGTCACCGAATGGCGTTTA3’
p561 *fepG* R	5’TGAAAAATCGCGCCACTGAC3’
p562 *fes* F	5’CCAGTCGATGCAGCGAATTG3’
p563 *fes* R	5’TCGCGTTCGGTGGGAATAAA3’
p564 *entA* F	5’TTTGCGACCGAAGTGATGGA3’
p565 *entA* R	5’CGTAAAATTCCCGCCGCATT3’
p566 *entF* F	5’CAGAATTACCCTCCGCCTGG3’
p567 *entF* R	5’TATCTGCTTGCGCTAGTCCG3’
p568 *copA* F	5’TCACTCAGGCACGGGTAAAC3’
p569 *copA* R	5’TTCCACCGCCTGCACTAAAT3’
p570 *gapA* F	5’AACGTGATCCGGCTAACCTG3’
p571 *gapA* R	5’GCGGTGATGTGTTTACGAGC3’

Purpose: Verification of Downstream Gene Expression	
p581 *entD* F	5’CGGATCGCTGCTGTTTATGC3’
p582 *entD* R	5’TATCTGCCAGTGCACAGCAA3’
p583 *fepC* F	5’GCGAACAGTTAACCCTGGGA3’
p584 *fepC* R	5’GTTGTCCGCCAGAAAGGGTA3’
p585 *ybdz* F	5’TGGCATTCAGTAATCCCTTCGA3’
p586 *ybdz* R	5’TGGGTAAAATTCGTCGGTGTCA3’
p587 *entF* F	5’AATTTTGGGCGCAAAGTATG3’
p588 *entF* R	5’CGAATCAACCTCTCCGGTTA3’
p589 *entH* F	5’AACGCCATTTAACGCTCGAC3’
p590 *entH* R	5’GCCCCTGTTCATCGAAAACG3’
p591 *fepE* F	5’CCCGATTATCCTCTGGCGTC3’
p592 *fepE* R	5’CTGGCATTGTCATCAACCGC3’
Purpose: Verification of Genetic Complementation	
p420 pGen F	5’GGCACTTGCTCACGCTCTG3’
p421 pGen R	5’GTGGTCACGCTTTTCGTTGG3’

Table 3. Copper-sensitive mutants identified in the secondary screen

Gene	Function	Location
ompC	Outer membrane porin C	OM
fepB	Ferric enterobactin ABC transporter periplasmic binding protein	PP
dcrB	Periplasmic bacteriophage sensitivity protein DcrB	PP
cueO	Multicopper oxidase CueO	PP
tonB	Ton complex subunit TonB	PP/IM
sdhD	Succinate:quinone oxidoreductase, membrane protein SdhD	IM
sdhA	Succinate:quinone oxidoreductase, FAD binding protein	IM
nuoF	NADH:quinone oxidoreductase subunit F	IM
sdhB	Succinate:quinone oxidoreductase, Fe-S cluster binding protein	IM
atpA	ATP synthase F1 complex subunit α	IM
atpC	ATP synthase F1 complex subunit ε	IM
atpH	ATP synthase F1 complex subunit δ	IM
copA	$copper^{+}$ exporting P-type ATPase	IM
atpD	ATP synthase F1 complex subunit β	IM
atpB	ATP synthase F0 complex subunit a	IM
fepD	Ferric enterobactin ABC transporter membrane subunit FepD	IM
exbD	Ton complex subunit ExbD	IM
exbB	Ton complex subunit ExbB	IM
tolR	Tol-Pal system protein TolR	IM
fepG	Ferric enterobactin ABC transporter membrane subunit FepG	IM

