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Role of CcoA and HmtA in Pseudomonas aeruginosa

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Role of CcoA and HmtA in *Pseudomonas aeruginosa*

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ABSTRACT

Copper homeostasis is unique as it requires high-affinity binding with molecules and copper sensing mechanisms to ensure a low level of free copper in both the cytoplasm and the periplasm. Though high levels of copper in living systems do not appear frequently, it is important that organisms tightly control copper concentration and strictly maintain copper homeostasis through transmembrane transporters and metallochaperone proteins. While the cellular copper efflux mechanism through the P-type ATPases has been studied, the process of copper uptake into the cytoplasm remains unclear. To understand how copper enters the cell, we conducted an experimental research on two potential copper importers CcoA (PA1848) and HmtA (PA2435) in this project. We also worked on characterizing their particular role played in copper transport through phenotypical and functional analyses, including metal sensitivity test, redox stress test, and copper tolerance test in both excess and deficient copper environments.

ACKNOWLEDGEMENTS

I would like to thank Professor José Argüello for giving me the opportunity to work in his lab during the past four years and providing me with this great research project that has allowed me to learn, think, and work as a scientist. I also need to give all my thanks to my supervisors, Dr. Julia Quintana Gonzalez and James McIsaac, for teaching me not only lab techniques, but also how to be a biochemist. Finally, I would like to thank Sarju Patel and Joshua Hall, whom I have worked alongside in the lab. I owe so much to these people. Their support and guidance have and will be invaluable to me as I move forward in my education and career.

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1. INTRODUCTION

Copper is an essential micronutrient required as a prosthetic group in enzymes (1). It functions in many biochemical processes, such as oxidative phosphorylation and photosynthesis in photosystem II located in the thylakoid membrane of chloroplasts in plants (1). The most common use of copper is binding to oxidative enzymes, which protect both cells and the bloodstream from adventitious free radicals (1, 2). Since the accumulation of copper in the cell is poisonous to all forms of life, free copper in the cytoplasm is normally maintained at a very low level (10^{-15} M) (1, 2). Under primarily anaerobic conditions, free copper can attack intracellular iron-sulfur centers of various proteins, attaching to the coordinating sulfur atoms due to its high affinity for thiolates (2, 3, 4). In aerobic conditions, it can also catalyze a Fenton-like reaction ($Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + HO^{\bullet} + OH^{-}$, $Cu^{2+} + O^{\bullet} \rightarrow O_2 + Cu^{+}$) (2) producing hydroxyl radicals, which can cause lipid peroxidation, protein oxidation, and DNA damage, which lead to membrane permeability and cell death (3, 4). Though high levels of copper in living systems do not appear frequently, it is important that organisms tightly control copper concentration and strictly maintain copper homeostasis through transmembrane transporters and metallochaperone proteins to prevent toxic effects and ensure the distribution required for cuproprotein synthesis (5).

Copper homeostasis is unique as it requires a high-affinity binding with molecules and copper sensing mechanisms to ensure a low level of free copper ions in both the cytoplasm and the periplasm (6). This process of metal distribution to cuproenzymes has been studied in gamma-proteobacteria (Gram-negative), such as *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Escherichia coli (*6, 7, 8). The copper homeostatic mechanisms were primarily characterized by evaluating copper tolerance and sensitivity in strains carrying mutations in key components of the homeostatic system (7). Cuproproteins, working together with sensors, transcriptional regulators,

transporters chaperones, and chelators, function and participate in both the influx and efflux system of copper transport (7). A copper homeostatic model has been proposed for a Gram-negative bacterial cell (Figure 1) (6) and defense mechanisms against excess copper are rather conserved in most Gram-negative and even Gram-positive organisms (1).

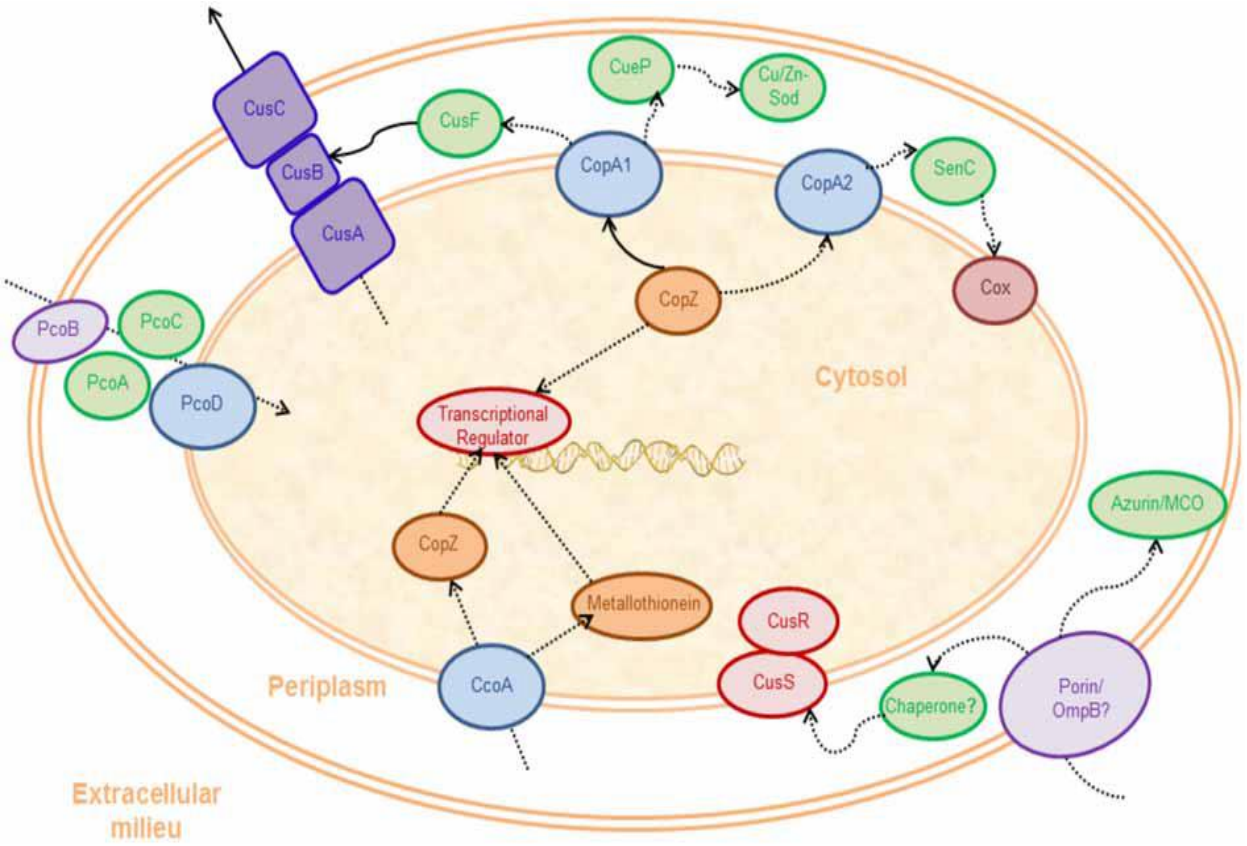


Figure 1. Scheme of systems participating in copper homeostasis in a Gram-negative bacterial cell. The drawing represents the major systems, and not all cuproproteins are depicted. Experimentally verified copper ion transfer and transport events are indicated with solid lines. Postulated copper fluxes are indicated with dotted lines. The colored shapes represent groups of proteins (various transcriptional regulators, various chaperones, etc.): Lavender: putative outer membrane transporters, green: periplasmic copper chaperones and cuproproteins, magenta: membrane cuproproteins, royal blue: Cus system, purple: inner membrane transporters, red: transcriptional regulators, orange: cytosolic copper chaperones. (6)

Nonetheless, there are still several questions to be answered, and some nodes of the models to be functionally characterized. For example, it is not yet fully understood how copper ions enter the cell.

