Searching for Cu Importers in *Salmonella* Typhimurium

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ABSTRACT

This study investigates the sensitivity of *Salmonella* mutants lacking a putative porin OMP gene to copper stress, aiming to understand if reduced porin expression affects copper influx and growth rates compared to wild-type strains exposed to copper sulfate ($CuSO_4$). Despite *Salmonella* Typhimurium's significant global health impact, with millions of cases annually, and its ability to survive in diverse environments, including host cells containing high copper levels, the specific role of porins in copper sensitivity remains unclear. Experimental methods involve screening *Salmonella* mutants lacking the porin OMP gene obtained from a mutant library and comparing their growth patterns to wild-type strains under varying copper concentrations using liquid cultures and solid media. Mutants lacking the porin OMP gene demonstrate similar growth patterns to wild-type strains when exposed to $CuSO_4$ in liquid cultures but in solid media revealed mutant STM14_0394 to have higher copper resistance than the wild-type. These findings emphasize the need for further research to understand copper resistance mechanisms in *Salmonella* and inform the development of targeted therapeutic interventions and preventive measures against *Salmonella* infections.

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TABLE OF CONTENTS

ABSTRACT....2
ACKNOWLEDGEMENTS....3
TABLE OF CONTENTS....4
LIST OF FIGURES....5
LIST OF TABLES....5
INTRODUCTION....6
METHODS....10
RESULTS....15
DISCUSSION....18
REFERENCES....21
APPENDIX....23

LIST OF FIGURES

Figure 1. Example of protein in copper homeostasis....8
Figure 2. Growth curves of WT and mutant strains of *Salmonella* mutants STM14_0043, STM14_0235, and STM14_0394 at varying concentrations of $CuSO_4$....15
Figure 3: Copper Sensitivity Assay of WT and mutant strains of Salmonella....16
Figure 2.1. Growth curves of WT and mutant strains of *Salmonella* mutants STM14_1777, STM14_1848, and STM14_3401 at varying concentrations of $CuSO_4$....24
Figure 2.2 Growth curves of WT and mutant strains of Salmonella mutants STM14_0638, STM14_1294, and STM14_2653 at varying concentrations of $CuSO_4$....24
Figure 2.3 Growth curves of WT and mutant strains of Salmonella mutants STM14_3348, STM14_4874, and STM14_5514 at varying concentrations of $CuSO_4$....25
Figure 2.4 Growth curves of WT and mutant strains of Salmonella mutants STM14_1777, STM14_1848, and STM14_3401 at varying concentrations of $CuSO_4$....25
Figure 2.5 Growth curves of WT and mutant strains of Salmonella mutants STM14_5099, STM14_4829, and STM14_4527 at varying concentrations of $CuSO_4$....26
Figure 2.6 Growth curves of WT and mutant strains of Salmonella mutants STM14_5119, STM14_2095, and STM14_0489 at varying concentrations of $CuSO_4$....26
Figure 2.7 Growth curves of WT and mutant strains of Salmonella mutants STM14_2797, STM14_2942, and STM14_5542 at varying concentrations of $CuSO_4$....27
Figure 2.8 Growth curves of WT and mutant strains of Salmonella mutants STM14_0207, STM14_4385, and STM14_0426 at varying concentrations of $CuSO_4$....27
Figure 2.9 Growth curves of WT and mutant strains of Salmonella mutants STM14_1183, STM14_2978, and STM14_1378 at varying concentrations of CuSO4....28
Figure 2.10 Growth curves of WT and mutant strains of Salmonella mutants STM14_0228, STM14_4965, and STM14_0802 at varying concentrations of $CuSO_4$....28
Figure 2.11 Growth curves of WT and mutant strains of Salmonella mutants STM14_5534, STM14_0682, and STM14_2713 at varying concentrations of $CuSO_4$....29
Figure 2.12 Growth curves of WT and mutant strains of Salmonella mutants STM14_3859, STM14_0376, and STM14_1898 at varying concentrations of $CuSO_4$....29
Figure 2.13 Growth curves of WT and mutant strains of Salmonella mutant STM14_2613 at varying concentrations of $CuSO_4$....30
Figure 3.1: Copper Sensitivity Assay of WT and remaining mutant strains of Salmonella....30

LIST OF TABLES

Table 1. Needed antibiotics for each *Salmonella* mutant....24

INTRODUCTION

Salmonella Typhimurium, referred to as *Salmonella* throughout this work, is a ubiquitous Gram-negative bacterium that stands as a significant global health concern, with its impact reverberating through both human and animal populations. *Salmonella,* a prominent genus among enteric pathogens, holds clinical significance globally, being a leading cause of both foodborne illnesses in developed nations and diarrheal diseases in developing countries, typically resulting from ingestion of contaminated food or water (1). Despite concerted efforts to mitigate its spread and impact, *Salmonella* continues to challenge public health systems, necessitating a deeper understanding of its biology, pathogenesis, epidemiology, and control strategies.

Salmonella, closely associated with *Escherichia*, comprises Gram-negative, rod-shaped bacteria that do not form spores and are part of the Enterobacteriaceae family (1). It exhibits remarkable adaptability, thriving in diverse environments ranging from soil and water to the gastrointestinal tracts of mammals, including humans. The bacterium possesses an extensive repertoire of virulence factors that facilitate its colonization, invasion, and survival within host organisms, contributing to the severity of infections it causes.

Each year, nearly 94 million instances of enteric salmonellosis and over 150,000 fatalities are documented globally, with the majority of cases being self-limiting and effectively managed with antimicrobial treatment (2). *Salmonella* infection in humans leads to two primary clinical syndromes: enteric or typhoid fever and colitis/diarrheal disease, characterized by symptoms such as fever, headache, abdominal pain, and transient diarrhea or constipation, with potential complications including fatal respiratory, hepatic, splenic, and neurological damage (1). Its ability to cause systemic infection distinguishes it from many other *Salmonella* serovars, underscoring the need for targeted therapeutic interventions and preventive measures.

Furthermore, the emergence of antimicrobial resistance among *Salmonella* strains, including Typhimurium, poses additional challenges to the effective management of infections and underscores the urgency of surveillance and stewardship efforts.

Research aimed at understanding how *Salmonella* manages copper stress stems from investigations conducted on *Salmonella* enterica subspecies enterica serovar Typhimurium(10). *Salmonella* adeptly regulates the expression of crucial virulence factors, allowing it to withstand elevated levels of copper within the vacuole (2). This pathogen demonstrates remarkable adaptability and survival capabilities in hostile environments, including those encountered within host cells, where exposure to high copper levels and redox stress is common. Numerous studies have focused on *Salmonella* and various mutant strains within macrophages to elucidate the functions of various regulators, transporters, and chaperones, as well as the role of copper handling in the cell envelope in virulence. During infection within host macrophages, *Salmonella* responds to increased copper levels within the *Salmonella*-containing vacuole, and mutants with reduced copper resistance exhibit diminished survival compared to the wild-type strain (2,10,11). Similar to other gram-negative bacteria, copper homeostasis in S. Typhimurium is regulated transcriptionally by envelope and cytoplasmic copper sensors, such as CueR (10).

Salmonella has developed unique strategies to survive in various environments. A potential factor could be porins as they are the most abundant proteins in the outer membrane of Gram-negative bacteria (4). Porins are a group of proteins situated in the outer membrane of Gram-negative bacteria that function as channels facilitating passive diffusion between the extracellular environment and the periplasm, enabling the exchange of metals such as copper, thus contributing to the maintenance of cell envelope integrity (6). These proteins play a crucial role in metal transport and membrane integrity (3,4,5). Porins represent the predominant proteins

within the outer membrane of Gram-negative bacteria, serving as the primary route for polar antibiotics and inhibitors to enter Enterobacter species (4). Gram-negative bacteria frequently evolve antimicrobial resistance, often characterized by a decrease in the quantity of porins (9). Porins, including OmpF, OmpC, PhoE, and OmpD, exist in *Salmonella* as homodimers, homodimers, and monomers, forming aqueous channels facilitating passive transport of hydrophilic molecules of low molecular weight, thus serving a dual function of nutrient uptake and waste product/toxic substance excretion in bacterial interactions with the extracellular environment (7). Outer membrane protein (OMP) structures are composed of a transmembrane motif that consists of β-strands that form a transmembrane β-barrel. These strands are connected by short loops in the periplasm and longer extracellular loops.