fepD	Ferric enterobactin ABC transporter membrane subunit FepD	IM
exbB	Ton complex subunit ExbB	IM
tolR	Tol-Pal system protein TolR	IM
yebA	Peptidoglycan DD-endopeptidase MepM	IM
gltA	Citrate synthase	cytosol
ubiF	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase	cytosol
gor	Glutathione reductase	cytosol
ubiX	Flavin prenyltransferase	cytosol
yfjG	Ribosome association toxin RatA	cytosol
astE	Succinylglutamate desuccinylase	cytosol
secB	SecB chaperone	cytosol
purA	Adenylosuccinate synthetase	cytosol
cpxR	DNA-binding transcriptional dual regulator CpxR	cytosol
ompR	Transcriptional dual regulator	cytosol
pdxH	Pyridoxine 5'-phosphate oxidase / pyridoxamine 5'-phosphate oxidase	cytosol
cyaA	Adenylate cyclase	cytosol
iscS	Cysteine desulfurase	cytosol
rpsF	30S ribosomal subunit protein S6	cytosol
ubiH	2-octaprenyl-6-methoxyphenol 4-hydroxylase	cytosol
rbfA	30S ribosome binding factor	cytosol
rpmJ	50S ribosomal subunit protein L36	cytosol

guaA	GMP synthetase	cytosol
lipB	lipoyl(octanoyl) transferase	cytosol
pnp	Polynucleotide phosphorylase	cytosol
rsgA	Ribosome small subunit-dependent GTPase A	cytosol
rimM	Ribosome maturation factor RimM	cytosol

[a]IM, inner membrane; PP, periplasm, and OM, outer membrane

Table 4. Copper-resistant mutants identified in the secondary screen

Gene	Function	Location[a]
csgF	Curli assembly component	OM
tsx	Nucleoside-specific channel-forming protein Tsx	OM
lpp	Murein lipoprotein	OM/PP
zntA	$Zn^{2+}/Cd^{2+}/Pb^{2+}$ exporting P-type ATPase	IM
ybgH	Dipeptide:H+ symporter DtpD	IM
ynaJ	DUF2534 domain-containing protein Ynaj	IM
nuoE	NADH:quinone oxidoreductase subunit E	IM
ydjN	Cystine/sulfocysteine:cation symporter	IM
yfdG	Putative bactoprenol-linked glucose translocase	IM
kdpF	K+ transporting P-type atpase subunit KdpF	IM
yqjF	DoxX family protein	IM
ybaK	Cys-tRNApro and Cys-tRNAcys deacylase	cytosol
rdgC	Putative component of the Rsx system	cytosol
sucC	Succinyl-coA synthetase subunit β	cytosol
pepT	Peptidase T	cytosol
rimJ	Ribosomal-protein-S5-alanine N-acetyltransferase	cytosol
cusR	Transcriptional activator CusR	cytosol
sucC	Succinyl-coA synthetase subunit β	cytosol
lon	Lon protease	cytosol
yehS	DUF1456 domain-containing protein YehS	cytosol

yciU	DUF440 domain-containing protein YciU	cytosol
ybiN	23S rRNA m6a1618 methyltransferase	cytosol
rpiA	Ribose-5-phosphate isomerase A	cytosol
yddK	Leucine-rich repeat domain-containing protein YddK	Secreted
ybfO	RHS repeats-containing protein YbfO	Unknown

[a]IM, inner membrane; PP, periplasm, and OM, outer membrane

Table 5. Mutants lacking genes involved in iron uptake/metabolism that were screened for copper sensitivity