The identification and functional characterization of the different proteins involved in maintaining copper concentration and mediating copper transportation will further our understanding of the copper homeostatic mechanisms (6, 7). The proposed system in Figure 1 has suggested that cytoplasmic copper-chaperones acquire copper ions from influx transporters and then distribute copper ions to efflux transporters, cuproproteins, and transcriptional regulators (6). Although the cellular copper efflux mechanism through the P-type ATPases has been studied (7), the process of uptaking copper into the cytoplasm remains unclear. Based on previous analyses of conserved sequences and phenotypical performances (7, 9), we hypothesize that CcoA (PA1848) and HmtA (PA2435) are potential copper influx transporters. We propose that the absence of the gene *ccoA* and *hmtA* in the mutant strains will lead to phenotypical and functional characteristics different from the wild-type strain under deficient and excess copper concentrations. Expression, separation, and purification of corresponding proteins can be conducted to understand the acquisition and distribution of copper by these potential transmembrane importers. This work aims to contribute to the characterization of two candidate cytoplasmic influx transporters, CcoA and HmtA, by analyzing mutant strains from *P. aeruginosa PAO1*, which lack the corresponding genes.

1.1 Pseudomonas aeruginosa

P. aeruginosa, a Gram-negative, rod-shaped bacterium that grows in soil, wetlands as well as on plant and animal tissues, belongs to the class of Pseudomonadaceae (10). For burn victims and catheterized patients with urinary tract infections or pneumonia, *P. aeruginosa* can be dangerous and life-threating because of its natural resistance to antibiotics causing the infection that leads to pulmonary failure and death (10, 11). It has been observed that *P. aeruginosa* has two

transmembrane copper transport ATPases, P_{1B}- type ATPases CopA1 and CopA2, in its copper homeostasis system (7, 12). It has also been demonstrated that *P. aeruginosa* can actively grow in the presence of elevated copper (13). Due to recent advances in whole-genome sequencing, *P. aeruginosa* has become an important tool to study and understand the uptake and the release of metal micronutrients, particularly in this project, to further the study of copper homeostasis.

1.2 Copper Efflux Transporters

Copper efflux transporters, Cu^+-ATPases, are responsible for cytoplasmic copper efflux in a bacterial system, which occurs in the presence of ATP when cytoplasmic copper-attached chaperones deliver the ion to the two-transmembrane transport sites (12). Cu^+-ATPases play key roles in copper homeostasis by participating in copper detoxification and cuproproteins assembly (7, 12). They are constituted by the eight transmembrane segments where transport metal binding sites are located and two Cys in H6 and four additional residues (a Tyr, an Asn, a Thr and a Ser) in H7 and H8 form two specific copper binding sites (Figure 2) (6, 13).

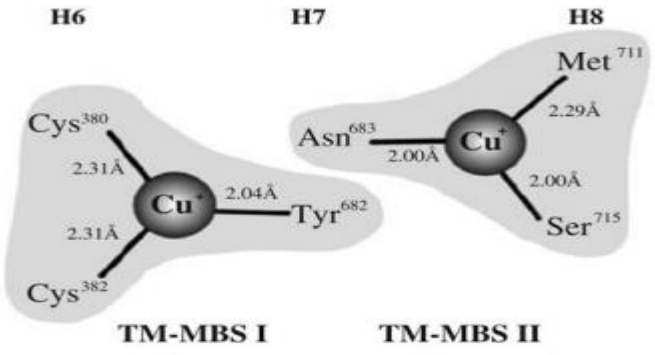


Figure 2. Scheme showing amino acid forming the transmembrane copper binding sites I and II. (6)

Following the catalytic phosphorylation of these metal binding enzymes, copper is translocated to the periplasmic region or extracellular milieus (1, 7). When the metal is released, the enzyme returns to the initial conformation while completing the classical E1/E2 Albers-Post cycle (Figure 3) (14), which describes the transport mechanism of all P-type ATPases. In the Albers-Post cycle mechanism, the enzyme adopts two major confomations E1/E2 in the absence of ATP (14).

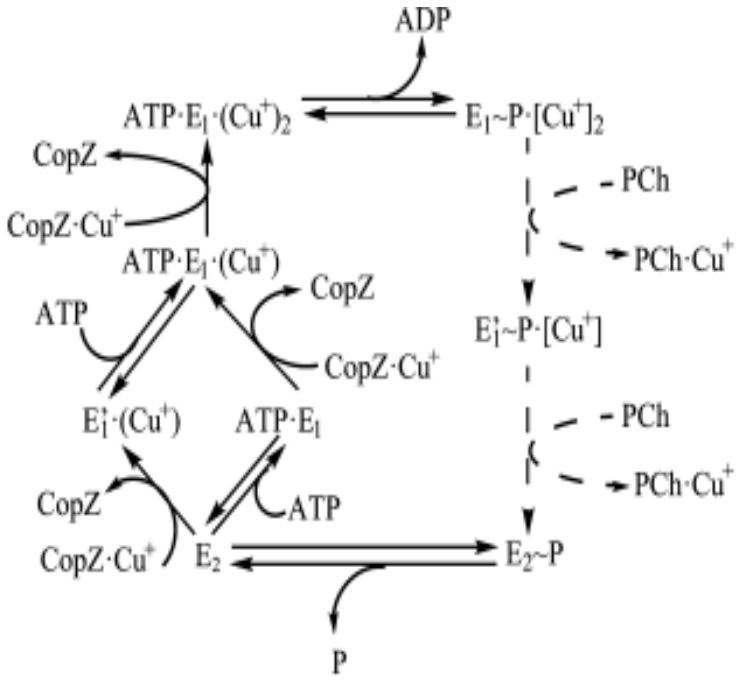


Figure 3. Scheme showing the classical E1/E2 Albers-Post cycle: Catalytic and transport cycle of Cu^+-ATPases. (14)

González-Guerrero el at. have studied the two Cu^+-ATPases, CopA1 (PA3920) and CopA2 (PA1549), present in *P. aeruginosa* (PAO1) (7). The mutation of *copA1* causes a lack of copper tolerance and virulence while the mutation of *copA2* leads to a decreased oxidase activity of the cell (7). Although two Cu^+-ATPases have identical transmembrane metal binding sites and two cytoplasmic metal binding domains in their N terminals, each of the ATPases plays distinct cellular functions and dynamic characteristics because of different transcriptional regulation or other factors (7). While both Cu^+-ATPases drive cytoplasmic Cu^+ efflux, these two *P. aeruginosa*

copper ATPases have been proved to be functionally inequivalent and to play distinct physiological roles.

