Cu^{+}-importing Phenotypes of ΔCuiT strain and the Expression Optimization of P_{1B}-ATPases in *Pseudomonas aeruginosa*

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Abstract

Copper is required as cofactor for many enzymes in bacteria, but excess copper can be toxic due to the ability of producing free radicals and disrupting Fe-S clusters in specific proteins. Therefore, bacteria need to maintain copper homeostasis. In *Pseudomonas aeruginosa*, the expression levels of various proteins are changed to alleviate copper stress conditions, and this suggests that the proteins must play a role in maintaining copper homeostasis. The roles of some upregulated proteins have been described recently. Chaperone CopZs distribute copper in the cytoplasm, while ATPases CopAs efflux the excess copper to the periplasm. However, the biochemical properties of CopAs have not been characterized yet. Downregulated transmembrane proteins are likely acting as copper importers, but these have received little attention. To address these questions, we focused our research on the copper-importing phenotypes of the putative CuiT importer and optimizing the expression of the P_{1B}-ATPases. The copper-importing phenotypes of CuiT were investigated by three methods (growth rate, cell survival, and copper uptake assays). Results showed that ΔCuiT strain accumulated less copper than WT within 10 min of incubation, while longer incubation (more than 12 hours) in the other two experimental approaches showed no phenotype. The optimizations of the sequences and expression conditions of both ΔN-CopA1 and ΔN-CopA2 were investigated. The results suggested that the expression of codon-optimized ΔN-CopA1 and ΔN-CopA2 was significantly improved, and the optimum conditions for ΔN-CopA1 and ΔN-CopA2 expression were obtained. In summary, the work here provides initial data supporting the copper-importing role of CuiT and optimum parameters for expressing for ΔN-CopA1 and ΔN-CopA2.

Keywords: CuiT, Copper importer, ATPases, Phenotype, Expression optimization, and *Pseudomonas aeruginosa*.

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List of Abbreviations

SOD - Superoxide dismutase

DNA - Deoxyribonucleic acid

GSH - Glutathione

ATP - Adenosine triphosphate

MER family - Mercury resistance regulator family

CsoR - Copper-sensitive operon repressor

MFS - Major facilitator family

qPCR - Quantitative polymerase chain reaction

TM-MBSs - Transmembrane metal binding sites

WT – Wild type

LB - Luria-Bertani medium

OD600 - Optical density 600

BCS - Bathocuproinedisulfonic acid disodium salt

DTT - Dithiothreitol

AAS - Atomic absorption spectroscopy

TEV - Tobacco etch virus

IPTG - Isopropylthio-β-galactoside

1. Introduction

1.1 Roles of copper in bacteria

Various metalloenzymes in bacteria need copper as cofactor. It has been estimated that these metalloenzymes account for around 0.3% of the bacterial proteome (1). The ability of copper being cofactor to these metalloenzymes is mainly associated to the redox potential generated from the interchange of Cu^{2+} (oxidized) and Cu^{+} (reduced) (1). For instance, the conversion cycle between Cu^{2+} and Cu^{+} in the active center of Superoxide dismutase (SOD) drives the toxic superoxide anion radicals into dioxygen and hydrogen peroxide, and thus, protects cells from oxidative stress and regulates the cell signaling through the local production of hydrogen peroxide (2). The redox center of cytochrome C oxidase, the terminal enzyme in respiratory chain, also needs copper to complete the electron transfer to transform dioxygen into water (3). Methane monooxygenases in Methanotrophic bacteria utilize copper to break the C-H bond in methane to generate methanol (4). These suggest that copper is essential for bacteria to perform various biological functions.

Despite the benefits of copper as cofactor in bacteria, excess copper can be detrimental to bacteria cells. Excess copper can initiate Fenton-like reaction and produce toxic reactive oxygen species (5). These oxygen species can damage the lipid, DNA, and proteins, leading to compromised physiological functions in bacteria (6). Other than the perspective of oxidative stress caused by excess copper, the strong affinity with thiolates by the reduced form of copper (Cu^{+}) also poses damage to the bacteria (7). Excess Cu^{+} can replace the iron in the active Fe-S cluster in Ferredoxin which acts as an electron carrier for various metabolic pathways (7,8). Excess copper can even affect the biogenesis of Fe-S clusters in *E. coli* by disrupting the sulfide-providing enzyme IscS *(8)*. Excess copper can consume the natural antioxidant peptide Glutathione (GSH) in bacteria and thus break the redox balance maintained by GSH (7,9).

In a nutshell, bacteria need copper as cofactor for various enzymes, but excess copper can compromise normal biological functions through different pathways (Figure 1). Therefore, bacteria must maintain copper homeostasis to survive.