Gene	Function	Location
fepA	Ferric enterobactin outer membrane transporter	OM
fecA	Ferric enterobactin outer membrane transporter	OM
cirA	Ferric dihyroxybenzoylserine outer membrane transporter	OM
fhuE	Ferric coprogen/ferric rhodotorulic acid outer membrane transporter	OM
fhuA	Ferrichrome outer membrane transporter/phage receptor	OM
fiu	Iron catecholate outer membrane transporter	OM
yncD	Putative TonB-dependent outer membrane receptor	OM
fhuD	Iron(III) hydroxamate ABC transporter periplasmic binding protein	PP
fepB	Ferric enterobactin ABC transporter periplasmic binding protein	PP
fecB	Ferric citrate ABC transporter periplasmic binding protein	PP
yncE	PQQ-like domain-containing protein YncE	PP
tonB	Ton complex subunit TonB	PP/IM
fhuC	Iron(III) hydroxamate ABC transporter ATP binding subunit	IM
exbB	Ton complex subunit ExbB	IM
fhuB	Iron(III) hydroxamate ABC transporter membrane subunit	IM
fepD	Ferric enterobactin ABC transporter membrane subunit FebD	IM
fepG	Ferric enterobactin ABC transporter membrane subunit FepG	IM
fepE	Polysaccharide co-polymerase family protein FepE	IM
fecD	Ferric citrate ABC transporter membrane subunit FecD	IM

fecE	Ferric citrate ABC transporter ATP binding subunit	IM
exbD	Ton complex subunit ExbD	IM
fecC	Ferric citrate ABC transporter membrane subunit FecC	IM
entE	2,3-dihydroxybenzoate-[aryl-carrier protein] ligase	IM/cytosol
entB	Enterobactin synthase component B	IM/cytosol
fhuF	Hydroxamate siderophore iron reductase	IM/cytosol
fepC	Ferric enterobactin ABC transporter membrane subunit FebD	IM/cytosol
entF	Apo-serine activating enzyme	IM/cytosol
acnA	Aconitate hydratase A	cytosol
entA	Enterobactin synthase component A	cytosol
feoA	Ferrous iron transport protein A	cytosol
ftnA	Ferritin iron-storage complex	cytosol
fes	Ferric enterobactin esterase	cytosol
fur	DNA-binding transcriptional dual regulator Fur	cytosol
entC	Isochorismate synthase EntC	cytosol
ydiE	PF10636 family protein YdiE	unknown

[a]IM, inner membrane; PP, periplasm, and OM, outer membrane

Table 6. Expression of downstream genes in KEIO mutants

KEIO Mutant	Downstream Gene	Expression (+/-)[a]
ΔfepG	*fepC*	+
Δfes	*ybdZ*	+
Δfes	*entF*	+
ΔentA	*entH*	+
ΔentF	*fepE*	+

