Copper Homeostasis is Important for Pathogenesis of *Pseudomonas aeruginosa* in *Caenorhabditis elegans*

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*This report represents the work of WPI undergraduate students submitted to the faculty as evidence of a degree requirement. WPI routinely publishes these reports on its website without editorial or peer review. For more information about the project’s program at WPI, see [http://www.wpi.edu/Academics/Projects](http://www.wpi.edu/Academics/Projects)*
Abstract

Maintaining a homeostatic balance of transition metal ions is important for all organisms, as they are both essential for many biological processes as well as toxic in excess. The opportunistic parasite *Pseudomonas aeruginosa* has shown that metal ion homeostasis, specifically the regulation of free copper, may be important to its pathogenesis. This study utilizes the free-living nematode *Caenorhabditis elegans* as a model organism to study this pathogenesis. *C. elegans* was exposed to transposon mutants of *P. aeruginosa* that pertain to the bacteria’s copper ion homeostasis system and its lethality was measured over a time course to infer the importance of these genes to *P. aeruginosa*’s pathogenesis. Here, it is shown that functional knockouts of a vast majority of these genes do cause a decrease in lethality, suggesting copper ion homeostasis is an important measure by which *P. aeruginosa* resists the innate immune responses of its host organisms.
Acknowledgments

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Introduction

Copper Homeostasis

Transition metals have many integral roles in biological processes. Copper, in particular, acts as an important cofactor in many different enzymes found in both prokaryotic and eukaryotic organisms. Crucial enzymatic activity involved in aerobic respiration could not occur without Cu\(^{+}\) binding to the active sites of these enzymes (1). For example, cytochrome c oxidase (also known as Complex IV), a major player in oxidative phosphorylation, requires Cu\(^{+}\) bound to its active site to facilitate transfer of electrons onto their final carrier, oxygen, in the electron transport chain (2). In general, Cu\(^{+}\) is bound to enzymes with oxidative functions to serve as a barrier to harmful free radical molecules that could endanger the cell (3).

Just as Cu\(^{+}\) is crucial for these biochemical processes to occur, it can also be very damaging to a cell if allowed to accumulate. For this reason, cytosolic Cu\(^{+}\) concentration is kept to minimal levels (3). When free Cu\(^{+}\) is allowed to accumulate, these ions, under anaerobic conditions, can attack the iron-sulfur centers of cytosolic proteins via attachment to coordinating sulfur atoms, owing in large part to the affinity of Cu\(^{+}\) for thiolate groups. Aerobically, Cu\(^{+}\) also has the ability to catalyze Fenton-like reactions, which in turn creates hydroxyl radicals that can cause a myriad of deleterious effects in the organism, and ultimately cell death (3, 4, 5).

Due to the importance of Cu\(^{+}\) in cellular function as well as its toxicity when left unsequestered, strict maintenance of Cu\(^{+}\) both inside and outside the cell is crucial. Homeostasis of Cu\(^{+}\) is unique in that it requires a system comprised of high-affinity molecular binding and copper-sensing mechanisms to ensure a low level of Cu\(^{+}\) in the cytoplasm and periplasm at all times (6). Metal distribution to cuproenzymes has been studied at length in Gram-negative bacteria such as Pseudomonas aeruginosa and Escherichia coli, with characterization occurring
primarily through evaluation of copper sensitivity in strains that contain mutations in key homeostatic genes (7). The homeostasis system itself is composed of transporters, regulators, chaperones, and cuproproteins that are involved in both copper influx and efflux (7). A proposed scheme for copper homeostasis in bacteria can be seen in Figure 1. The system itself is fairly conserved among bacteria, both Gram-negative and Gram-positive (7).

**Figure 1: Proposed Mechanism of Copper Homeostasis in Gram-Negative Bacteria.** The drawing represents major systems and not all cuproenzymes are depicted. Colored shapes represent groups of proteins: transporters (lavender), periplasmic chaperones and cuproenzymes (green), membrane cuproenzymes (magenta), Cus system (royal blue), inner membrane transporters (purple), regulators (red), cytosolic chaperones (orange) (6).