1.3 Copper Influx Transporters

Little is known about how most bacteria acquire copper (1). Although several exporters have been identified, metal importers have almost entirely eluded researchers. Only one copper-dependent importer has been identified in eukaryotes, the Ctr (Cu transporter) proteins located in the plasma membrane (15, 16). Homologues of Ctr proteins have not been found in bacteria (16). Some studies have suggested that P_{1B}- type ATPases might work as copper importers, yet all found P_{1B}- type ATPases export metals from the cytoplasm (12, 17).

1.4 CcoA (PA1848)

CcoA (PA1848), probable member of the major facilitator superfamily transporter, is proposed to function as a metal influx transporter (Pseudomonas Database). According to the Pseudomonas Database, its classification as influx transporter is based on the presence of its conserved amino acid motif, structural feature, and the sequence. CcoA protein has a molecular weight of 40.8 kDa (Pseudomonas Database). According to Figure 1 (6), the scheme of the system participating in copper homeostasis in Gram-negative bacterial cell, CcoA was proposed to import copper into the cytoplasm (6). This novel transporter has been recently discovered because of its necessity for the metalation of *cbb3-COX* and *cbb3* assembly in *Rhodobacter capsulatus* (14). *R. capsulatus* CcoA is the only bacterial copper importer that has been documented (6). Similar to other functional major facilitator superfamily transporters, the study has predicted that CcoA has twelve transmembrane helices, which are divided into two subdomains of six helices each and separated by a large cytoplasmic loop (6). The conserved motif, mentioned previously, includes

several transmembrane Met rich regions perhaps associated with copper binding (6). Mutation of *R. capsulatus* ccoA leads a decrease in the total copper content of *R. capsulatus* and a reduced ability to assemble cytochrome oxidase (19). Though few studies (6, 19) have been conducted to analyze the function of copper influx transporters to identify their roles in copper homeostasis, a focused study on CcoA can provide particular knowledge and further understanding towards the complete transport scheme particularly in *P. aeruginosa*.

1.5 HmtA (PA2435)

HmtA (PA2435) has been characterized as one of the heavy metal translocating P-type ATPases in *P. aeruginosa* (Pseudomonas Database). Some P-type ATPases in *P. aeruginosa* (PAO1) have been functionally characterized (9). They are putative ion specific ATPases, including PA1429 (Ca^{2+}), PA4825 (Mg^{2+}), PA1634 (K^{+}), and PA2435 (Zn^{2+} and Cu^{2+}) (9, 20). Experimental evidence has demonstrated that HmtA, heavy metal transporter A, transports copper and zinc (20). The deletion of the chromosomal copy of the gene coding for HmtA resulted in a *P. aeruginosa* strain that was mildly more resistant to copper and zinc, which potentially suggested its involvement in metal uptake (9, 20). However, when the amino acid sequences were aligned, the transmembrane motif of HmtA was found to contain the same conserved sequences of both Zn^{2+}/Cd^{2+} and Cu^{+}/Ag^{+} exporters, such as *A. fulgidus* CopA, *R. radiobacter* CopA1, and ZntA within either N or C terminals (9). Hypothesized as a potential P-type ATPases for copper import, the essential functions of HmtA (PA2435) are still under discussion and remain uncovered.

In order to further understand copper homeostasis in bacteria, it is important to answer the biological question of how copper enters into the cell. Conducting an experimental research on CcoA and HmtA can probably provide the solution to the question. Using *P. aeruginosa* as an

efficient tool, this project conducts the study on these two potential copper importers and to characterize their specific roles in copper transportation through phenotypical and functional analyses. Growth curves of cultures grown in excess and deficient copper environments, metal sensitivity assays, and redox stress assays were designed and conducted to understand the difference between *CcoA* and *HmtA* specific gene mutated strain and wild-type strain. Gene specific primers were designed in order to clone, transform, purify, and express the proteins for the copper binding and accumulation assays. This report provides experimental data and a basic understanding of the roles of CcoA and HmtA through phenotypic and functional performance in absence and presence of these two genes in copper homeostasis of the bacteria *P. aeruginosa*.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 *Pseudomonas aeruginosa* Strains

Pseudomonas aeruginosa PAO1 wild type strain, ΔcopA1, ΔPA1848 (*CcoA*), and ΔPA2435 (HmtA) insertional mutant strains were obtained from the Comprehensive *P.aeruginosa* Transposon Mutant Library at the University of Washington Genome Center (7). All four strains were confirmed by colony PCR. Cells were grown aerobically at 37°C in Lysogeny broth (LB) or minimal media (13, See Appendix). Wild type strain was supplemented with 100 µg ml^{-1} ampicillin while mutant strains were treated with 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} tetracycline.

2.1.2 *Escherichia coli* Strains

For routine cloning applications, the experiment used *E. coli* strain TOP10: F-*mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ (*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) endA1 *nup*G. They were grown in LB medium (1% Triptone, 0.5% Yeast Extract, 1% NaCl) at 37ºC with 200 rpm shaking. For protein expression, the experiment used *E. coli* strain BL21 (DE3) pLysS: *E. coli* B F^{-} *DCM ompT hsd*S (r_B^{-} m_B^{-}) gal λ (DE3) [pLysS]. They were grown in LB medium at 37ºC with 200 rpm shaking.

2.1.3 Primers

Primers were designed and ordered from Sigma Aldrich. Stocks were stored as 100 µM solutions in distilled water at -20°C. Working stocks were prepared at 10 µM concentration from the original stocks. All primers used in this project are listed below in Table 1.

Table 1. List of primers used in this Report. They are organized by order of appearance in the project. The amplified products and the corresponding annealing temperature are also specified.

Name	Sequence (5'-3')	Amplified product
HmtA_F Nde I	CTTCATATGAGCGAGCCGACCGCCCAC	*hmtA*
HmtA_R Hind III	CATAAGCTTGGACTGAAAATACAGGTTTTC GGCAGCAGCCGGTGTACCCTCCTCTTCGCG TCCCTCCACGGCCGG	
CcoA_F Nde I	CTTCATATGCTGCTACGCAACCCGACCCTG	*ccoA*
CcoA_R Hind III	CATAAGCTTGGACTGAAAATACAGGTTTTC GGCAGCAGCGCGCGACAGCGTC	

2.2 Methods

2.2.1 Bacterial Methods

Metal Sensitivity Test

A. Growth under a deficient copper environment

The LB or minimal liquid media cultures were again inoculated at 0.1 OD_{600} with overnight cultures of the various *P. aeruginosa* strains; half of the cultures were supplemented with 0.5 mM Bathocuproine Disulfonic Acid Disodium Salt (BCS) (Sigma-Aldrich, Sigma-B1375). Cells were grown for 16 hours and OD_{600} was measured.