[a]Based on RT-qPCR

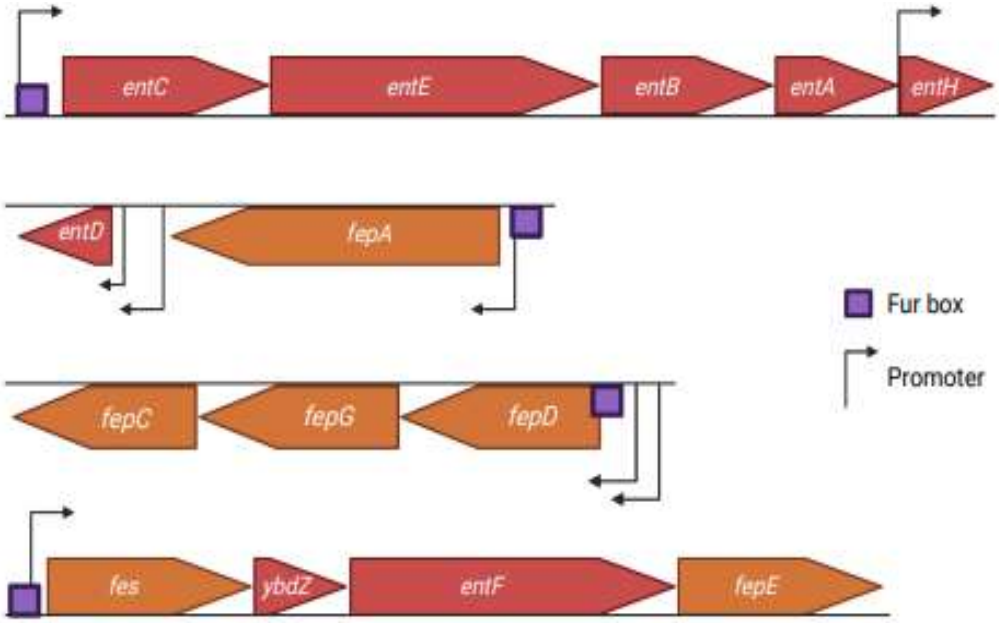

Figure 1. Operons involved in enterobactin production and uptake in *E. coli*. Schematic of gene organization as operons including the location of promoters and Fur binding sites was generated in Biorender, based on publicly available information from EcoCyc.org

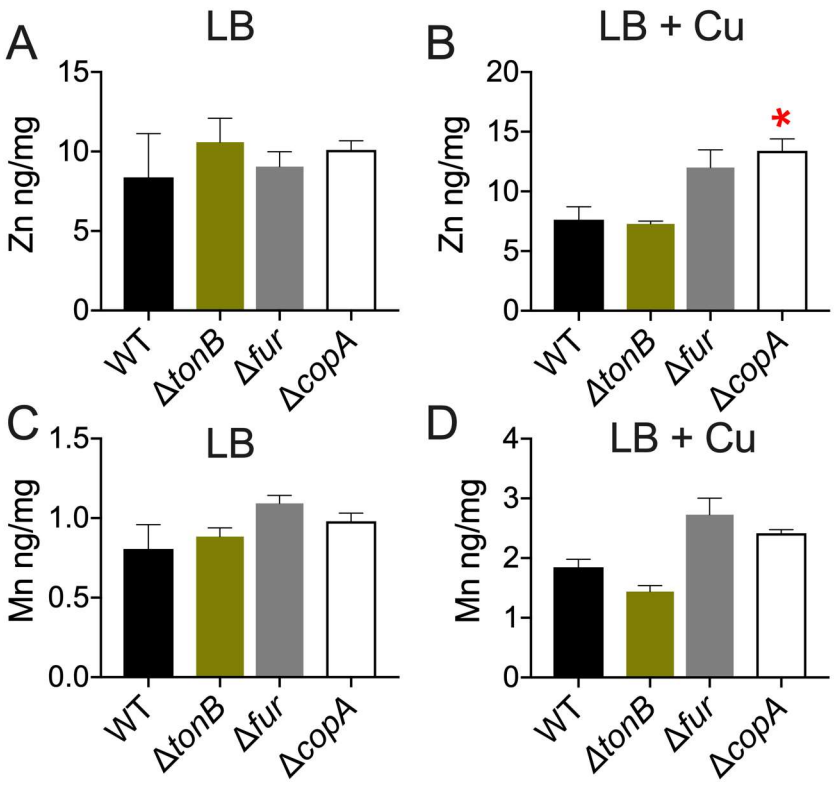

Figure 2. Concentration of select transition metals in *E. coli*. Wild-type (BW25113), *ΔtonB, Δfur,* and *ΔcopA* mutant strains were cultured in LB or LB supplemented with 3 mM $CuSO_4$. Cell pellets were digested and analyzed by ICP-MS or -OES to quantify Zn (**A** and **B**), and Mn (**C** and **D**). Mean+SEM from three biological replicates is presented here.