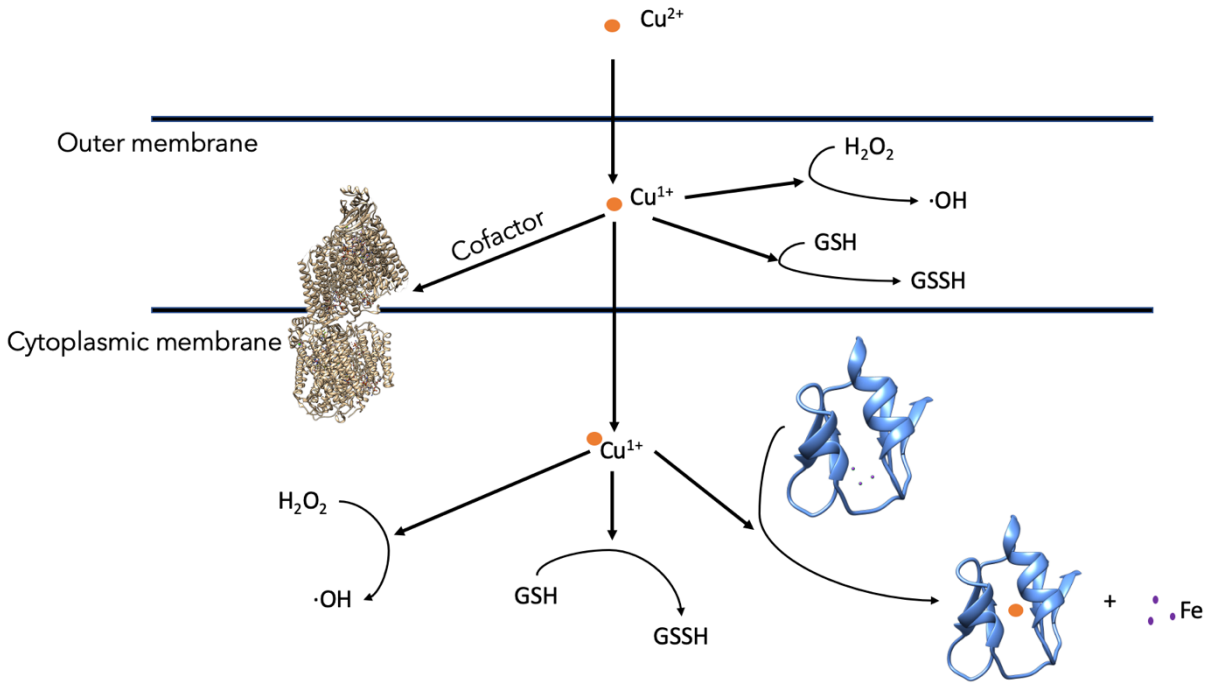

Figure 1 The double-edged sides of copper in bacteria
Copper is essential to various enzymes as a cofactor, but copper can be toxic when excess due to the interchange of oxidized and reduced state of copper in the bacterial cells. The gold color represents the structure of Cytochrome C oxidase, the light blue represents the structure of Ferodoxin.

1.2 Mechanisms of maintaining copper homeostasis in bacteria

How do bacteria maintain copper homeostasis? There are three possible strategies: limiting copper entry, speed up copper efflux, and buffering the excess copper in cytoplasm and periplasm (7). Since bacteria membranes are ion impermeable lipid bilayers, the copper influx and outflux are mediated by membrane transporters. Copper-exporting transporters in bacteria are P_{1B}-type ATPases, and they have 8 transmembrane segments (10). CopA Cu transporting ATPases utilize

the energy from ATP hydrolysis to pump the excess Cu^+ out through the classical Albers-Post cycle (10,11). Briefly, initially CopA adopts the E1 inward conformation, then change the conformation to outward E2 upon copper binding and ATP hydrolysis, then release the copper and move back to E1 inward conformation (10).

It is believed that there is no free copper in cells due to the high affinity of copper-sensing proteins (12-14). The copper-sensing proteins initiate the process of maintaining copper homeostasis by activating or repressing the transcription of copper-handling proteins to efflux the excess copper in cells (12). These copper-sensing proteins include the transcriptional activator CueR of the MER family (13,15,16) and the transcriptional repressors CsoR (17,18) and CopY (19,20) members of the ArsR family (21). In bacteria, the copper in cytoplasm or periplasm must be buffered by soluble proteins when the copper levels are maintained by these highly sensitive copper-sensing proteins (13). CopZs are reported to distribute copper in cytoplasm (12,13,20). CopZs have a classic βαββαβ ferredoxin-like folding pattern with an invariant GXXCXXC Cu^+-binding motif (12). In *E. coli*, CopZ delivers Cu^+ directly to the transmembrane segments in CopA and this is driven by the electrostatic interaction between the negative region of CopZ and the positive patch of CopA (22). In periplasm, bacteria also have various proteins to distribute excess copper. In *E. coli*, the tripartite complex CusCBA effluxes copper to the extracellular environment and the CusCBA is composed of a proton-substrate carrier CusA, outer membrane pore CusC and the linker protein CusB in periplasm (12,23). CusB also can directly receive copper from the periplasm chaperone CusF (24).