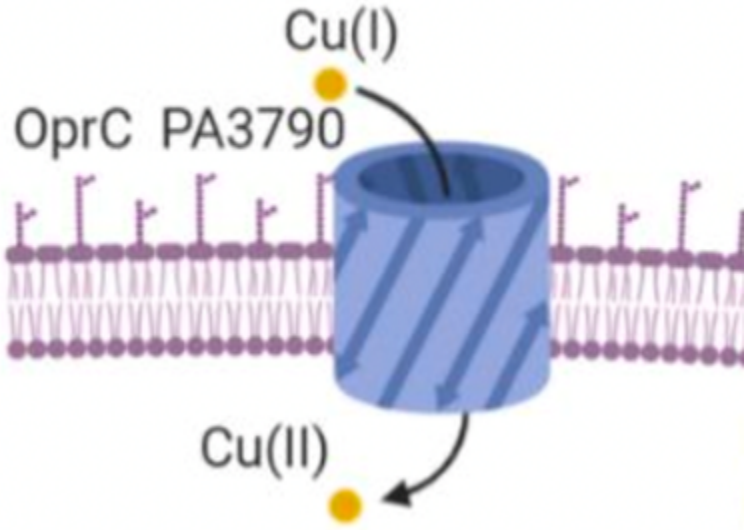

Figure 1. Example of protein in copper homeostasis. Copper is represented by the yellow dots. (8)

As of now the understanding is that as in other gram-negative bacteria, copper homeostatic systems in *Salmonella* are maintained by transcriptional control by envelope and

cytoplasmic Cu sensors, including CueR (1). But the new focus is in porin-mediated transport which likely accounts for the majority of copper movement in bacteria, necessitating an increased copper efflux rate alongside reduced permeability. The exchange of Cu+ with the extracellular environment may happen through passive diffusion facilitated by porins, as demonstrated by the copper resistance observed in an E. coli mutant lacking porins when exposed to antimicrobial copper surfaces (8). Copper importers are specific transport proteins that enable the uptake of copper ions into the cell. Copper is an essential element for cellular processes, but in excess, it can be toxic. Therefore, the regulation of copper import is vital for the survival of *Salmonella* (2). Therefore research is needed where reduced porin expression may impede copper influx, potentially resulting in a higher initial growth rate observed in mutants compared to $CuSO_4$-treated wild-type strains.

METHODS

Growth Curves

All mutant strains were found from BEI Resources, NIAID, NIH: S. enterica subsp. enterica, Strain 14028s (Serovar Typhimurium) Single-Gene Deletion Mutant Library. All mutant strains were selected based on having porin leaving 42 potential poring mutants based on the 252 STM OMP. 20 were ordered and stocks were placed in storage and another 17 were found using BEI and ordered.

Antibiotic Stock Solutions:

The antibiotic stock solutions were prepared by dissolving 60 μg/mL kanamycin in deionized (DI) water and 20 μg/mL chloramphenicol in ethanol. Both solutions were filter sterilized and stored at -20ºC.

Mutant Strains and Growth Conditions

Luria broth (LB) plates with the appropriate amount of antibiotic were prepared by adding the respective antibiotic (Table #1) to liquid LB and agar after autoclaving and cooling, before pouring the plates. LB growth plates without antibiotics were prepared as controls. The effectiveness of the antibiotics was tested by plating wild-type (WT) bacteria, and absence of growth was confirmed. Each mutant was streaked on the corresponding plate and placed into an incubator and grown for 24 hours. 20 mutants were available and grown. Another 17 mutants were ordered and grown under the same conditions.

Preparation of Overnight Cultures:

The day before the experiment, overnight cultures were prepared by isolating a single bacterial colony from needed mutant and inoculating in a cell culture tube with 5 mL LB medium

containing the appropriate antibiotic. The cultures were then incubated overnight in a rolling incubator at 37°C. The wild type (WT) and 6 mutants were able to fit on the 96 well plate at a time so only 6 mutants were grown overnight at a time. Stocks of each mutant were made and stored at -80°C with 60% glycerol.

Preparation of Fresh Cultures:

On the day of the experiment, fresh cultures were prepared by transferring 100 µL of the overnight culture into a 5 mL LB medium, again with the appropriate antibiotic. The cultures were then incubated for 1-2 hours until the optical density at 600 nm (OD-600) reached a range suitable for accurate measurement by the spectrophotometer (typically 0.5-0.7).

Preparation of $CuSO_4$ Solutions:

A 50mL 200mM $CuSO_4$ stock solution was made. While the cultures were incubating, solutions of $CuSO_4$ were prepared at twice the final concentration needed in the wells. Solutions of 0, 4, 8, and 12 mM $CuSO_4$ were prepared in LB medium. These solutions were not reused day-to-day; only the 200 mM $CuSO_4$ stock solution was reused to prepare more dilute concentrations again.

Dilution of Fresh Cultures:

Once the fresh cultures reached the desired OD-600 range (0.5-0.7), they were diluted to an OD-600 of 0.1 using LB medium. The final volume in each tube was adjusted to 3 mL to achieve a final OD-600 of 0.05 in the microplate wells. The tubes containing the fresh cultures were kept on ice to prevent further bacterial growth.

Preparation of 96-Well Plates:

For each well in the 96-well plates, 100 µL of the respective $CuSO_4$ solution was added first, followed by 100 µL of the diluted bacterial culture. Controls containing only LB were prepared similarly, with the addition of the appropriate antibiotic to the LB before distributing among the

wells. Three replicates of each condition were prepared as per the arrangement diagram provided.

Microplate Reader Assay:

The prepared 96-well plates were subjected to overnight analysis using a microplate reader. The data from the microplate reader was taken and formatted into graphs the WT and mutant growth curves.

Copper Sensitivity Assay

Preparation of Solid media

Copper-Luria agar plates were prepared to assess the growth response of [your organism/species] to varying concentrations of copper. The copper concentrations were prepared using a stock solution of 200 mM $CuSO_4$. Eight concentrations were selected: 0 mM, 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, and 8 mM. Each concentration was replicated eight times to accommodate for WT and mutant placement. Luria broth/agar (LB agar) was used as the growth medium. LB agar powder was dissolved in distilled water and autoclaved to sterilize.

After cooling, appropriate volumes of the copper solutions were added to the molten LB agar to achieve the desired concentrations. The agar was thoroughly mixed, avoiding causing bubbles, to ensure even distribution of copper ions. Petri dishes were arranged in a sterile environment and molten copper-LB agar was poured into each Petri dish to a uniform depth.

Plates were left undisturbed to solidify and cool. Each plate was labeled with the corresponding copper concentration and other relevant information for identification. Prepared plates were

stored in a cool, dark place until further use, to prevent degradation of the copper ions and maintain sterility.

Preparation of Overnight Cultures:

The day before the experiment, overnight cultures were prepared by isolating a single bacterial colony from needed mutant and inoculating in a cell culture tube with 5 mL LB medium containing the appropriate antibiotic. The cultures were then incubated overnight in a rolling incubator at 37^{o}C.

Preparation of Fresh Cultures:

On the day of the experiment, fresh cultures were prepared by transferring 100 µL of the overnight culture into a 5 mL LB medium, again with the appropriate antibiotic. The cultures were then incubated for 1-2 hours until the optical density at 600 nm (OD-600) reached a range suitable for accurate measurement by the spectrophotometer (typically 0.5-0.7). The OD-600 was then adjusted to 0.5 for the WT and each mutant and placed in ice to stop growth.

Serial Dilution and Spot Plating

Measurements were initially obtained at a standardized OD-600 of 0.5. Subsequently, serial 1:10 dilutions of the OD-600 standardized cultures were prepared for both the WT and each mutant strain. These dilutions were performed in microcentrifuge tubes and immediately placed on ice to maintain the integrity of the cultures. Following dilution, aliquots of each dilution were spot plated onto the prepared copper-Luria agar plates using a micropipette, with 5 µL of culture applied to each plate. This process was repeated for the WT and each strain across the entire range of copper concentrations.

Incubation

Following the completion of spotting, the plates were allowed to air dry to ensure proper adhesion of the inoculum to the agar surface. Once dried, the plates were flipped over and placed in the incubator set at 37°C and left to grow overnight. After the designated incubation period, the plates were removed from the incubator, and colony growth was visually assessed and images were recorded for subsequent analysis.

RESULTS

Growth curves were analyzed to assess the sensitivity of mutated strains lacking the gene corresponding to a potential porin OMP to copper. The growth curves indicated no significant differences in growth patterns between the mutated strains and the wild-type strain when treated with copper. Figure 2 depicts the growth curves of the wild-type strain and the mutated strains in the presence of varying concentrations of $CuSO_4$. Despite the absence of the porin gene in the mutated strains, their growth behavior closely mirrored that of the wild-type strain across all concentrations of $CuSO_4$ tested.