APPENDIX B

SUPPLEMENTARY INFORMATION FOR CHAPTER 4: COMPARATIVE TRANSCRIPTIONAL PROFILING OF UROPATHOGENIC *E. COLI* UNDER PHYSIOLOGICALLY-RELEVANT COPPER STRESS REVEALS A ROLE FOR FEOB IN COPPER HOMEOSTASIS

Table 1. Nucleotide and Protein Similarity to CFT073

BW25113 Protein	**DNA Sequence Percent Identity**	**Amino Acid Percent Identity**
FeoA	99.56%	100%
FeoB	97.80%	99.74%
FeoC	93.67%	97.44%
CopA	96.33%	98.92%
CusC	96.51%	98.91%

Table 2. Bacterial strains and plasmids used in this study

Strain/Plasmid	Description[a]	Source
E. coli		
BW25113	Wild-type strain	KEIO library (1)
BW25113 *ΔfeoB*	*ΔfeoB::npt*	KEIO library (1)
BW25113 *ΔfeoA*	*ΔfeoA::npt*	KEIO library (1)
BW25113 *ΔfeoC*	*ΔfeoC::npt*	KEIO library (1)
BW25113 *ΔcopA*	*ΔcopA::npt*	KEIO library (1)
BW25113 pCA24N	Empty vector control	This study
BW25113 *ΔfeoB*/pCA24N	Empty vector control	This study
BW25113 *ΔfeoB*/p*feoB*	*feoB* complementation	This study
Uropathogenic *E. coli*		
CFT073	Wild-type strain	(2)
CFT073 *ΔcueR*	*ΔcueR::npt*	This study
CFT073 *ΔcusRSCFBA*	*ΔcusRSCFBA::npt*	This study
CFT073 *ΔfeoB*	*ΔfeoB::cat*	This study
CFT073 *ΔcopA*	*ΔcopA::npt*	(4)
Plasmids		
pCA24N	middle-copy number vector	(5)
p*feoB*	*feoB* complementation*::cat*	This study

[a]*npt*, neomycin phosphortransferase; and *cat*, chloramphenicol acetyl transferase

Table 3. Oligonucleotide primers used in this study

Primer ID[a]	Sequence
Purpose: Verification of KEIO library mutants	
p725 *feoB* diagnostic F	5’ TCTGGTCTCATGTCGCTGTC 3’
p726 *feoB* diagnostic R	5’ CAGCATGGCGTTAATCATTG 3’
Purpose: Construction and Verification of CFT073 mutants	
p723 *feoB* KO F	5’ATCTGGCCTTATTAGAAGTGGAAGCGGTTTCCTGTTAATACGGTGATAACAACAGTGTAGGCTGGAGCTGCTTC3’
p724 *feoB* KO R	5’CTTCCATACGGCCCCGTAACGCCAGCAAATCACGCACCTGAATAAGTGAAGCCAATGGGAATTAGCCATGGTCC3’
p360 pkd3 Cm Cassette F	5’GTGTAGGCTGGAGCTGCTTC3’
P361 pkd3 Cm Cassette R	5’ ATGGGAATTAGCCATGGTCC3’
p725 *feoB* diagnostic F	5’TCTGGTCTCATGTCGCTGTC3’
p726 *feoB* diagnostic R	5’CAGCATGGCGTTAATCATTG3’
Purpose: RT-qPCR	
p759 BW25113 *feoB* F	5’ GCTGACTATCTTCCTCGCCC 3’
p760 BW25113 *feoB* R	5’ CGCTCAGGAAAATGCTGACG 3’
p568 BW25113 *copA* F	5’TCACTCAGGCACGGGTAAAC3’
p569 BW25113 *copA* R	5’TTCCACCGCCTGCACTAAAT3’
p585 BW25113 *cusC* F	5’TGGCATTCAGTAATCCCTTCGA3’

p586 BW25113 *cusC* R	5’TGGGTAAAATTCGTCGGTGTCA3’
p733 CFT073 *feoB* F	5’GCCGCATACAGCTACCTGAT3’
p734 CFT073 *feoB* R	5’TTGCCAGCAGTTCGACATCT3’
p250 CFT073 *copA* F	5’GCTACTGCTTTCCCGTCAAC3’
p251 CFT073 *copA* R	5’TGACCAACAGGTCGATACCA3’
p535 CFT073 cusC F	5’GCGCAATTGCAAATAGCCGAA3’
p536 CFT073 cusC R	5’CTGTTCCAGCGCCAGAACATT3’