B. Growth under an excess copper environment

The LB liquid media cultures were inoculated at 0.1 OD_{600} with overnight cultures of the various *P. aeruginosa* strains and supplemented with the desired $CuSO_4$ concentration as indicated in the figures (1 mM, 2 mM, 4 mM, and 6 mM). Cells were grown 16 hours and OD_{600} was measured. All experimental data were obtained from a total of three biological replicates, where each replicate included three technical repeats.

Metal Tolerance Test (Disc Diffusion Assay)

The LB liquid cultures were inoculated at 0.1 OD_{600} from overnight cultures. When cultures reached 0.5 OD_{600}, 100 µL were added to 4 mL soft agar (LB with 50% agar supplemented with 100 µg ml^{-1} ampicillin for wild type strain and with 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} tetracycline for mutant strains) and poured into LB agar plates (no antibiotics). While soft agar solidified, 10 µL 0.8 M, 1 M, or 2 M $CuSO_4$ or $ZnSO_4$ solution were added to 6 mm filter discs. When the discs absorbed all the solution, they were placed on the plate. Plates were incubated in 37°C for 16 or 24 hours. The diameters of circular inhibition zones were measured.

H_2O_2 Sensitivity Test

The LB liquid cultures were inoculated at 0.1 OD_{600} with overnight cultures of the various *P. aeruginosa* strains. When 0.6 OD_{600} was reached, cultures were split, a half was supplemented with 30 mM H_2O_2 (Fisher Chemical) for 15, 30, and 45 minutes and the other left untreated. 10 µL cultures were placed on LB agar plates and were grown at 37°C overnight. After the treatment, cell survival was enumerated using viable cell count. Survival rate was estimated as the ratio of number of cells in the presence and the absence H_2O_2 (13).

Tert-Butyl Hydroperoxide Sensitivity Test

The LB liquid cultures were inoculated at 0.1 OD_{600} with overnight cultures of the various *P. aeruginosa* strains. When an OD_{600} value of 0.6 was reached, cultures were split, a half was supplemented with 100 mM Tert-Butyl Hydroperoxide (Sigma-Aldrich, B2633) for 30, 60, 90, and 120 minutes and the other left untreated. 10 µL cultures were placed on LB agar plates and were grown at 37°C overnight. After the treatment, cell survival was enumerated using viable cell count. Survival rate was estimated as the ratio of number of cells in the presence and the absence Tert-Butyl Hydroperoxide.

Pyoverdin (PVD) Measurements

PVD from culture supernatants at $T_{16hours}$ was measured at OD_{405} normalized by the cell density of bacterial cultures (OD_{600}) using a spectrophotometer (21).

2.2.2 Nucleic Acid Methods

Standard PCR

Four *P. aeruginosa* strains used in the experiment were confirmed by PCR, using the *Taq* DNA polymerase system. Genomic DNAs and mutant strains' DNAs were resuspended in 10 µL of sterile water and used as DNA templates for PCR. PCR reactions were performed in a final volume of 25 µL containing 1 µL of each dNTP, 1 µL of each primer, 0.125 µL of *Taq* DNA polymerase, and 2.5 µL of 10X reaction buffer (1X is 75 mM Tris-HCl pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM ammonium sulfate). Amplification cycles consisted of an initial step of 5 minutes at 95ºC, followed by 30 cycles of 1 minute at 95ºC, 1 minute at 55ºC, 2 minutes 30 seconds at 68ºC, with a final extension step of 6 minutes at 68ºC.

Amplification of *CcoA* and *HmtA*

PCR amplifications of *CcoA* and *HmtA* were first performed with the *Taq* DNA polymerase system. Genomic DNA were resuspended in 10 µL of sterile water and used as DNA template for PCR. PCR reactions were performed in a final volume of 25 µL containing 1 µL of each dNTP, 1 µL of each primer, 0.125 µL of *Taq* DNA polymerase, and 2.5 µL of 10X reaction buffer (1X is 75 mM Tris-HCl pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM ammonium sulphate). Amplification cycles consisted of an initial step of 5 minutes at 95ºC, followed by 30 cycles of 1 minute at 95ºC, 1 minute at 55ºC, 2 minutes 30 seconds at 68ºC, with a final extension step of 6 minutes at 68ºC. After obtaining positive amplification results of *CcoA* and *HmtA*, high fidelity Q5 polymerase system was used in *CcoA* and *HmtA* amplification. The Q5 Amplification cycles consisted of an

initial step of 8 minutes at 95ºC, followed by 30 cycles of 1 minute at 95ºC, 30 s at 63ºC, 1 minute at 72ºC, with a final extension step of 3 minutes at 72ºC.

PCR products were purified using NucleoSpin Gel and PCR Purification Kit and Zyppy Plasmid Miniprep Kit. Amplified genes were cloned into the pET-30b (+)/ His vector (Invitrogen, Carlsbad, CA, USA) and transformed into *E. coli* BL21 (DE3) pLysS, following manufacturer instruction. Positive transformants were tested by colony PCR before sequencing.

2.2.3 Protein Methods

Bacterial transformation

The DNA concentrations of PCR purified products were checked by NanoDrop 2000c (Thermo Scientific) and ligated into vectors using 1:3 and 1:6 ratio per 10 µL reaction at room temperature overnight. For each transformation, 4 µL of ligation products was added to 100 µl of chemically competent TOP10 *E. coli* cells and incubated on ice for 30 minutes, followed by heat shock at 42°C for 45 seconds and incubation on ice for 10 minutes.

The cells were allowed to recover in 0.75 ml LB liquid media with Kanamycin and then incubated for 1h at 37°C with shaking (200-250 rpm). Cell pellets were spun down and resuspended in 100 µL LB liquid media. Cells were plated on LB-agar plates containing appropriate antibiotics and incubated at 37°C overnight to select the transformed colonies.

Selected colonies were grown and checked with PCR, followed by sequencing for gene verification. Positive results with the corresponding ligation products were transformed into competent cell *E. coli* BL21 (DE3) pLysS.

Protein Expression

Transformed *E. coli* BL21(DE3) pLysS were grown in 5 mL overnight cultures with 15 µg ml^{-1} kanamycin and 34 ug ml^{-1} chloramphenicol, which diluted to 0.1 OD_{600} in 25 mL

flask. When the cell cultures grew to 0.6 OD_{600}, they were induced by IPTG (100mM original Isopropyl β-D-1-thiogalactopyranoside stock to obtain 1 mM in 5 mL cultures) and incubated 3 hours after induction under different temperature at 37°C. 1 ml of the culture was centrifuged for pellets and stored in -20°C.