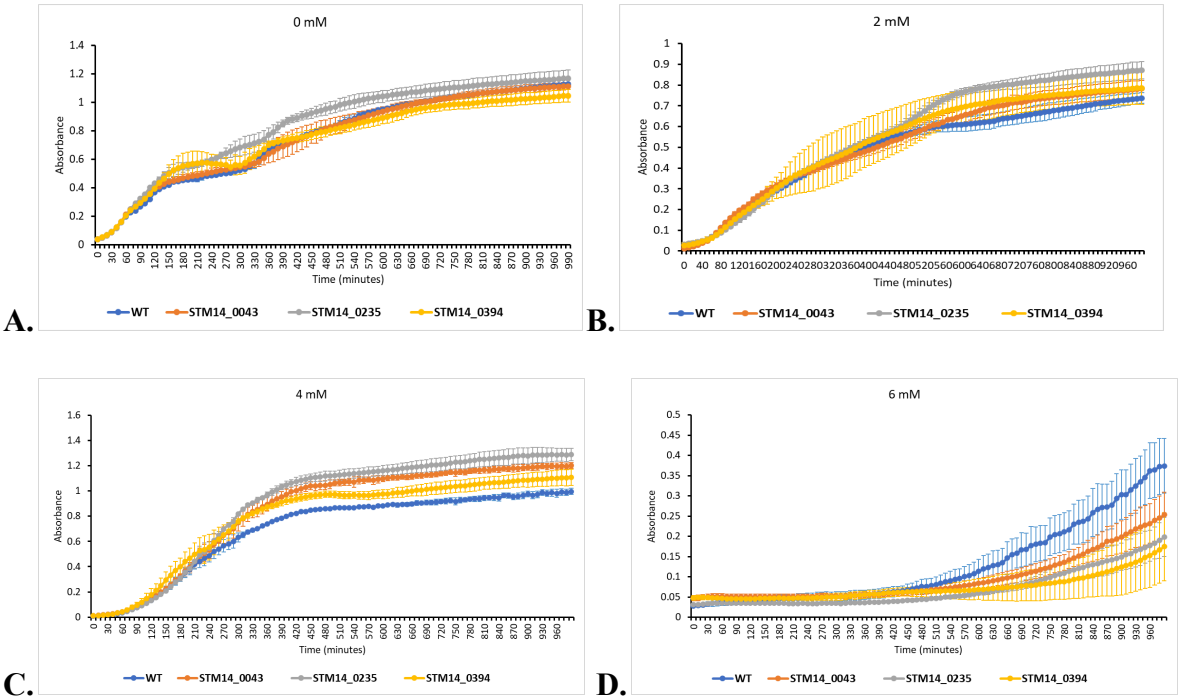

Figure 2. Growth curves of Salmonella WT and mutant strains of *Salmonella* at varying concentrations of $CuSO_4$. All 37 mutants were tested. While similar results were obtained with all strains, a few examples are shown (STM14_0043, STM14_0235, and STM14_0394). Remaining growth curves are found in the appendix. A. 0 mM **B.** 2mM **C.** 4mM **D.** 6mM

No notable deviations in growth were observed between the mutated strains and the wild-type strain in the absence of $CuSO_4$, suggesting comparable growth rates under normal conditions. 4mM displayed the mutants with a higher growth than the WT but not an immediate spike. 6mM displayed that the bacteria did not grow well. For all 37 mutant strains none of the growth curves displayed any immediate growth over the WT. An example of the growth curves can be found in Table 2.1. There is variation in some curves and speed but none overwhelmingly displayed an immediate growth over the WT. These findings suggest that the absence of the porin gene did not significantly affect the sensitivity of *Salmonella* strains to copper, as evidenced by the similar growth patterns observed between the mutated strains and the wild-type strain across different concentrations of $CuSO_4$.

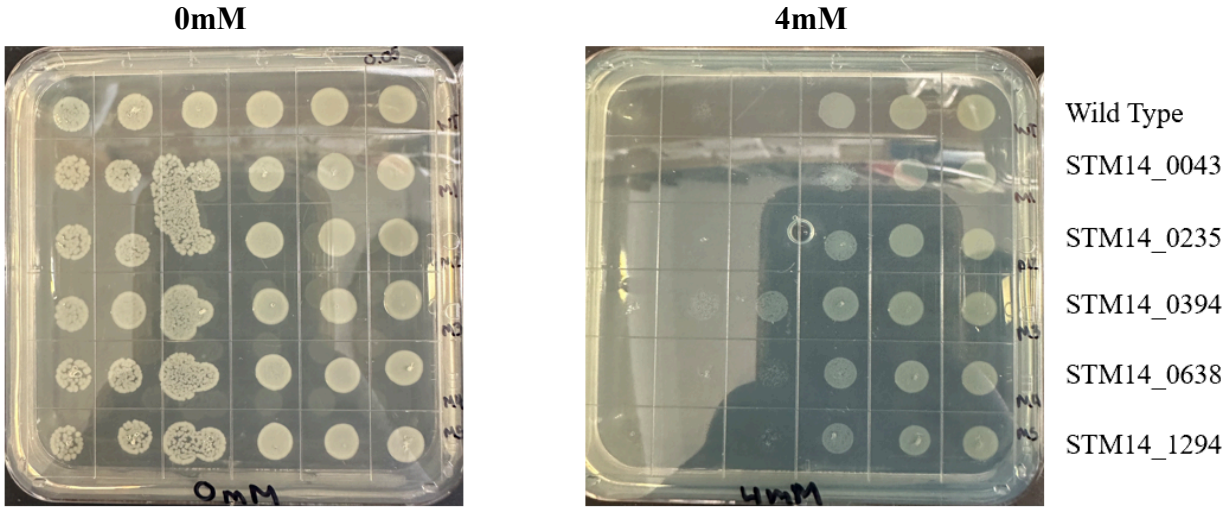

Figure 3: Copper Sensitivity Assay of WT and mutant strains of Salmonella. Serial 1:10 dilutions WT and mutant strains were spot plated on LB and varying concentrations of $CuSO_4$. While all 37 were tested, examples STM14_0043, STM14_0235, STM14_0394, STM14_0638, STM14_1294 are shown. Strains exposed to 0 mM and 4 mM $CuSO_4$ are shown. All strains had similar sensitivity except for mutant STM14_0394 which showed a higher resistance to copper and can be found in the appendix.

In the copper sensitivity assay, the mutant strain STM14_0394 displayed enhanced growth compared to the wild-type strain at higher concentrations of copper (Fig. 3B). This

observation suggests that the mutation in the STM14_0394 strain confers increased resistance to copper stress. Conversely, all other mutant strains either exhibited similar sensitivity to copper as the wild-type strain or displayed heightened sensitivity. These findings indicate that the absence of the porin gene in most mutant strains does not significantly alter their sensitivity to copper.

DISCUSSION

Salmonella Typhimurium remains a formidable global health threat, affecting both human and animal populations worldwide. The complexity of *Salmonella* infections, coupled with its ability to cause a wide range of clinical manifestations, underscores the urgent need for a comprehensive understanding of its biology, pathogenesis, and control strategies. This study explored the role of porins in copper sensitivity among *Salmonella* strains. Porins, essential outer membrane proteins, facilitate the passage of nutrients and ions across the bacterial membrane. Additionally, they play a crucial role in metal transport, including copper, thereby contributing to cellular homeostasis and membrane integrity.

Based on the growth curve results, porin-mediated transport is not the primary mechanism governing copper influx in *Salmonella*. Despite the absence of the porin gene, *Salmonella* mutants exhibited similar growth patterns to wild-type strains when exposed to varying concentrations of copper. This unexpected resilience to copper stress suggests the existence of alternative mechanisms for copper uptake or resistance in these bacteria.

The discovery that mutant strain STM14_0394 exhibited enhanced growth over the wild-type strain at higher copper concentrations raises intriguing questions about the role of porins in copper sensitivity. While most mutant strains displayed similar or increased sensitivity to copper compared to the wild-type strain, the unexpected resilience of STM14_0394 suggests the presence of alternative mechanisms for copper uptake or resistance. Further investigation into the genetic basis of this enhanced copper resistance phenotype in STM14_0394 could uncover novel insights into copper homeostasis in Salmonella.

The identification of STM14_0394 as an outlier with enhanced copper resistance highlights the importance of genetic diversity in bacterial populations and the potential for the emergence of adaptive traits under selective pressure. Future studies elucidating the specific genetic determinants underlying the copper resistance phenotype of STM14_0394 could provide valuable insights into novel targets for antimicrobial intervention strategies. Further research would be to research the copper concentration within the mutated strain STM14_0394 but that was outside the scope of my research.

This project sheds light on the intricate interplay between bacterial physiology and environmental stressors. The ability of *Salmonella* strains to adapt to copper-induced stress highlights the remarkable resilience of these pathogens and underscores the challenges in developing effective antimicrobial strategies. Understanding the mechanisms underlying copper resistance in *Salmonella* is crucial for the development of targeted therapeutic interventions and preventive measures. Additionally, elucidating alternative pathways involved in copper transport and resistance mechanisms may pave the way for novel antimicrobial strategies to combat *Salmonella* infections.

However, this project has certain limitations that warrant consideration. The growth curve assay may not have been sensitive enough to detect subtle differences in growth patterns between the mutants and wild-type strains under copper stress conditions. The copper sensitivity assay, on the other hand, may have provided a more direct measure of the mutants' resistance to copper by specifically assessing their ability to survive and grow in the presence of high copper concentrations. The focus on a specific set of mutations may not fully capture the complexity of copper resistance mechanisms in *Salmonella*. Future studies incorporating a broader range of mutants and exploring additional regulatory pathways are needed to provide a comprehensive

understanding of copper homeostasis in these bacteria. Further research is warranted to unravel the intricate interplay between bacterial physiology and environmental stressors, ultimately informing the development of effective antimicrobial strategies against *Salmonella* infections.

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APPENDIX

Table 1.1 Needed antibiotics for each *Salmonella* mutant strain.