Table 4. Genes with differential regulation in UPEC CFT073 under Cu stress compared to control conditions

Gene	Function	Log_2 Fold Change
cusF	Copper/silver export system periplasmic binding protein	8.957230563
cusB	Copper/silver export system membrane fusion protein	6.767822925
cusC	Copper/silver export system outer membrane channel	6.369358034
cusA	Copper/silver export system RND permease	4.622627734
C_RS04530	Fels-2 prophage: probable prophage lysozyme	1.639698488
C_RS04515	Putative capsid completion protein	1.624452725
copA	Copper-exporting P-type ATPase CopA	1.613731974
fhuA	Ferrichrome outer membrane transporter/phage receptor	1.599677767
C_RS04500	Putative capsid scaffolding protein	1.559528337
C_RS04555	Putative Phage baseplate assembly protein V	1.544539728
C_RS04510	Terminase, endonuclease subunit	1.529435435
ccp	Cytochrome c peroxidase	1.423953329
mntS	Manganase accumulation protein MntS	1.41624791
C_RS04595	Putative tail fiber component of prophage	1.395376482
yojI	Hypothetical ABC transporter ATP-binding protein yojI	1.392013784
cyuA	Putative L-cysteine desulfidase CyuA	1.325647442
C_RS04505	Phage major capsid protein, P2 family	1.307816064
plaP	Putrescine:H+ symporter PlaP	1.2899779

C_RS04535	Putative membrane protein	1.28557852
yoeI	Membrane protein YoeI	1.279364578
cusR	DNA-binding transcriptional activator CusR	1.184137139
C_RS04590	Probable major tail sheath protein	1.167713064
sitA	Iron/manganese ABC transporter substrate-binding protein SitA	1.133034273
feoA	Ferrous iron transport protein A	1.079028087
suhB	Inositol-phosphate phosphatase	1.064682401
cueO	Multicopper oxidase CueO	1.038083869
C_RS04565	Phage baseplate assembly protein	1.004295266
C_RS04495	Terminase, ATPase subunit	1.001764661
agn43.1	Autotransporter adhesion Ag43	0.992515187
C_RS04575	Probable variable tail fibre protein	0.969028827
ahpC	Alkyl hydroperoxide reductase, AhpC component	0.944119778
rimO	Ribosomal protein S12 methylthiotransferase RimO	0.940466839
exbB	Biopolymer transport exbB protein	0.939004138
mgtA	Mg(2+) transport ATPase, P-type 1	0.925716468
ydiY	Hypothetical protein ydiY precursor	0.822102309
rapA	RNA polymerase-binding ATPase and RNAP recycling factor	0.810746239
feoB	Fe2+ transporter FeoB	0.804181418

rluA	23S rRNA pseudouridine746 and tRNA pseudouridine32 synthase	0.764810543
apt	Adenine phosphoribosyltransferase	0.762173325
priB	Primosomal replication protein N	0.679578373
C_RS16955	Hemolysin B	0.671703702
rluB	23S rRNA pseudouridine2605 synthase	0.652812476
rnb	RNase II	0.644453008
rpsR	30S ribosomal protein S18	0.620205596
cusS	Sensor histidine kinase CusS	0.619412804
rpsN	30S ribosomal protein S14	0.568962906
rpsF	30S ribosomal protein S6	0.570355565
ansA	L-asparaginase 1	0.563723777
ttcA	tRNA cytosine32 2-sulfurtransferase TtcA	0.56211702
flgD	Basal-body rod modification protein flgD	-0.607304269
flgC	Flagellar basal-body rod protein FlgC	-0.618027382
fliD	Flagellar filament capping protein	-0.653918515
fliK	Flagellar hook-length control protein	-0.662652103
C_RS11080	Flagellin	-0.690510916
flgK	Flagellar hook-filament junction protein 1	-0.740109352
flgI	Flagellar P-ring protein	-0.770791515
hdeD	Acid-resistance membrane protein	-0.797831924
flgE	Flagellar hook protein FlgE	-0.801382541