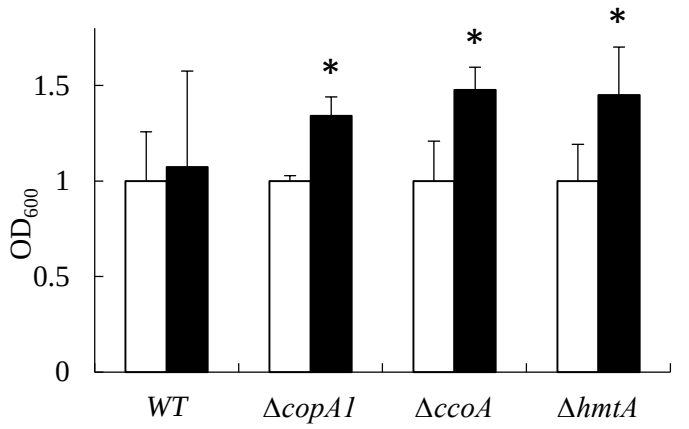
Dot Blot

Dot Blot experiments were performed to demonstrate the expression of heterologous genes. The cell pellets were resuspended in 200 µl lysis buffer (5% SDS and 20% glycerol) and sonicated at 40 Watts for 20 seconds. When everything dissolved after sonication, 10 uL of each sample was blotted on a nitrocellulose membrane along with untransformed *E. coli* BL21 (DE3) pLysS as a negative control and 5 µl of His-tag TEV protein as a positive control. The membrane was dried thoroughly before being soaked in buffer containing 50 mM Tris pH 7. 4 and 0.2 M NaCl (Buffer 229). The membrane was blocked with Buffer 229 + 5% nonfat dry milk (blocking solution) for 20 minutes. The blocked membrane was then subjected to incubation with the primary antibody (rabbit anti-His, 1:5000 dilution in blocking solution) for 2 hours. The membrane was then rinsed twice with Buffer 229 + 0.05% Tween-20 for 5 minutes before another rinse in Buffer 229 for 5 minutes. After the rinse, the membrane was subjected to incubation with the secondary antibody (goat anti-rabbit·HRP conjugate, 1:5000 dilution in blocking solution) for 1 hour. The membrane was rinsed and washed again following the previous step. The membrane was exposed to Pierce ECL Western Blotting Substrate solution (Thermo Scientific) and visualized using Bio-Rad Gel/Chemi Doc.

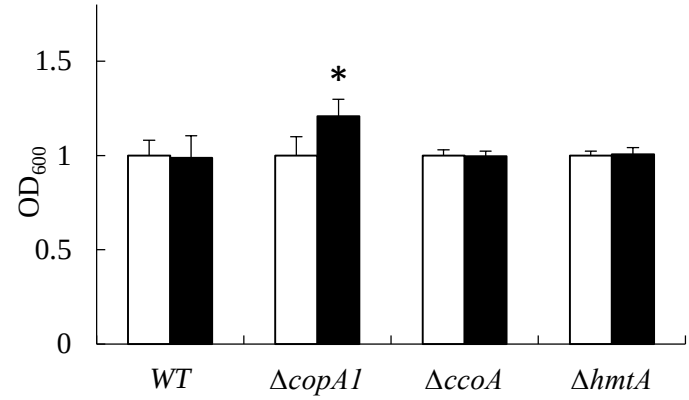
3. RESULTS

To understand the distinct role in the biochemical process that CcoA and HmtA play in *P. aeruginosa*, we examined the phenotypical performance of *CcoA* and *HmtA* mutant strains together with wild-type strain and previously well-identified copper exporter P_{1B}-ATPase CopA1 under identical experimental conditions. The experiment was initiated by monitoring the response of the mutant strains to copper deficiency. Compared with the growth of wild-type strain, there was a significant increase in the growth of mutant strains (*CopA1*, *CcoA*, and *HmtA*) after 16 hours BCS treatment in LB media, according to Figure 4A. *CopA1*, *CcoA*, and *HmtA* mutant strains grew better under the copper-deficient environment, compared with the bacteria growth without BCS treatment. However, there was no significant difference in the growth of the *P. aeruginosa* strains under the copper-depleted environment in Minimal media, except a slight increase in the growth of *CopA1* mutant strain (Figure 4B). Interestingly, there were color changes in both *CcoA* and *HmtA* mutant strains after 16 hours' incubation and, pyoverdine (PVD) content was measured from the culture supernatant. As expected, a significantly increased relative PVD concentration was determined within *CcoA* mutant strain in Figure 4C. The figure also indicated that the PVD content of *HmtA* mutant strain was relatively high, which was close to the concentration of PVD in *CopA1* mutant strain.

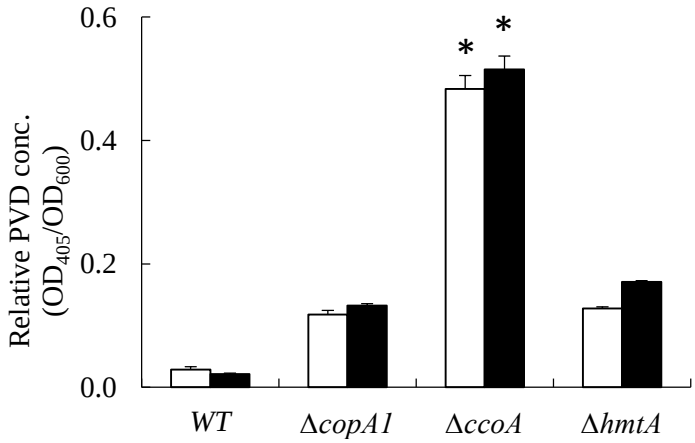
Figure 4. Role of CcoA and HmtA in Low Cu Tolerance.



A. Effects of deficient concentrations of copper (II) on the growth of *P. aeruginosa* PAO1 wild-type, *CopA1* mutant, *CcoA* mutant and *HmtA* mutant with no BCS treatment (□) and 0.5 mM BCS treatment (■). Turbidity (OD_{600}) was determined after 16 hours of growth in LB liquid medium at 37°C. *p < 0.01 *vs.* growth of mutants with no BCS treatment.



B. Effects of deficient concentrations of copper (II) on the growth of *P. aeruginosa* PAO1 wild-type, *CopA1* mutant, *CcoA* mutant and *HmtA* mutant with no BCS treatment (□) and 0.5 mM BCS treatment (■). Turbidity (OD_{600}) was determined after 16 hours of growth in the Minimal liquid medium at 37°C. *p < 0.01 *vs.* growth of *CopA1* mutant with no BCS treatment.