Antibiotic	Mutant Strain
60 µg/mL Kanamycin	STM14_0043 STM14_0235 STM14_0394 STM14_0638 STM14_1294 STM14_2653 STM14_3348 STM14_4874 STM14_5514 STM14_1777 STM14_1848 STM14_3401 STM14_5099 STM14_2095 STM14_0489 STM14_2797 STM14_2942 STM14_5542 STM14_1378 STM14_0228 STM14_4965 STM14_0802 STM14_5534 STM14_0682 STM14_2713 STM14_3859 STM14_0376
20 µg/mL Chloramphenicol	STM14_4829 STM14_4527 STM14_5119 STM14_0207 STM14_4385 STM14_0426 STM14_1183

	STM14_2978 STM14_1898 STM14_2613

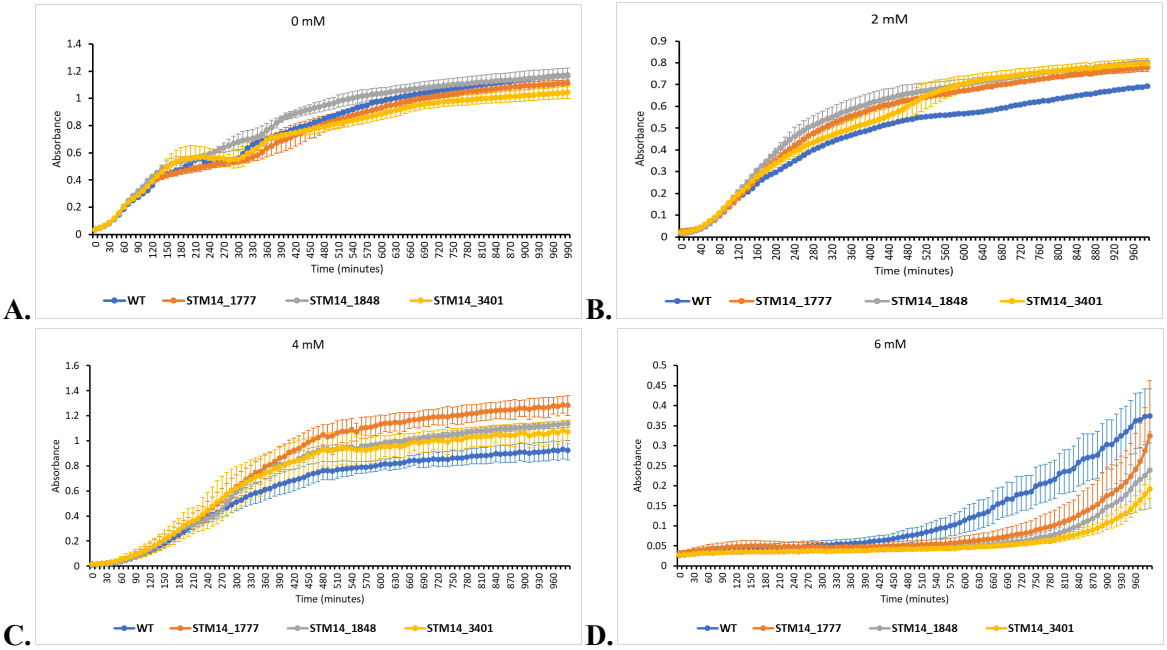


Figure 2.1 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_1777, STM14_1848, and STM14_3401 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

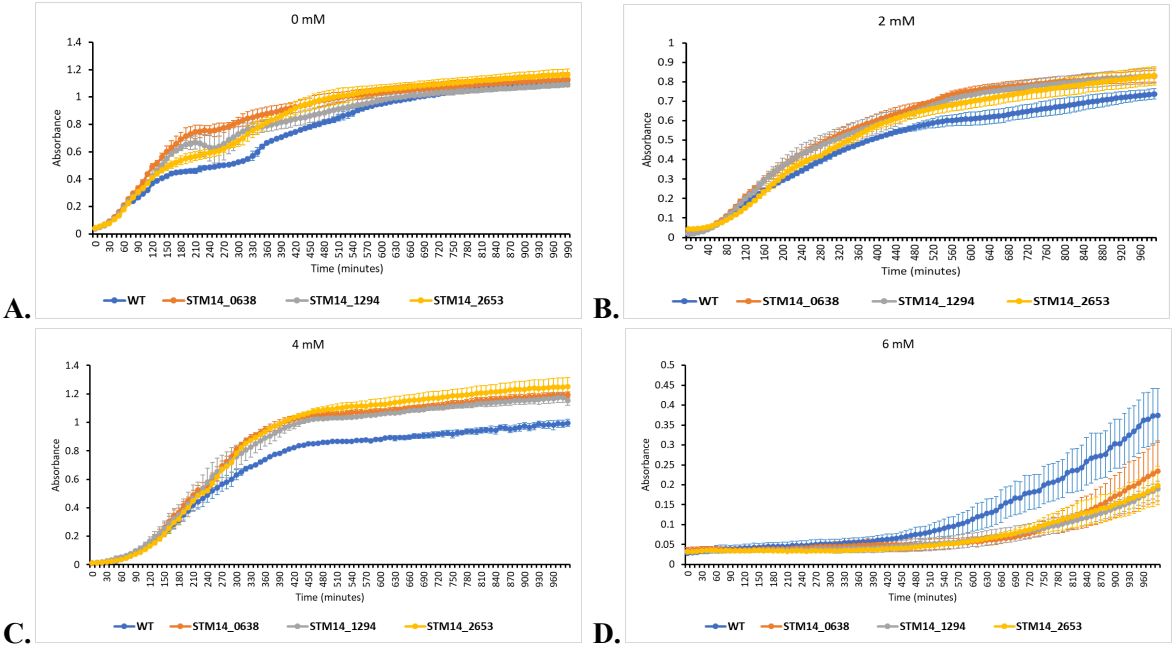


Figure 2.2 Growth curves of WT and mutant strains of Salmonella mutants STM14_0638, STM14_1294, and STM14_2653 at varying concentrations of $CuSO_4$ A. 0 mM **B.** 2mM **C.** 4mM **D.** 6mM

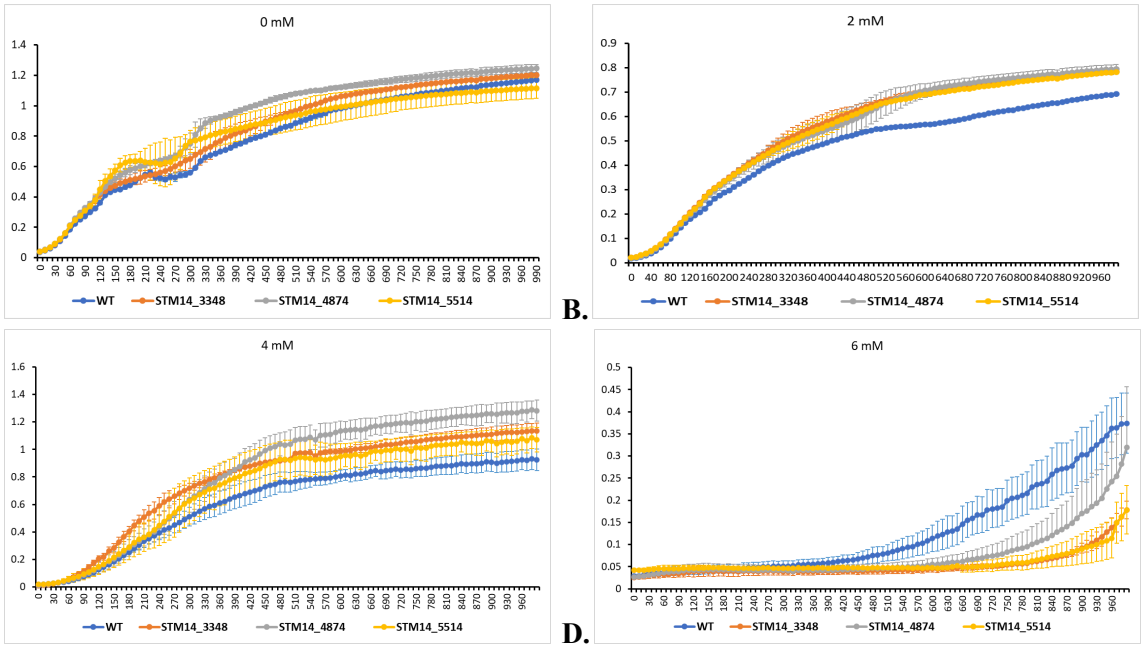


Figure 2.3 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_3348, STM14_4874, and STM14_5514 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