flgH	Flagellar L-ring protein	-0.809483086
fliS	Flagellar biosynthesis protein FliS	-0.81081275
motB	Motility protein B	-0.824931345
hdeA	Periplasmic acid stress chaperone HdeA	-0.847573296
flgJ	Putative peptidoglycan hydrolase FlgJ	-0.852355945
flgG	Flagellar basal-body rod protein FlgG	-0.86884634
flgL	Flagellar hook-filament junction protein 2	-0.874489878
ghoT	Toxin of the GhoTS toxin-antitoxin system	-0.888787982
flgF	Flagellar basal-body rod protein FlgF	-0.918645776
pdeH	c-di-GMP phosphodiesterase PdeH	-0.933532564
nrfE	Putative cytochrome c-type biogenesis protein NrfE	-0.936863115
tar	Methyl-accepting chemotaxis protein Tar	-0.940953548
nrfF	Putative formate-dependent nitrite reductase complex subunit NrfF	-0.981237832
nrfD	Cytochrome c nitrite reductase subunit NrfD	-0.98134771
cheR	Chemotaxis protein methyltransferase	-1.029066159
hdeB	Periplasmic acid stress chaperone HdeB	-1.036047046
fliT	Flagella biosynthesis regulatory protein FliT	-1.066125482
zntA	Zn2+/Cd2+/Pb2+ exporting P-type ATPase	-1.128403422
rdcB	Regulator of diguanylate cyclase RdcB	-1.137893401
hcp.1	Protein S-nitrosylase	-1.216255778
hcr	NADH oxidoreductase	-1.275093292

Table 5. Genes with differential regulation in CFT073 *ΔcueR* under Cu stress compared to control conditions

Gene	Function	Log_2 Fold Change
cusF	Cu(+)/Ag(+) efflux RND transporter periplasmic metallochaperone CusF	10.51797039
cusB	Cu(+)/Ag(+) efflux RND transporter periplasmic adaptor subunit CusB	8.78366627
cusC	Cu(+)/Ag(+) efflux RND transporter outer membrane channel CusC	7.618701337
cusA	Cu(+)/Ag(+) efflux RND transporter permease subunit CusA	6.223296775
zraP	Zinc resistance sensor/chaperone ZraP	5.255672695
cusR	Copper response regulator transcription factor	1.914674287

Table 6. Genes with differential regulation in CFT073 *Δcus* under Cu stress compared to control conditions

Gene	Function	Log2 Fold Change
zraP	Zinc resistance sensor/chaperone ZraP	7.945200071
cpxP	Cell-envelope stress modulator CpxP	3.260055856
copA	Copper-exporting P-type ATPase CopA	2.735141409
cdgI	GGDEF domain-containing protein	2.298238677
yebE	Tellurite resistance TerB family protein	1.625815429
cueO	Multicopper oxidase CueO	1.586071736
degP	Serine endoprotease DegP	1.512075313
spy	ATP-independent periplasmic protein-refolding chaperone Spy	1.408237451
yccA	FtsH protease modulator YccA	1.356926225
yliI	Aldose sugar dehydrogenase YliI	1.253057544
htpX	Protease HtpX	1.234383361
chaA	Sodium-potassium/proton antiporter ChaA	1.187757987
yqjA	DedA family protein	1.070210804
yqaE	YqaE/Pmp3 family membrane protein	1.032777431
tomB	Hha toxicity modulator TomB	1.031681084
ldtC	L,D-transpeptidase LdtC	0.985704614
mzrA	EnvZ/OmpR regulon moderator MzrA	0.95576531