**Pseudomonas aeruginosa**

The Gram-negative, rod-shaped bacterium *P. aeruginosa*, shown in Figure 2, is an opportunistic pathogen found in soil, water, and skin flora throughout the world (8). *P. aeruginosa* is the primary cause of bacterial infections in hospitals, owing primarily to the
bacterium’s penchant for infecting compromised immune systems (9). *P. aeruginosa* also possesses extensive and advanced antibiotic resistance mechanisms, making it a species of significant medical relevance (10). Infection typically occurs in the airway, urinary tract, burns, and wounds, in addition to other blood infections (9).

![P. aeruginosa Visualized Using Scanning Electron Microscopy](image)

**Figure 2: *P. aeruginosa* Visualized Using Scanning Electron Microscopy (S. Gschmeissner)**

Pathogenesis in *P. aeruginosa* occurs in a variety of ways. The bacteria can secrete exotoxins that inactivate eukaryotic elongation factor 2, which in turn prevents the host cell from synthesizing proteins and eventually leads to cell death (11). Quorum sensing is also a major mode of pathogenesis, allowing the collective bacteria to regulate the expression of advantageous genes when infecting host cells, such as the production of virulence factors as well as the switch from planktonic growth to biofilm phenotype (12).

In addition to its methods of direct pathogenesis, as mentioned above, *P. aeruginosa* also has several antimicrobial resistance pathways in place that allow it to persist in host organisms. One such mechanism is the aforementioned switch to a biofilm phenotype, which can protect the
bacteria from adverse environmental factors and lower the efficacy of some traditional antibiotic therapies (12). *P. aeruginosa* also possesses the ability to resist antimicrobial innate immune responses generated by the host organism. Following macrophage phagocytosis, the bacteria are exposed to different cytotoxic molecules ranging from oxidative species to transition metal ions, like Cu$^{+}$, so as to induce bacterial death (13, 14). Because of its ability to regulate this copper via the copper homeostasis system, *P. aeruginosa* is able to survive these antimicrobial responses. Whether or not the homeostasis system itself is important for direct pathogenesis remains to be described. This study aims to characterize the copper homeostasis system’s role in *P. aeruginosa* virulence.

**Caenorhabditis elegans**

The free-living nematode *C. elegans* (Figure 3) can be found in temperate soil environments and feed on microbes and decaying matter (15). A majority of the population of these nematodes is comprised of hermaphrodites capable of self-fertilization. Males exist as well, though they are completely dispensable for reproduction (16). *C. elegans* can grow to be over 1 mm long and have an average lifespan of approximately 3 weeks (15). After hatching, *C. elegans* goes through several larval stages that last roughly 48 h before reaching maturity and becoming fertile (15).
Figure 3: *C. elegans* Viewed Under a Microscope. Hermaphroditic nematode is pictured (17).

*C. elegans* is one of the most extensively studied organisms in the world and thus has been deemed a model organism. It was the first multicellular organism to have its entire genome and connectome (neuronal “wiring diagram”) completed (18). These nematodes are also incredibly simple organisms, as they lack respiratory and circulatory systems (19). Additionally, they have a genetically determined, fixed number of cells that does not change from adulthood onward, a phenomenon known as eutely (20). The number of cells does not change following the larval stages, and any subsequent increase in size is solely due to changes in size of the cells (20).

As previously mentioned, *C. elegans* are used extensively in research as model organisms. One particular ability of interest the nematode possesses is its use as a simple, effective model for pathogenesis. In addition to being such a well-defined organism, it has been ascertained that *C. elegans* also employs some of the same innate and adaptive immune responses as humans and other higher organisms (21). As a result, numerous studies have described the susceptibility of *C. elegans* to many of the same bacteria that can infect humans as well, including *P. aeruginosa* (22). This study hopes to make use of this reliable pathogenesis
model to first develop a system of assaying the nematodes against any strain of *P. aeruginosa*. Once this system is established, it will subsequently be utilized to study the effects that mutations in Cu\(^+\) homeostasis genes have on the virulence of *P. aeruginosa*. 
Materials and Methods

Strains

*P. aeruginosa:* Bacterial strains utilized in this study are listed in Table 1. All mutant strains were grown at 37°C in LB medium supplemented with the appropriate antibiotics.