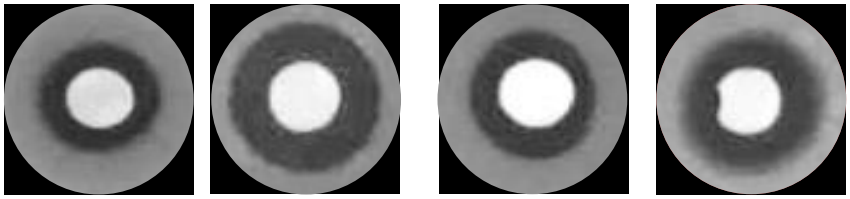


C. Pyoverdine (PVD) content of *P. aeruginosa* strains exposed to 0.5 mM BCS treatment (■). Pyoverdine (PVD) from culture supernatants was normalized by the cell density of bacterial cultures using a spectrophotometer. Data are the mean ± SE of three independent experiments. *p < 0.01 *vs.* wild-type.

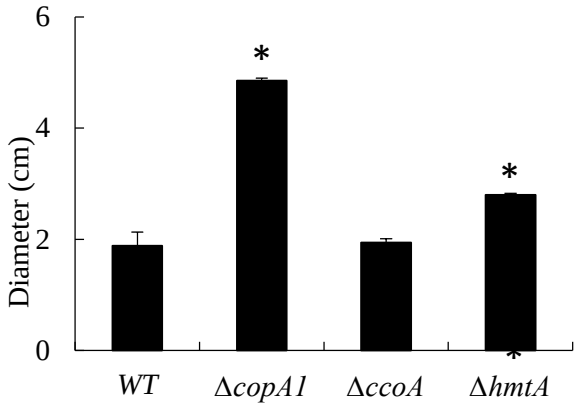
Previous researches have proposed that CcoA and HmtA could participate in copper acquisition. A recent paper also mentioned that *HmtA* gene contains conserved domains that could potentially bind with both copper and zinc ions. In order to verify this statement, the following experiments were conducted to continuously examine the metal sensitivity of *CcoA* and *HmtA* mutant strains exposed to either high copper or zinc concentration.

Photos of inhibition zones treated with 1 M $CuSO_4$ solution after 16 hours of incubation at 37°C are shown in Figure 5A. Differences in the diameters of inhibition zones were observed when the four bacteria strains were grown in LB media with excess copper. The results of challenging these strains with a high copper concentration in plates after 16 and 24 hours were indicated in Figure 5B and 5C. As shown in Figure 5B, the *CopA1* mutant demonstrated a significantly increased average diameter of the inhibition zones, and the *HmtA* mutant showed a relatively higher average diameter compared with the wild-type strain. Figure 5B indicated that the *CcoA* mutant strain was as insensitive as the wild-type strain when treated with 2M $CuSO_4$ solution through the measurement of the average diameters. In Figure 5C, compared with the wild-type strain, *CcoA* and *HmtA* mutant strains showed a significant insensitivity towards high copper concentration as a positive control after 24 hours of incubation, while the *CopA1* mutant strain remained highly sensitive. However, no significant difference between four strains was observed while treating these bacteria strains with a high concentration of zinc (2 M) after 24 hours (Figure 5D).

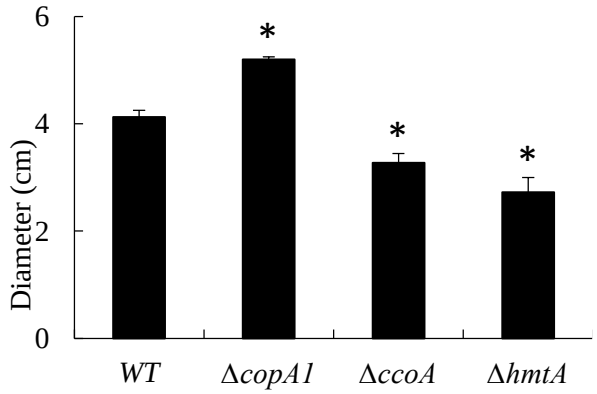
Figure 5. Role of CcoA and HmtA in Metal Sensitivity.



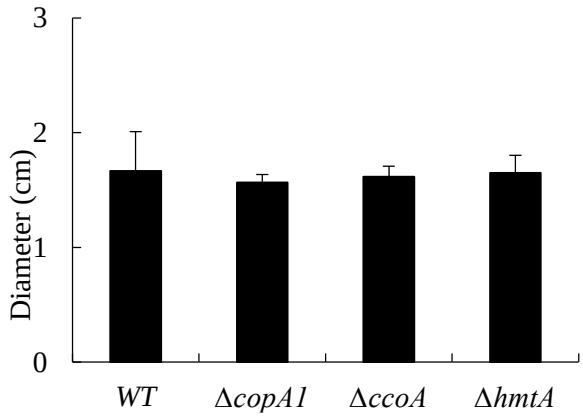
A. Cu^{2+} sensitivity (1 M) of *P. aeruginosa* PAO1 wild-type, *CopA1* mutant, *CcoA* mutant and *HmtA* mutant on LB/Agar plates. Pictures were taken after 16 hours of growth at 37°C.



B. Cu^{2+} sensitivity (2 M) of *P. aeruginosa* PAO1 wild-type, *CopA1* mutant, *CcoA* mutant and *HmtA* mutant on LB/Agar plates. The diameter was measured after 16 hours of growth on at 37°C. * $p < 0.01$ *vs.* wild-type.



C. Cu^{2+} sensitivity (2 M) of *P. aeruginosa* PAO1 wild-type, *CopA1* mutant, *CcoA* mutant and *HmtA* mutant on LB/Agar plates. The diameter was measured after 24 hours of growth on at 37°C. * $p < 0.01$ *vs.* wild-type.



D. Zn^{2+} sensitivity (2 M) of *P. aeruginosa* PAO1 wild-type, *CopA1* mutant, *CcoA* mutant and *HmtA* mutant on LB/Agar plates. The diameter was measured after 24 hours of growth on at 37°C.

Further experiments were conducted for a better understanding of the role of CcoA and HmtA regarding the tolerance of high copper concentration in LB liquid media. A previous research has showed that *CopA1* mutant strain does not grow in the presence of high copper concentration. The experiment repeated the previous experimental results for wild-type and *CopA1* mutant strains, and in contrast, showed that *CcoA* and *HmtA* mutant strains were slightly influenced by high copper concentration in LB liquid media and had similar phenotypical performances (Figure 6).