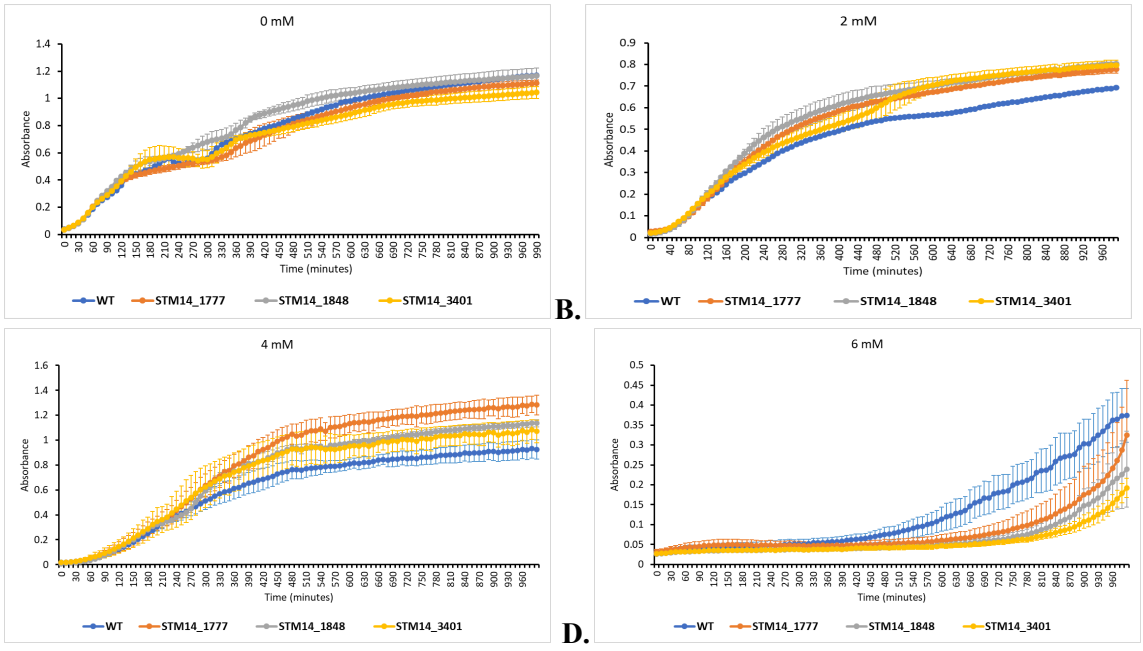


Figure 2.4 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_1777, STM14_1848, and STM14_3401 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

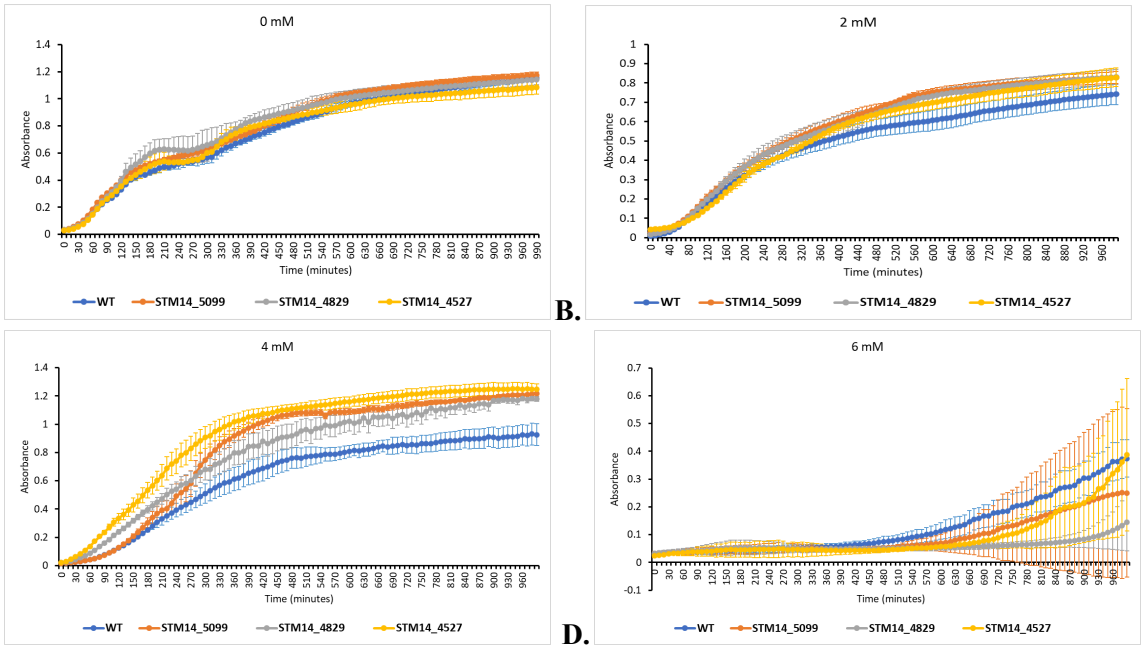


Figure 2.5 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_5099, STM14_4829, and STM14_4527 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

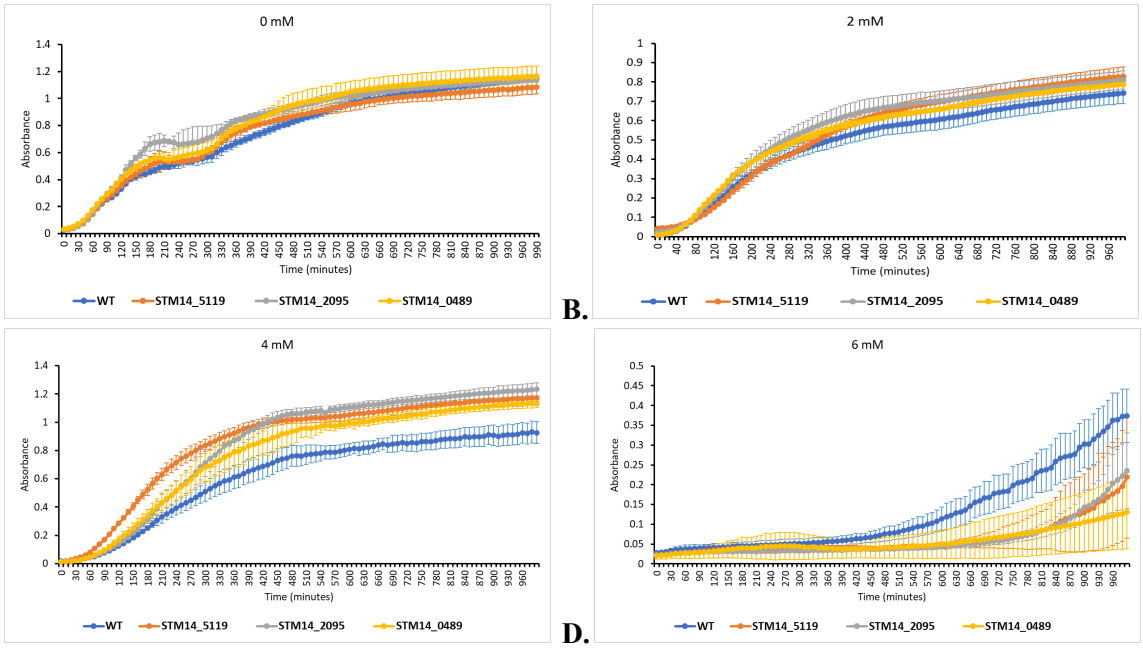


Figure 2.6 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_5119, STM14_2095, and STM14_0489 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

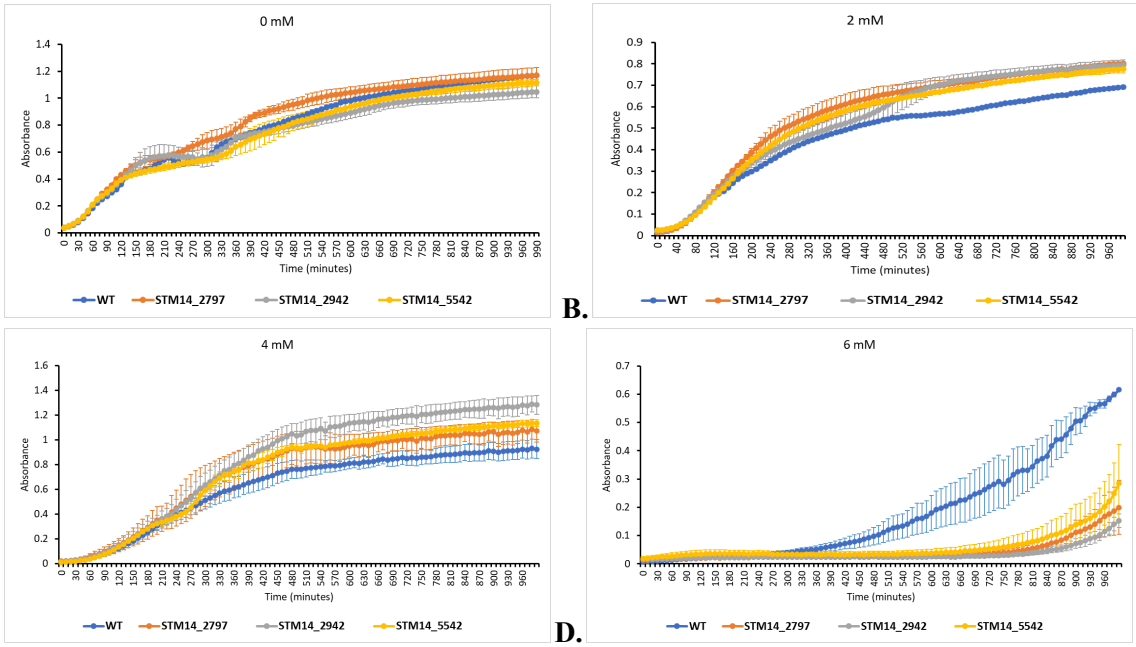


Figure 2.7 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_2797, STM14_2942, and STM14_5542 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