*E. coli:* The *E. coli* strain OP50 was utilized in this study (generously gifted by Dr. Olsen). OP50 is a uracil auxotroph that serves as the primary food source for *C. elegans*. OP50 was grown at 37°C in LB medium.

*C. elegans:* The *C. elegans* strains N2 (WT, generously gifted by Dr. Olsen) and HH142 (obtained from the Caenorhabditis Genetics Center at the University of Minnesota) were used for assay purposes in this study. HH142 is a temperature-dependent strain that is fertile when maintained at 15°C and infertile when maintained at temperatures equal to or exceeding 25°C. This strain was used to bypass the need to transfer *C. elegans* in virulence assays every 24 hr due to egg-laying. *C. elegans* strains were maintained on NGM plates seeded with OP50 *E. coli*. The HH142 strain was maintained at 15°C and the N2 strain was maintained at 20°C. *C. elegans* growth protocols described below were obtained from Dr. Olsen’s laboratory and the WormBook.

Media

*Nematode Growth Medium (NGM):* 3 g of NaCl, 2.5 g of peptone, 3 g of KH₂PO₄, and 0.5 g of K₂HPO₄ were dissolved in 1 L of distilled water. 6.25 g of agar were added to each of four 250 mL aliquots of media and autoclaved. After autoclaving, 250 µL of 1 M MgSO₄, 250 µL of 1 M
CaCl₂, and 250 µL of cholesterol (8 mg/mL) were added aseptically to each 250 mL aliquot of NGM solution. NGM was stored at 4°C.

**1X M9 Buffer, pH ~7.2:** 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 5 g of NaCl, 1 mL of 1 M MgSO₄ were dissolved in 1 L of distilled water. pH was adjusted to 7.2, autoclaved, and stored at room temperature.

**Luria Broth (LB) Media:** 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl were dissolved in 1 L of distilled water, autoclaved, and stored at room temperature.

**LB Agar:** 15.5 g of Luria broth base and 4.5 g of NaCl were dissolved in 1 L of distilled water. 3 g of agar were added to each of four 250 mL aliquots of media and autoclaved. LB agar was stored at room temperature.

**C. elegans Freezing Solution:** 150 mL of glycerol, 2.9 g NaCl, 3.4 g of KH₂PO₄, and 2.8 mL of 1 M NaOH were dissolved in distilled water so as to create a 30 percent glycerol solution. Solution was autoclaved and 150 µL of 1 M MgSO₄ was added aseptically. Freezing solution was stored at room temperature.

**C. elegans Bleaching Solution:** 2.5 mL of bleach, 1.25 mL of 5 M KOH, and 8.75 mL of 1X M9 buffer were combined in a 15 mL Falcon tube.

**Methods**

**Freezing C. elegans Stocks:** Animals on a recently starved 100 mm NGM plate containing a high density of L1-stage *C. elegans* were collected by washing the plate twice with M9 buffer. This suspension was then centrifuged at 1760 rcf for 1 min and the supernatant was discarded. The pelleted *C. elegans* were then resuspended in 3 mL of M9 buffer. 3 mL of *C. elegans*
freezing solution was added and the mixture was then separated into six 1 mL aliquots stored at -80°C.

**Thawing C. elegans Stocks:** A 1 mL aliquot of *C. elegans* was removed from -80°C storage and thawed to room temperature. Mixture was transferred to a centrifuge tube and centrifuged at 1760 rcf for 1 min. Supernatant was discarded and the pelleted *C. elegans* were resuspended in 1 mL of M9 buffer. Mixture was centrifuged again at 1760 rcf for 1 min. Supernatant was discarded, and the pelleted *C. elegans* were once more resuspended in a small volume (~100 µL) of M9 buffer and transferred to an OP50-seeded 100 mm NGM plate.