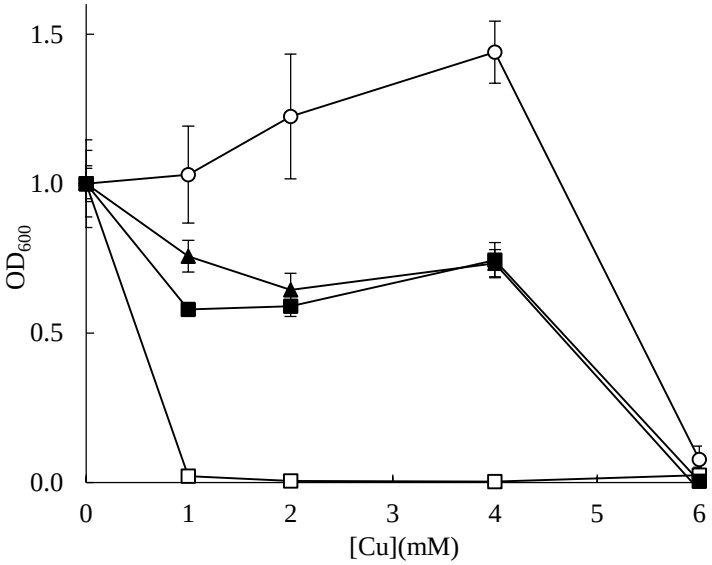
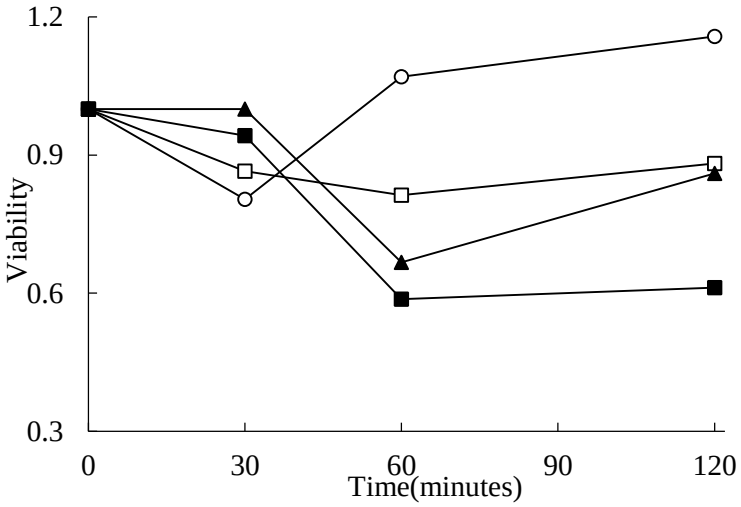


Figure 6. Role of CcoA and HmtA in High Copper tolerance. Effects of increasing concentrations of Cu^{2+} on the growth of *P. aeruginosa* PAO1 (wild-type, ○), *CopA1* mutant (□), *CcoA* mutant (▲) and *HmtA* mutant (■). Turbidity (OD_{600}) was determined after 16 hours of growth in LB liquid medium at 37°C.

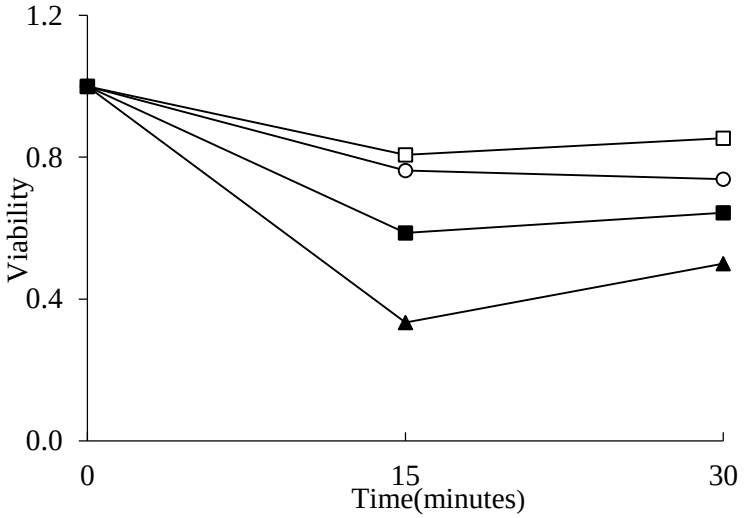
There is evidence indicating CcoA is involved in the assembly of cytochrome oxidase, a copper-requiring redox enzyme in the bacteria. The experiments were then conducted to reveal the phenotypical performance of *CcoA* and *HmtA* mutant strains regarding oxidative stress tolerance. In order to determine the role of CcoA and HmtA, the response of both mutant strains to oxidative stressors was measured through cell viability. Figure 7A showed that, with the treatment of Tert-butyl peroxide throughout 120 minutes, *P. aeruginosa* strains did not have significant differences

in growth. Figure 7B indicated that the experiments with the treatment of hydrogen peroxide repeated previously published experimental results, which the wild-type, *HmtA* and *CopA1* mutations strains showed no phenotypical difference under oxidative stress. However, *CcoA* indicated a significantly decreased cell viability and grew poorly in the presence of H_2O_2 within 30 minutes, due to the potential susceptibility to H_2O_2.

Figure 7. Role of *CcoA* and *HmtA* in oxidative stress tolerance.



A. Cell survival of *P. aeruginosa* PAO1 (wild-type, ○), CopA1 mutant (□), CcoA mutant (▲) and HmtA mutant (■), after treatment with 100 mM tert-Butyl hydroperoxide in LB medium for the indicated times. Cell viability is shown as the ratio of cfu of treated/untreated cultures.



B. Cell survival of *P. aeruginosa* PAO1 (wild-type, ○), *CopA1* mutant (□), *CcoA* mutant (▲) and *HmtA* mutant (■), after treatment with 30 mM H_2O_2 in LB medium for the indicated times. Cell viability is shown as the ratio of cfu of treated/untreated cultures.

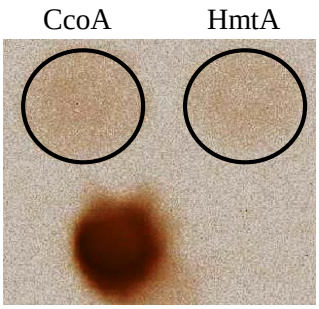


Figure 8. Protein Expression of CcoA and HmtA in *E. coli*. Protein expression in *E. coli* BL21 (DE3) pLysS expressing *P. aeruginosa* CcoA and HmtA immunostained with anti-His-tag antibody in the Dot Blot.

In order to conduct further research on CcoA and HmtA proteins, *CcoA* and *HmtA* genes were cloned from *P. aeruginosa* genomic DNA, ligated into the vector pET 30b (+), and transformed into *E. coli* BL21 bacteria strain. Since these two tested protein constructs had His tags, Dot Blot with the corresponding antibody was used to show that both CcoA and HmtA could be expressed in *E. coli* strain demonstrated in Figure 8, using a TEV protein as a positive control. However, the protein expression level was extremely low.