srkA	Stress response kinase SrkA	0.937846373
lpxP	kdo(2)-lipid IV(A) palmitoleoyltransferase	0.894188721
ppiA	Peptidylprolyl isomerase A	0.889096686
sbmA	Peptide antibiotic transporter SbmA	0.852794006
ygiB	DUF1190 family protein	0.847726283
ygiC	Glutathionylspermidine synthase family protein	0.844076211
dgcZ	Diguanylate cyclase DgcZ	0.826973147
ftnB	Non-heme ferritin-like protein	0.809209659
cpxA	Sensor histidine kinase CpxA	0.796783766
exbB	Biopolymer transport exbB protein	0.794873428
ahpC	Alkyl hydroperoxide reductase, AhpC component	0.777560166
ybbA	Hypothetical ABC transporter ATP-binding protein ybbA	0.766729421
tnpA.10	IS200/IS605-like element IS200C family transposase	0.760550356
cpxR	DNA-binding transcriptional dual regulator CpxR	0.757176339
yoaE	CNNM family cation transport protein YoaE	0.74678649
ybbP	ABC transporter permease	0.739438502
dsbA	Thiol:disulfide oxidoreductase DsbA	0.737725968
sltY	Soluble lytic murein transglycosylase	0.708132363
dacC	D-alanyl-D-alanine carboxypeptidase DacC	0.68788028
tqsA	Autoinducer 2 exporter	0.648513918
feoA	Ferrous iron transport protein A	0.638695411
tnpA.7	IS200/IS605-like element IS200C family transposase	0.600836341

exbD	Biopolymer transport exbD protein	0.589556224
yecD	Hypothetical isochorismatase family protein yecD	0.575989276
amiA	N-acetylmuramoyl-L-alanine amidase A	0.574632477
opgD	Glucan biosynthesis protein D	0.562084135
hemF	Coproporphyrinogen III oxidase	0.490371274
feoB	Fe2+ transporter FeoB	0.480910888
yfcE	Phosphodiesterase	-0.519613378
flhD	Flagellar transcriptional regulator FlhD	-0.522120998
nrfD	Cytochrome c nitrite reductase subunit NrfD	-0.563833849
ydhV	Aldehyde ferredoxin oxidoreductase	-0.581087148
dtpB	Dipeptide/tripeptide permease DtpB	-0.591859045
ydhW	YdhW family putative oxidoreductase system protein	-0.633912999
ydhT	Cytochrome c nitrite reductase pentaheme subunit	-0.638307325
nrfB	c552 subunit	-0.652213035
nrfA	Ammonia-forming nitrite reductase cytochrome	-0.656597864
nhaB	Na(+)/H(+) antiporter NhaB	-0.680788091
emrA	Multidrug efflux MFS transporter periplasmic adaptor subunit EmrA	-0.684786189
yfcD	NUDIX hydrolase YfcD	-0.696867677
ydhX	4Fe-4S dicluster domain-containing protein	-0.742706664
cdiA	Contact-dependent inhibition effector tRNA nuclease	-0.75523836
yiaY	L-threonine dehydrogenase	-0.784390595

ydhU	Thiosulfate reductase cytochrome B subunit	-0.829000058
ybaK	Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase YbaK	-0.950154087
ykgJ	YkgJ family cysteine cluster protein	-1.05204964
C_RS05655	Hemagglutinin repeat-containing protein	-1.140964506
ompF	Porin OmpF	-1.866431214
C_RS01575	ShlB/FhaC/HecB family hemolysin secretion/activation protein	-2.01295143
yagU	YagU family protein	-2.252610782

Table 7. Ferrous iron transporter-deficient mutants phenotype during Cu stress response

BW25113 mutant	Phenotype
ΔfeoA	NC
ΔfeoB	Resistant
ΔfeoC	NC
ΔmntH	NC
ΔzupT	NC
ΔefeU	NC
ΔefeO	NC
ΔefeB	NC

NC: No Change from wild type