**C. elegans Bleaching:** An NGM Plate containing desired density of *C. elegans* was washed twice with M9 buffer and the collected suspension was centrifuged at 1760 rcf for 1 min. Supernatant was discarded and pelleted *C. elegans* were resuspended in 12.5 mL of *C. elegans* bleach solution and allowed to disintegrate for 5-7 min with periodic vortexing. Bleaching of the animals present on the plate ensured only those eggs not yet laid survived the process, as the carcasses of the *C. elegans* carrying these eggs protected them from bleaching. Suspension was then centrifuged at 1760 rcf for 1 min and the supernatant was discarded. Pelleted *C. elegans* eggs were resuspended in M9 buffer and centrifuged at 1760 rcf for 1 min (1 wash cycle) for 3 wash cycles. After the final wash, the pellet was resuspended in 4 mL of M9 buffer and allowed to incubate overnight with constant agitation. Incubation temperature was dependent on *C. elegans* strain (see **Strains: C. elegans**).

**Plate Quenching:** Any NGM plates containing *C. elegans* starved of OP50 were washed twice with M9 buffer and the collected suspension was centrifuged at 1760 rcf for 1 min. Supernatant was discarded and the pelleted *C. elegans* were resuspended in 300 µL of M9 buffer and transferred to a freshly-seeded NGM plate containing OP50.
**NGM Chunking:** In order to perform large-scale transfer of *C. elegans*, or “chunking”, a spatula was first sterilized by submerging its flat end in a 70% EtOH solution and heating it over a flame. Once cooled, the spatula was then used to cut out sections of an NGM plate containing a large density of *C. elegans*. This “chunked” section of agar was then placed upside down onto a new OP50-seeded NGM plate and the *C. elegans* were allowed to migrate to the new plate overnight before removing the chunked agar.

**Seeding NGM plates:** The bacterial culture of choice and accompanying NGM plates to be seeded were both prepared one day prior (see *Pouring Agar Plates* and *Preparing Liquid Bacterial Cultures*). NGM plates were seeded by pipetting liquid bacterial culture directly onto the plate (50 μL of culture for a 60 mm plate and 100 μL for a 100 mm plate). For all 100 mm plate seedings, sterile culture beads were used to evenly distribute the bacterial lawn on the surface of the agar. Distribution was not necessary for 60 mm plates. Seeded plates were allowed to incubate at room temperature overnight prior to use.

**Preparing Liquid Bacterial Culture:** Bacterial culturing was performed aseptically in its entirety. A single colony from a bacterial culture grown on LB agar was first picked and transferred to a sterile culture tube containing LB media (5 mL of media for all *P. aeruginosa* strains and 3 mL of media for OP50 *E. coli*). The appropriate working concentration of antibiotic was added to the LB media prior to transfer of bacterial colony (antibiotics were not necessary for OP50 cultures). The liquid culture was then allowed to incubate overnight at 37°C with constant agitation. Liquid cultures were then utilized for stock creation or NGM plate seeding.

**Preparing Bacterial Stocks:** Bacterial stock creation was performed aseptically in its entirety. Following incubation of a liquid bacterial stock (see *Preparing Liquid Bacterial Culture*), 700
µL of bacterial culture was added to each of two cryogenic vials containing 300 µL of a 50 % glycerol solution, creating a 15 % glycerol bacterial stock. All stocks were stored at -80°C.

**Thawing Bacterial Stocks:** For thawing bacterial stocks into liquid media, the frozen bacterial stock of choice was placed on ice and a culture pick was used to scrape a small amount of thawed culture onto the tip of the pick. This culture was then transferred to LB media and grown as described in **Preparing Liquid Bacterial Culture**, however no antibiotics were added to the media. For thawing bacterial stocks onto solid agar, the same process was followed, only the frozen culture was streaked onto an LB agar plate with no antibiotics and allowed to incubate at 37°C overnight prior to use.

**Agar Plate Pouring:** Plate pouring was performed aseptically. A 250 mL aliquot of either NGM or LB agar was microwaved for 4 min or until all solid agar had liquefied. Any necessary antibiotics were added to the agar while still hot and gently swirled to mix so as to not form any air bubbles. Once the agar had cooled just enough to hold, it was poured by hand into either 60 mm or 100 mm Petri dishes. The poured plates were allowed to dry and solidify and were stored at room temperature.