4. DISCUSSION

The overall goal of this study was to develop a further understanding of copper homeostasis in *P. aeruginosa* and to investigate the role of CcoA and HmtA in copper acquisition. In order to achieve this goal, different experiments, including metal sensitivity text, redox stress sensitivity text, and pyoverdine measure, were designed and conducted to study CcoA and HmtA through the phenotypical analyses of gene specific knock-out mutant strains. The plasmids with *CcoA* and *HmtA* gene insertion were also transformed into *E. coli* BL21 bacteria strain that could be induced followed by the protein expression. Some of the experimental data have made CcoA a strong candidate for copper acquiring in *P. aeruginosa*. Some interesting results also suggest that both CcoA and HmtA could be the probable importers functioning in the copper homeostasis of *P. aeruginosa*.

Copper depletion does not show a significant influence on the phenotypical performance of *CcoA* and *HmtA* mutant strains. To access the impact of copper deficiency on the phenotypical performance of *CcoA* and *HmtA* gene mutant strains, BCS has been used to eliminate copper in both LB media and Minimal media. The *CopA1* mutant strain, which lacks an important copper exporter CopA1, grows better under the low copper environment in both liquid LB and Minimal media. The mutant strains, lacking proposed copper importers CcoA and HmtA, should hypothetically grow less than the wild-type strain with BCS treatment. However, the data have demonstrated that *CcoA* and *HmtA* mutant strains grow even slightly better than the wild-type strain under the deficient copper environment. Since the composition of LB media is neither well-defined nor constant, the experiment was conducted using the same conditions in Minimal media (See Appendix) in order to clarify the outcome. The *CcoA* and *HmtA* mutant strains show a similar level of growth to the wild-type strain while the *CopA1* mutant strain still displays a marked

increased growth in BCS treated Minimal media. One of the possible explanations for obtaining non-affected growth of *CcoA* and *HmtA* mutant strains is that *P. aeruginosa* contains more than one pathway to acquire copper under copper depletion, which Figure 1 has already proposed.

The high pyoverdine content in mutant strains could be a hint for copper acquisition in *P. aeruginosa*. Pyoverdine, one of the major siderophores of *P. aeruginosa* mutant strains under copper deficiency, binds to copper ions and, in theory, might promote copper uptake. The production of siderophores in the mutant strains could potentially allow the mutant strains, which lack importer, to survive in a low copper environment, since a proposed mechanism suggested that some *P. aeruginosa* mutant strains produce more siderophores while reacting to copper depletion. The measurement of extremely high pyoverdine content in the *CcoA* mutant strain indicate that CcoA could obtain a copper uptake function.

The excess copper environment does not significantly affect the growth of *CcoA* and *HmtA* mutant strains. Lacking the potential candidates to import copper through the cell membrane, the *CcoA* and *HmtA* mutant strains show their insensibility towards the treatment of a concentrated copper solution (2 M $CuSO_4$) after 16 and 24 hours growing in the plates. However, the average diameter of the inhibition zones produced by the control *CopA1* mutant strain significantly differ with the other strains, indicating a high sensitivity towards the excess copper environment. Previous research has showed that *CopA1* mutant strain is intolerant of the high copper environment. Therefore, the copper tolerance test in LB liquid media was used to determine the copper tolerance of mutant strains lacking CcoA and HmtA under the same experimental conditions. In Figure 6, both *CcoA* and *HmtA* mutant strains are slightly influenced by high copper concentration. However, *CcoA* and *HmtA* mutant strains still grow in the excess copper environment, while *CopA1* mutant strain does not grow at all with the excess copper treatment due

to the lack of copper exporter for copper detoxification. The figure also illustrates a similar function in between the phenotypical performances of the *HmtA* and *CcoA* mutant strains. The relatively high tolerance of the *CcoA* and *HmtA* mutant strains to excess copper concentration in LB media could potentially associate with the function of copper uptake.

There is no evidence showing that HmtA participates in zinc uptake. Four tested strains were also treated with concentrated zinc solution (2 M $ZnSO_4$). Although it has been suggested that HmtA contains conserved regions that can potentially uptake both zinc and copper, the experimental data could not support this hypothesis. Both CcoA and HmtA mutant strains did not show a notable sensitivity toward excess zinc environment.

CcoA could potentially help *P. aeruginosa* overcome the oxidative stress. Previous research has suggested that *CcoA* plays a role in assembly of cytochrome c oxidase. In the oxidative stress tolerance experiment, *CcoA* mutant strain shows a small cell viability, which indicates that *CcoA* mutant strain is sensitive to H_2O_2 stress. However, both tert-butyl peroxide and hydrogen peroxide were used as oxidative stressors, but there is no significant phenotypical performance in tert-butyl peroxide experiment.

Experiments including *HmtA* and *CcoA* gene transformation into *E. coli* competent cells and protein expression were also conducted in this project (Figure 8). Although HmtA and CcoA proteins could be slightly expressed after protein induction, the level of the protein expression is extremely low, which could not be used for the future protein purification and further research on protein activities. In order to solve the problem of having a little expression of desired proteins, both *CcoA* and *HmtA* genes could be ligated into the pBAD plasmid, a plasmid often used by insoluble transmembrane proteins. The plasmids with *CcoA* or *HmtA* genes could then be transformed into *E. coli* BL21 competent cells after the positive sequencing result. In addition,

more phenotypical analyses, such as virulence test and copper accumulation assay, could be done in the future researches to study and understand the role of CcoA and HmtA regarding the copper homeostasis in *P. aeruginosa* continuously.

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APPENDIX

Minimal Medium Preparation (13)

MOPS medium was used to study the effects of particular nutrient sources on *P. aeruginosa* growth and virulence. To make MOPS medium the following solutions will need to be prepared.

10X MOPS Stock

Component	Stock	Volume for 500 ml stock	Concentration at 1X
MOPS	1 M (pH 7.5)	200 ml	40 mM
Tricine	1 M (pH 7.5)	20 ml	4 mM
$FeSO_4$	18.4 mM	5 ml	0.01 mM
NH_4Cl	1.9 M	25 ml	9.52 mM
$CaCl_2$	53 mM	50 µl	0.5 µM
$MgCl_2$ (hexahydrate)	512 mM	5 ml	0.52 mM
NaCl	5 M	50 ml	50 mM
Micronutrients	100X	5 ml	see table

100X Micronutrient stock for MOPS

Component	mg / 100 ml	Stock Concentration
Ammonium molybdate tetrahydrate	0.3 mg	3 µM
Boric acid	2.4 mg	400 µM
Cobalt chloride	0.7 mg	30 µM
Cupric sulfate	0.3 mg	10 µM
Manganese chloride	1.6 mg	80 µM
Zinc sulfate	0.3 mg	10 µM

1X Modified MOPS Media

Component	Stock	Volume for 500 ml of 1X	Final Concentration
10X MOPS Stock	see above	50 ml	see above
Deionized water	N/A	400 ml	N/A
Carbon source	1 M	10 ml	20 mM
$CaCl_2$	53 mM	300 µl	32 µM
K_2SO_4	27.5 mM	5 ml	0.29 mM
K_2HPO_4	172.8 mM	5 ml	1.32 mM
$FeCl_2$	8 mM	500 µl	8 µM