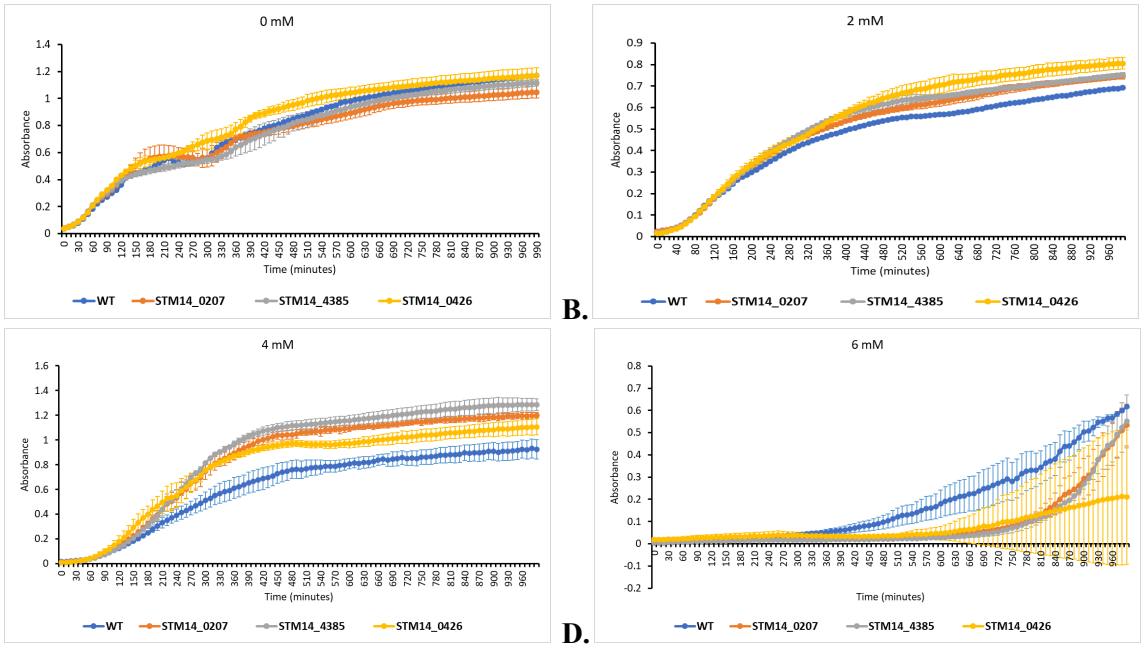


Figure 2.8 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_0207, STM14_4385, and STM14_0426 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

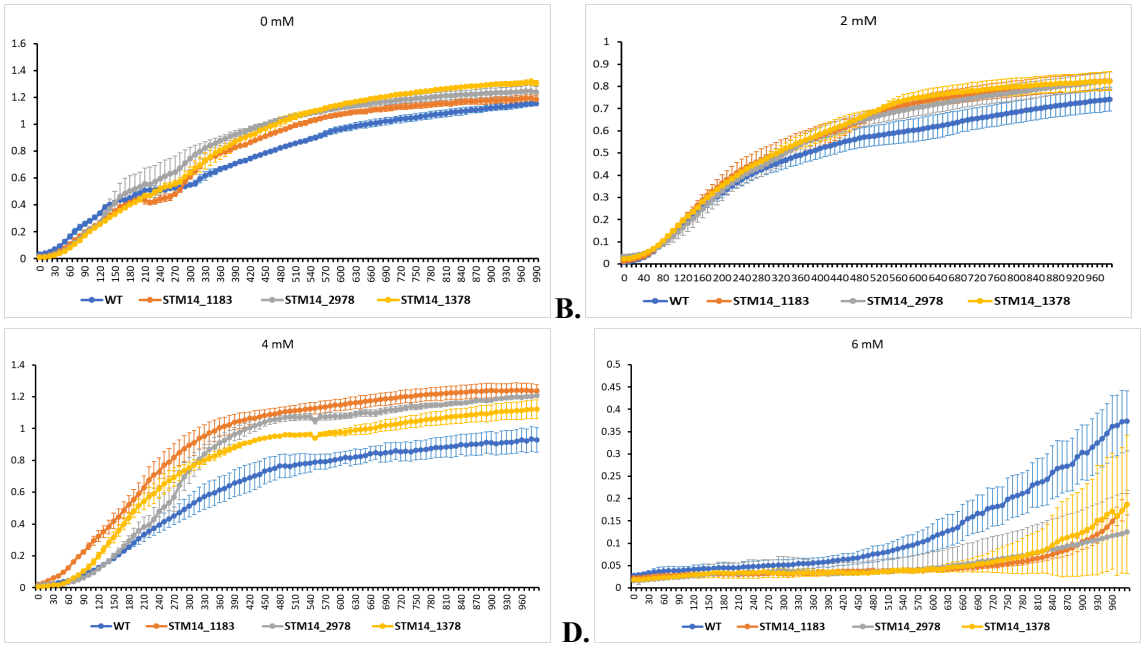


Figure 2.9 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_1183, STM14_2978, and STM14_1378 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

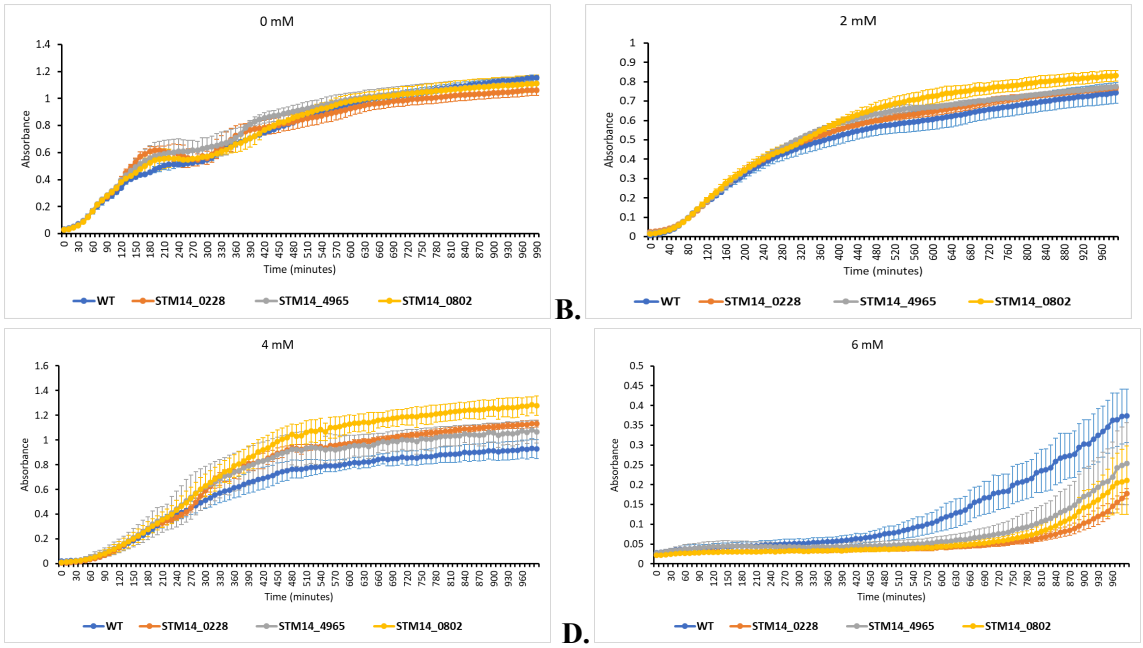


Figure 2.10 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_0228, STM14_4965, and STM14_0802 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

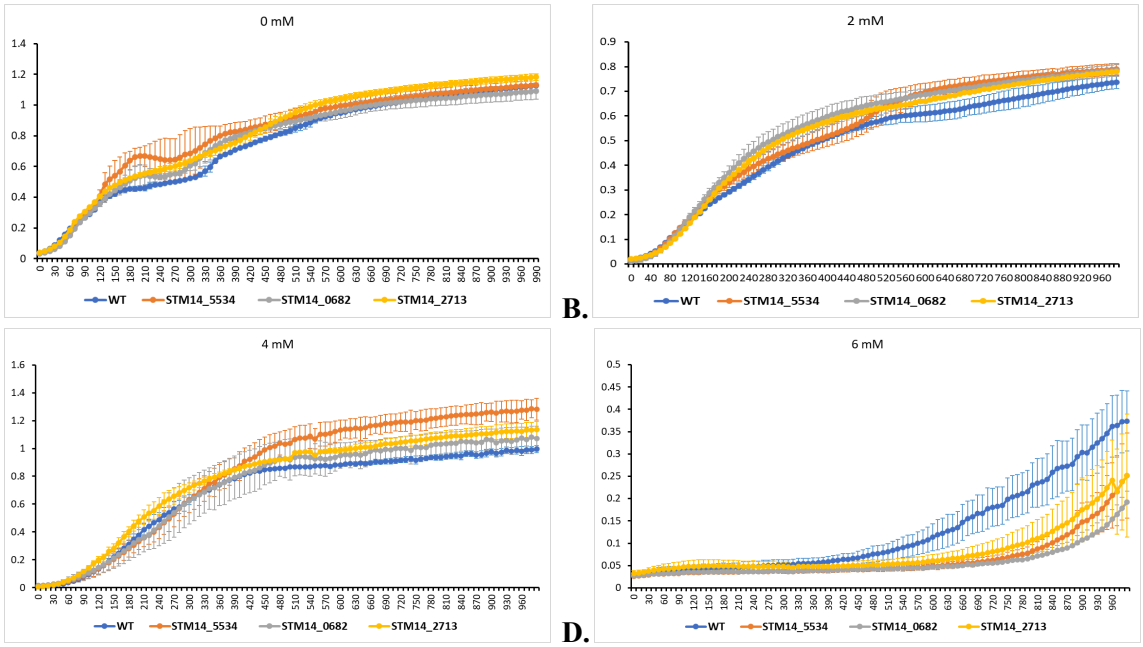


Figure 2.11 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_5534, STM14_0682, and STM14_2713 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