**C. elegans Virulence Assay:** One day prior to start of assay, 60 mm NGM plates were seeded with the bacterial strains to be tested (OD₆₀₀ of the liquid culture used to seed each plate were ensured to be above 2.00) and *C. elegans* were bleached and allowed to incubate overnight (see **C. elegans Bleaching**). An appropriate volume of the *C. elegans* and M9 buffer mixture was determined so that there were approximately 30 to 40 L1-stage *C. elegans* per plate, and each plate to be tested was seeded with *C. elegans* by pipetting the aforementioned volume directly onto the plate. Initial worm count per plate was recorded and all plates were allowed to incubate at 25°C over the course of several days. Counts on the number of living and deceased *C. elegans*
were recorded every 12 to 24 hr. *C. elegans* were considered deceased when they no longer responded to touch stimuli, and all deceased *C. elegans* were subsequently removed from their plates. Each bacterial strain tested was performed in triplicate.

**Table 1: *P. aeruginosa* Strains Utilized in This Study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA01</td>
<td>Wild Type</td>
<td>This study</td>
</tr>
<tr>
<td>ISphoA</td>
<td>ΔcopA1</td>
<td>Jacobs et al. (23)</td>
</tr>
<tr>
<td>ISphoA</td>
<td>ΔcopA2</td>
<td>Jacobs et al. (23)</td>
</tr>
<tr>
<td>ISBamHI</td>
<td>ΔcopZ1</td>
<td>Novoa-Aponte and Argüello</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unpublished results</td>
</tr>
<tr>
<td>ISPvuII</td>
<td>ΔcopZ2</td>
<td>Novoa-Aponte and Argüello</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unpublished results</td>
</tr>
<tr>
<td>lacZwp03q2F01, phoAwp08q2B07, lacZwp02q3E10</td>
<td>ΔcueR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Held et al. (24)</td>
</tr>
<tr>
<td>lacZwp05q2G04, lacZwp04q1A12</td>
<td>Δccoa</td>
<td>This study</td>
</tr>
<tr>
<td>phoAwp01q3H10, phoAwp09q4G04</td>
<td>ΔoprC</td>
<td>Nuñez Mir unpublished results</td>
</tr>
<tr>
<td>lacZwp09q3F06</td>
<td>ΔcuiT</td>
<td>Nuñez Mir unpublished results</td>
</tr>
</tbody>
</table>
Results

Using *C. elegans* as a pathogenesis model required the establishment of a system for cultivating the worms as well as the development of an assay to test *P. aeruginosa*’s virulence. The former was accomplished through optimization of protocols, graciously provided by the laboratories of Professor Olsen and Professor Rao, to suit the needs of our experiments. We were able to grow, maintain, store, and assay *C. elegans* simultaneously using these modified protocols. Evidence of healthy growth of *C. elegans* in our lab can be observed in Figure 4. Maintenance protocols used were described in Methods.

![Image: HH142 Strain C. elegans Cultured in This Study. C. elegans were grown on NGM plates seeded with OP50 E. coli.]

Creation of an assay method for these experiments required similar adaptation. Tan et al. (22) described a kill curve assay using *C. elegans* as an infection model for *P. aeruginosa*. This method was used as the framework for the development of the kill curve assay utilized in these experiments. Use of N2 *C. elegans* was quickly halted, as assays were found to be cumbersome owing to the proclivity for egg laying the strain displays, which in turn jeopardized accurate measurements of the worms if daily transfers to new plates were not performed. In its place,
HH142 strain *C. elegans* were employed, as their fertility to could be manipulated using simple temperature modulation. The adapted kill curve assay, described in *C. elegans Virulence Assay*, was utilized for all subsequent experiments.