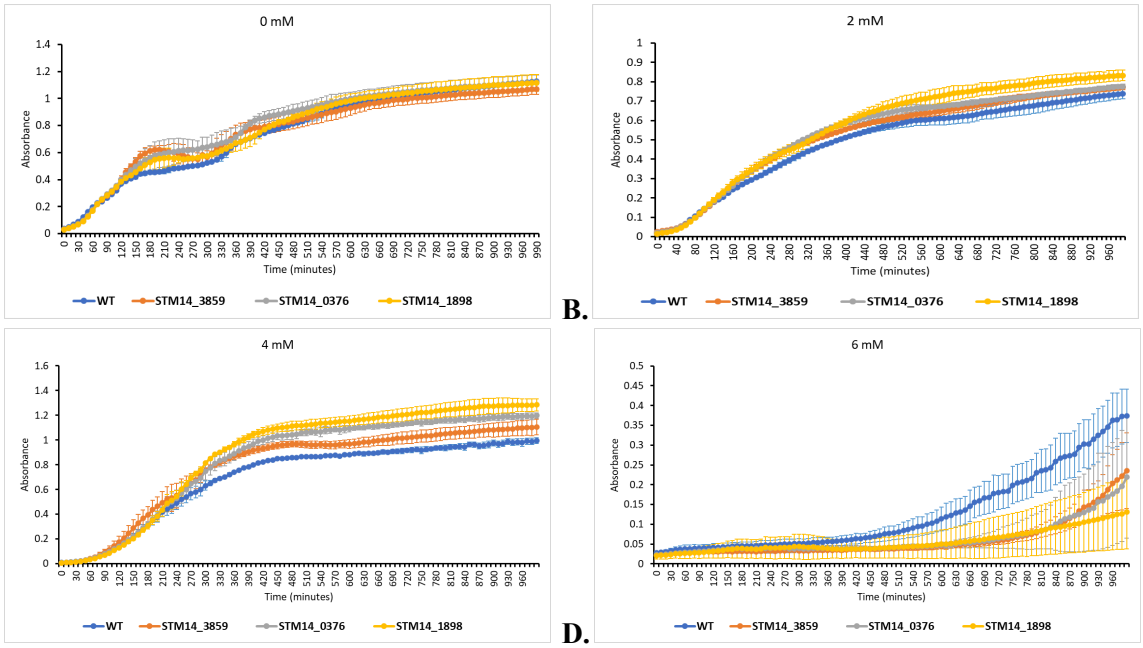


Figure 2.12 Growth curves of WT and mutant strains of *Salmonella* mutants STM14_3859, STM14_0376, and STM14_1898 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM

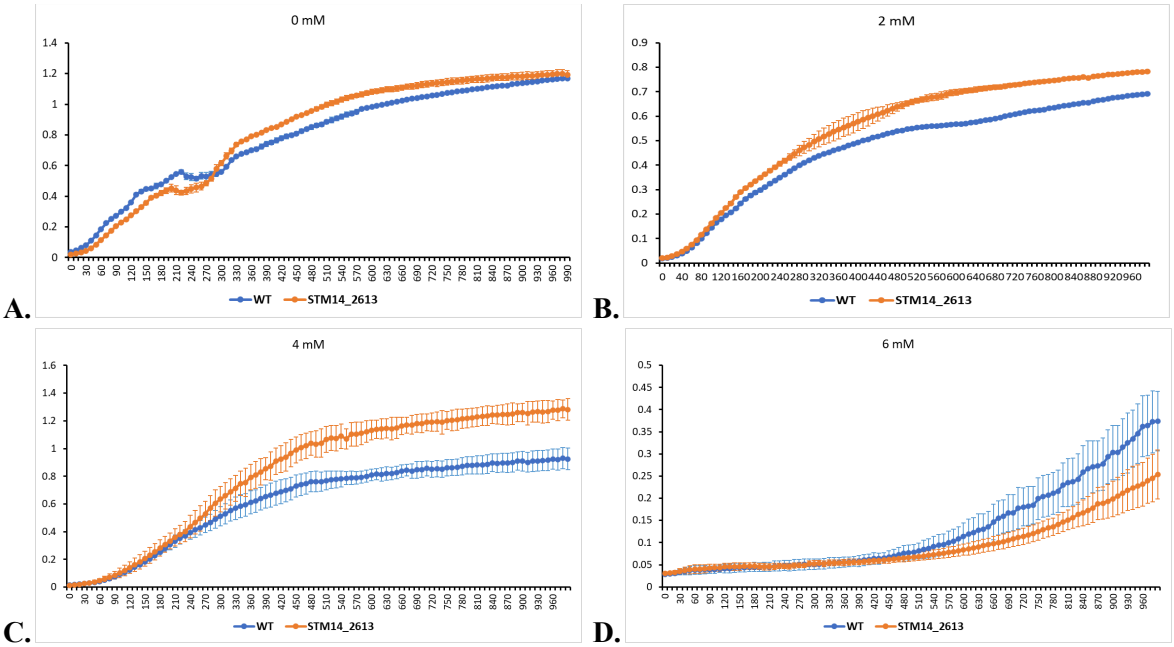
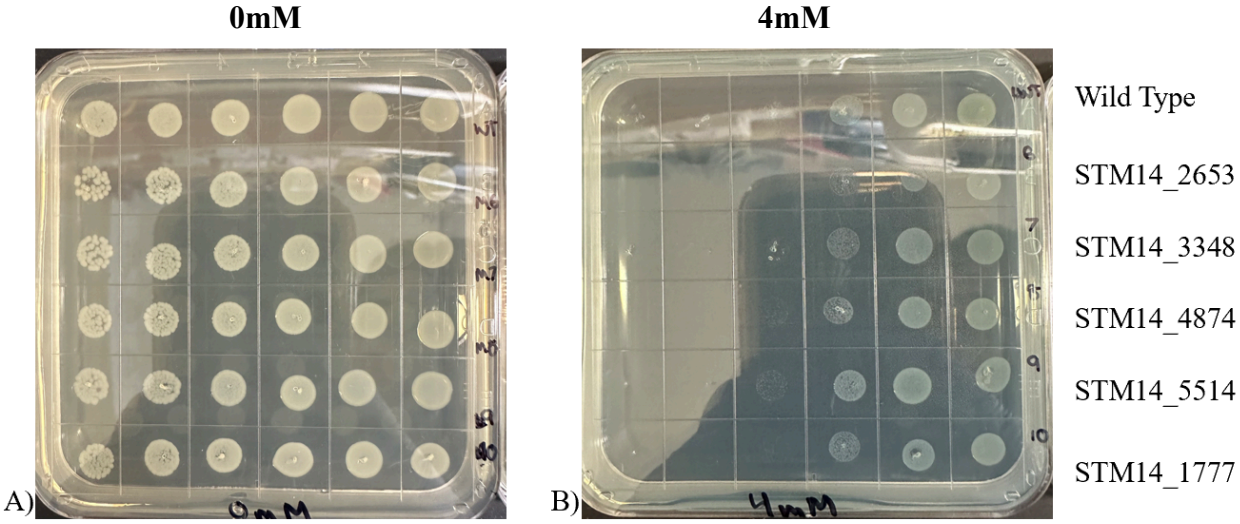
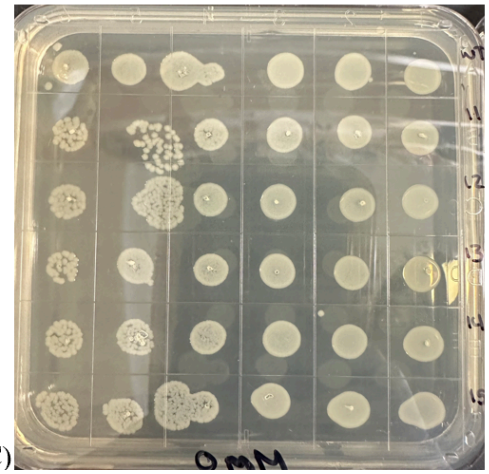


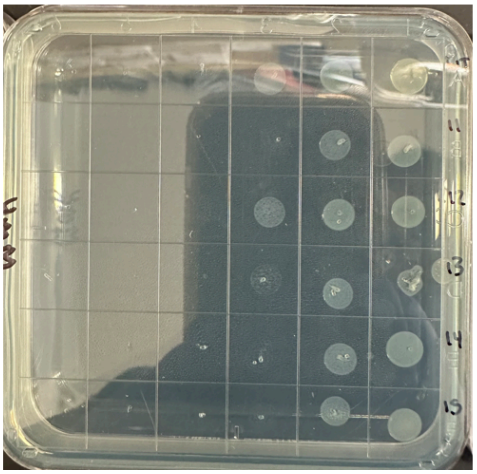
Figure 2.13 Growth curves of WT and mutant strains of *Salmonella* mutant STM14_2613 at varying concentrations of $CuSO_4$ A. 0 mM**B.** 2mM **C.** 4mM **D.** 6mM