The HH142 strain was tested against PA01 *P. aeruginosa* and the bacteria displayed complete lethality in a very consistent 192-hour timeframe (Figure 5). Lethality measurements yielded a sigmoidal curve to the following equation:

\[
y = 100 + \frac{-100}{1 + \left(\frac{x}{K_{1/2}}\right)^m}
\]

The variable \(m\) represents an arbitrary constant, while \(K_{1/2}\) refers to the time required for 50 percent lethality to be observed. This \(K_{1/2}\) was used in all subsequent experiments to characterize virulence.
Figure 5: *P. aeruginosa* is Virulent in HH142 Strain *C. elegans* and This Virulence is Comparable to That of N2 strain *C. elegans*. (Left) PA01 *P. aeruginosa* tested against HH142 *C. elegans* produces sigmoidal curve that $K_{1/2}$ can be calculated from (Right) Both HH142 and N2 strain *C. elegans* produce similar sigmoidal curves. OP50 *E. coli* was used as a negative control. Mean value of data points displayed ± standard error, with an n=3.

While HH142 *C. elegans* were proven to be susceptible to *P. aeruginosa*, it was unclear whether the observed virulence was consistent with that of N2 *C. elegans*. In order to justify use of the mutant HH142 strain, an assay of N2 *C. elegans* was performed. As expected, there was little difference between the two strains in terms of their susceptibility to *P. aeruginosa* (Figure 5). This confirmed that HH142 *C. elegans* were viable replacements for N2 *C. elegans* for the purposes of these experiments.

After development of a reliable kill curve assay, the focus of the experiments shifted towards the virulent effects of transposon mutant *P. aeruginosa* strains on *C. elegans*. Specifically, those mutants pertaining to the bacteria’s copper homeostasis system were investigated. This system, also present in many other species of bacteria, is composed of a myriad of genes, as can be seen in Figure 6. The genes chosen for study were selected based on their level of current characterization and their presumed relevance to the function of the copper homeostasis system. The list of transposon mutants tested can be seen in Table 1.
Figure 6: Genes Involved in Copper Homeostasis for *P. aeruginosa*. Genes marked with a red rectangle indicate those transposon mutants tested via kill curve assay. For the purposes of this study, *PA5030* is synonymous with *cuiT* (23).

Experiments were first performed on the copper chaperone mutants *ΔcopZ1* and *ΔcopZ2*. The $K_{1/2}$ of both mutants was shown to be slightly less than that of wild type *P. aeruginosa*. Interestingly, both mutants displayed strikingly similar kill curves (Figure 7A). The copper importer ATPases mutants *ΔcopA1* and *ΔcopA2* were next tested for virulence. *ΔcopA2* showed a moderate decrease in lethality. Surprisingly, however, *ΔcopA1* displayed near comparable virulence to the wild type (Figure 7B). The efflux transporter mutants *ΔoprC, ΔcuiT, and ΔccoA* all showed a general trend of decreased virulence in *C. elegans. ΔoprC* and *ΔcuiT* were particularly nonvirulent, as the lethality of both mutants was even less than the negative control OP50 *E. coli* (Figure 7C). Finally, the mutated regulator *ΔcueR* was tested and, as expected, displayed a decreased virulence as compared to the wild type (Figure 7D).
Figure 7: Virulence of Copper Homeostasis Mutants in C. elegans. (A) Cu$^+$ chaperone mutants ΔcopZ1 and ΔcopZ2 showed slightly decreased virulence (B) Decreased virulence can be seen in the effluxer ΔcopA2, though there appears to be a comparable virulence to wild type for ΔcopA1 (C) Importer mutants ΔoprC, ΔcuiT, and ΔccoA displayed decreased virulence, with ΔoprC and ΔcuiT displaying an almost complete loss (D) The mutated regulator ΔcueR showed decreased virulence. OP50 E. coli was used as a negative control. Mean value of data points displayed ± standard error, with an n=3.
**Discussion**

One of the primary goals of this study was the establishment of a system for testing virulence in various mutant strains of *P. aeruginosa*. The selection of *C. elegans* as a model for this virulence had multiple contributing factors. They are inexpensive and much easier to maintain than traditional animal models such as mice. Additionally, the virulence assays performed on the nematodes could be executed in a much shorter time frame than other models like plants (*Arabidopsis thaliana*). These factors made *C. elegans* the ideal model. The system itself is new to our laboratory, and optimizations of the process in conjunction with the ones already made could make the process even more efficient. Lastly, the kill curve assay developed is not limited to *P. aeruginosa* strains and can modified as seen fit. Other bacterial strains can be tested using this system, though small modifications will most likely need to be made on a case-by-case basis.

Wild type *P. aeruginosa* has been proven to be virulent in *C. elegans* in many studies in the past (22). However, these studies often make use of N2 *C. elegans* and, to our knowledge, the HH142 strain utilized in our experiments had not been characterized as such. It was unclear based on the literature whether or not this particular strain had exhibited any bacterial resistance. Using the newly-designed kill curve assay, it was confirmed that not only are HH142 *C. elegans* susceptible to wild type *P. aeruginosa*, they also display a similar virulence pattern as wild type *C. elegans*, thereby validating their use (Figure 5).

The virulence of the transposon mutants of *P. aeruginosa* listed in Table 1 were then tested against HH142 *C. elegans*. The mutated genes possessed by these strains were selected, as previously mentioned, based on their potential importance to functionality of the copper homeostasis system with *P. aeruginosa*. What was observed amongst the mutants tested was a
general trend of decreased virulence. This suggests that, while the copper homeostasis system in bacteria may not be directly responsible for any virulent behavior, it is important for allowing the bacteria to survive antimicrobial responses by the host organism so other virulence pathways can take effect.

One exception to the trend described above was virulence pattern observed in the ΔcopA1 mutant. This strain displayed a comparable virulence to wild type P. aeruginosa (Figure 7B). This was surprising, as previous studies in our lab had described copA1 as having an essential role in virulence pathways (7). The CopA1 protein is expressed in response to high Cu\(^{\text{+}}\) and is responsible for maintaining the cellular Cu\(^{\text{+}}\) quota and provides the bacteria with tolerance to the transition metal (7). This conflicting data requires further investigation.

The virulence patterns of the ΔcopZ1 and ΔcopZ2 mutants are strikingly similar, with near-identical K\(_{1/2}\) values (Figure 7A). The CopZ1 and CopZ2 chaperones both shuttle Cu\(^{\text{+}}\) around the cytoplasm to different membrane Cu\(^{\text{+}}\)-ATPases (Novoa-Aponte and Argüello unpublished results). Although they are described as having distinct targets, the similarity in virulence for both knockouts may suggest the presence of a degree of redundancy in the chaperone system. That is, deactivation of one chaperone may cause the other to compensate in its place, albeit with a decrease in virulence. Future studies with a double mutant knockout of copZ1 and copZ2 may offer more insight into this phenomenon.

The transporters oprC and cuiT are down-regulated during a Cu\(^{\text{+}}\) response (Figure 6). Assuming the antimicrobial response being initiated by C. elegans involves the exposure of bacteria to transition metal ions, it was somewhat surprising to observe a near absence of virulence when these genes are mutated, given they have a very limited role in copper homeostasis in the presence of excess Cu\(^{\text{+}}\) (Figure 7C). This points to a possible role in other
virulence pathways separate from the copper homeostasis system. These proteins are known to transport Cu\(^+\) to other cuproproteins (25). One such cuproprotein in particular is superoxide dismutase (SOD), an important enzyme in most organisms that is responsible for the eradication of reactive oxygen species (ROS) (26). Disfunction in either of these transporters could result in a Cu\(^+\) deficiency SOD and an inability to process ROS by the cell. Much in the same way as exposure to excess Cu\(^+\) is utilized as an antimicrobial response, so too can the host organism use ROS to combat infecting bacteria. Thus, mutation of oprC and cuiT could potentially be debilitating to the bacteria’s ability to adapt to host immune responses.

The assay itself, as currently constituted, does have its limitations. Phenotypical analysis of the mutated genes can only give so much insight into the biochemical processes and physical interactions at work. A reporter gene such as GFP would help visualize where the *P. aeruginosa* mutants were localizing during a *C. elegans* infection. The use of small interfering RNAs (siRNAs) for repressing immune response genes in *C. elegans* may also help to gain a better understanding of the role of copper homeostasis, or other systems, in virulence pathways in *P. aeruginosa*. Regardless, the assay system and results described in this report offer a simple and effective method for characterizing host-pathogen interactions.
References