C)



D)



Wild Type

STM14_1848

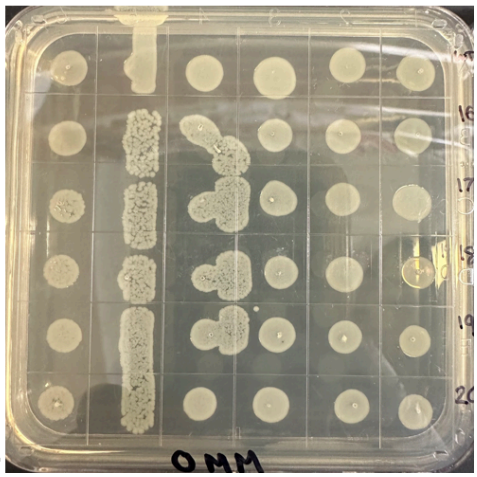
STM14_3401

STM14_5099

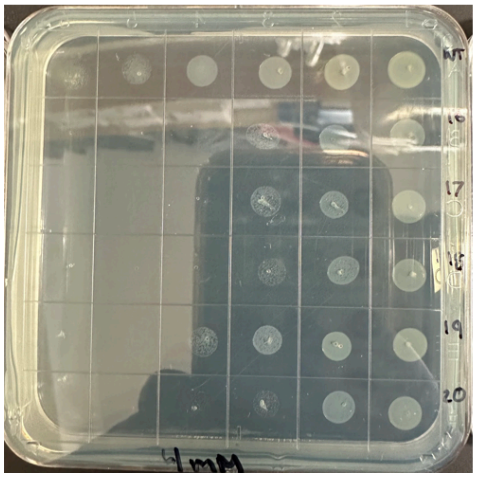
STM14_4829

STM14_4527

E)



F)



Wild Type

STM14_5119

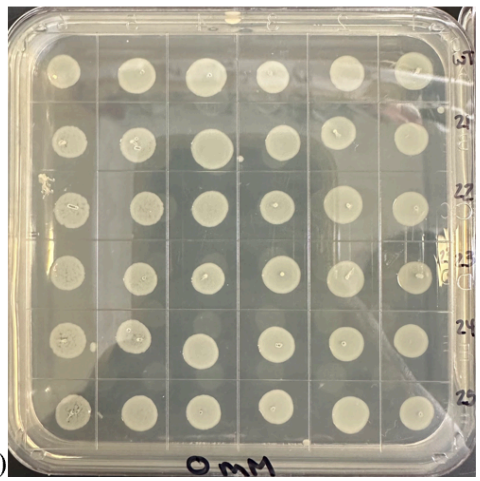
STM14_2095

STM14_0489

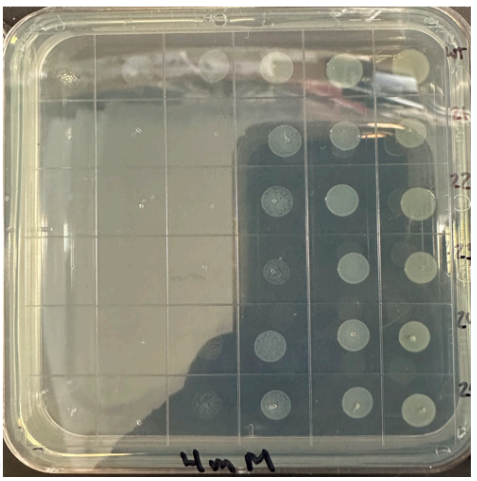
STM14_2797

STM14_2942

G)



H)



Wild Type

STM14_5542

STM14_0207

STM14_4385

STM14_0426

STM14_1183

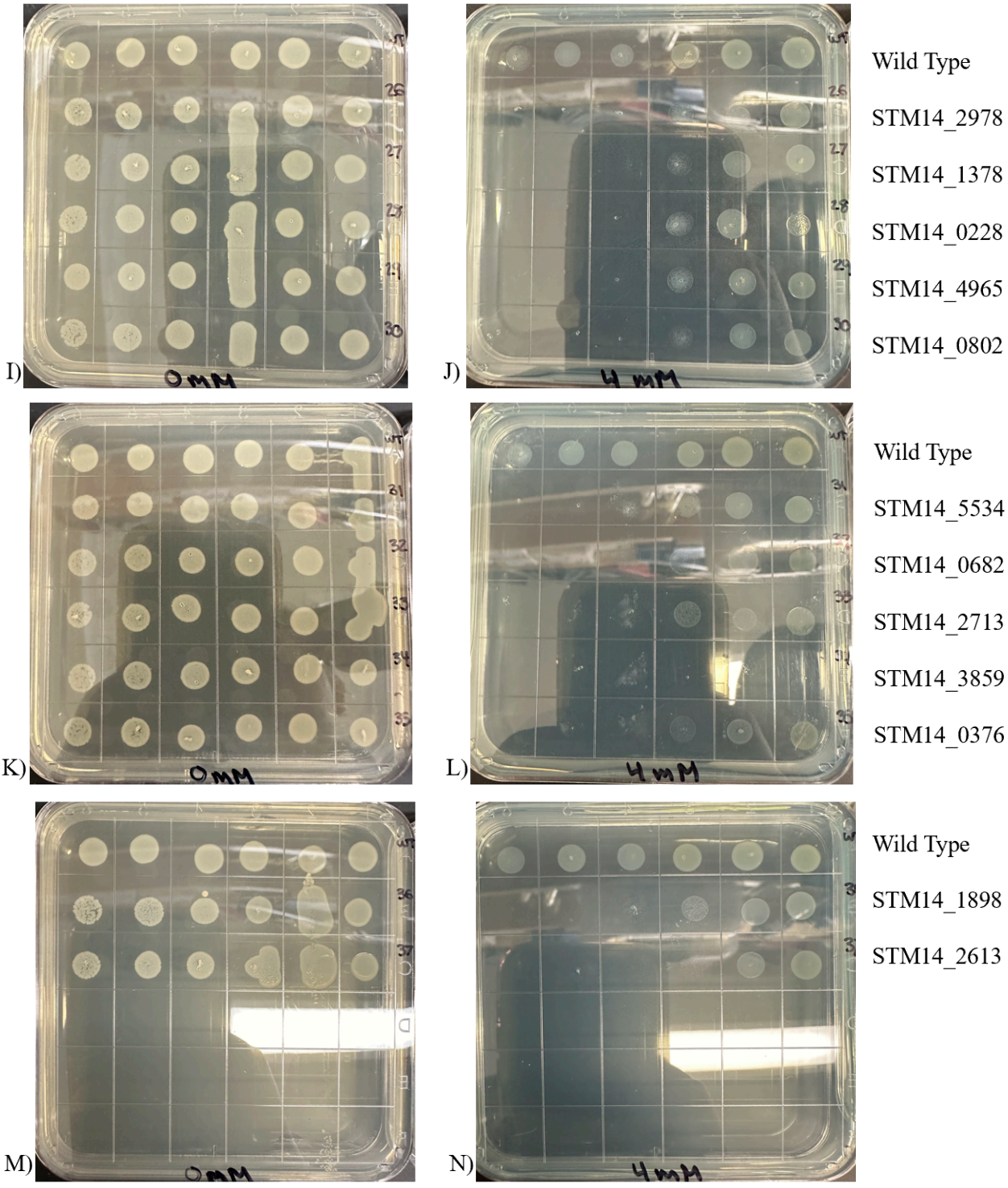


Figure 3.1: Copper Sensitivity Assay of WT and mutant strains of Salmonella. Serial 1:10 dilutions WT and mutant strains were spot plated on LB and varying concentrations of $CuSO_4$. Strains exposed to 0 mM and 4 mM $CuSO_4$ are shown. **A.** 0mM WT and STM14_2653, STM14_3348, STM14_4874, STM14_5514, and STM14_1777. **B.** 4mM WT and STM14_2653, STM14_3348, STM14_4874, STM14_5514, and STM14_1777. **C.**0mM WT and STM14_1848, STM14_3401, STM14_5099, STM14_4829, and STM14_4527. **D.**4mM WT and STM14_1848,

STM14_3401, STM14_5099, STM14_4829, and STM14_4527. **E.**0mM WT and STM14_5119, STM14_2095, STM14_0489, STM14_2797, andSTM14_2942. **F.**4mM WT and STM14_5119, STM14_2095, STM14_0489, STM14_2797, andSTM14_2942. **G.**0mM WT and STM14_5542, STM14_0207, STM14_4385, STM14_0426, and STM14_1183. **H.**4mM WT and STM14_5542, STM14_0207, STM14_4385, STM14_0426, and STM14_1183. **I.**0mM WT and STM14_2978, STM14_1378, STM14_0228, STM14_4965, and STM14_0802. **J.**4mM WT and STM14_2978, STM14_1378, STM14_0228, STM14_4965, and STM14_0802. **K.**0mM WT and STM14_5534, STM14_0682, STM14_2713, STM14_3859, and STM14_0376. **L.**4mM WT and STM14_5534, STM14_0682, STM14_2713, STM14_3859, and STM14_0376. **M.**0mM WT and STM14_1898 and STM14_2613. **N.**4mM WT and STM14_1898 and STM14_2613.