Another way to buffer the excess copper is achieved by storage proteins. A cysteine-rich protein, MymT, can bind up to six Cu^+ in *Mycobacterium tuberculosis*, and under copper stress condition the expression level can be increased up to 1000-fold above normal expression (25). This suggests

the copper-storage role of MymT for coping excess copper in cells. Later, a new family of copper-storage proteins, Csps, has been found in *Methylosinus trichosporium* and Csp3 was predicted to be able to bind up to 19 Cu^{+} in the crystal structure (26). In addition, various cuproenzymes might play a role in maintaining copper homeostasis and these enzymes include Cu-Zn SOD, Cytochrome C oxidase, and CueO etc (12,27). A proportion of copper is likely received by these enzymes to perform biological functions. Cu-Zn SOD binds copper to eliminate toxic free radicals (28,29). Cytochrome C oxidase facilitate the respiratory activity with the aid of copper (30,31). CueO oxidizes the Cu^{+} to less toxic Cu^{2+} since Cu^{+} is believed to not only participate in the production of free radicals but also in the binding of thiolates in proteins (32-34).

Overall, bacteria maintain copper homeostasis through the teamwork of various proteins, including transporters, chaperones, sensor proteins, storage proteins, and enzymes (Figure 2).

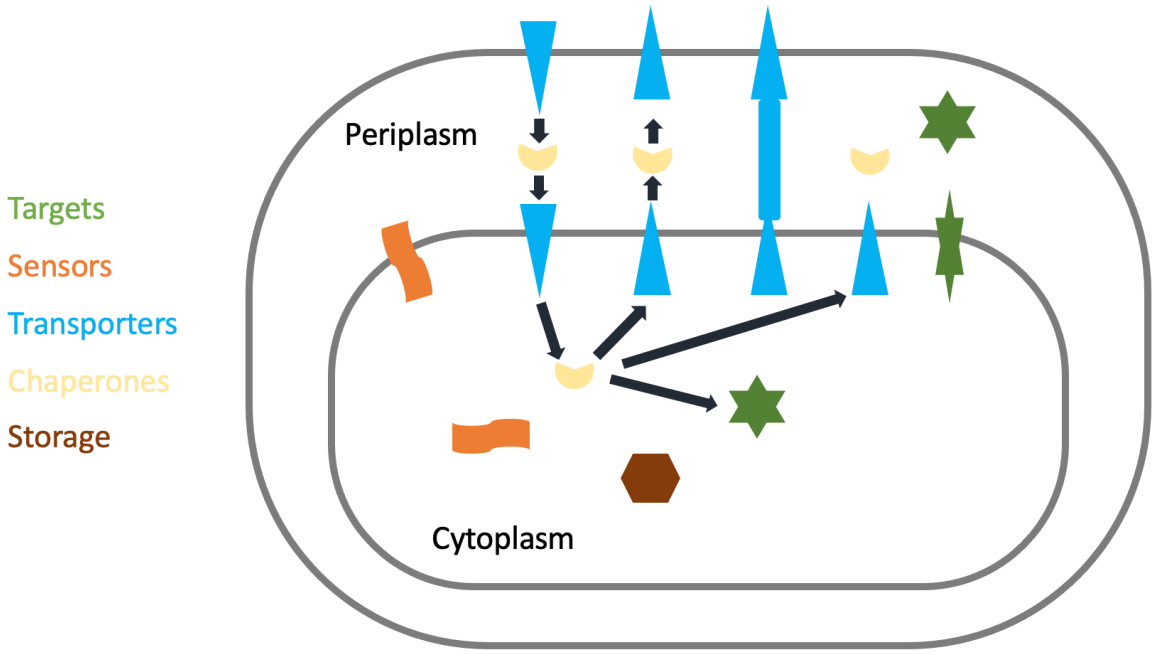

Figure 2 Mechanisms of Copper homeostasis in bacteria
The copper influx, efflux, and distribution in bacteria are achieved through the interplay of transporters, chaperones, sensors, storage proteins, and target proteins.

1.3 Mechanisms of maintaining copper homeostasis in *P. aeruginosa*

P. aeruginosa is a common pathogen that can be extremely dangerous to immunocompromised people. The diseases caused by this bacterium include healthcare-associated infections, including pneumonia, bloodstream infections, urinary tract infections, and surgical site infections (35). It is estimated that 32000 people in United States were hospitalized in 2017 due to *P. aeruginosa* infections, with a death rate of around 8%. This bacterium generated $767 million cost in just one year based on the data from Centers for Disease Control (CDC). The discovery of multidrug resistant strains of *P. aeruginosa* makes the treatment of the bacterium infection more difficult (36). Therefore, elucidation of copper homeostasis in *P. aeruginosa* can provide insights on the infection treatment or on the designing of effective antibiotics.

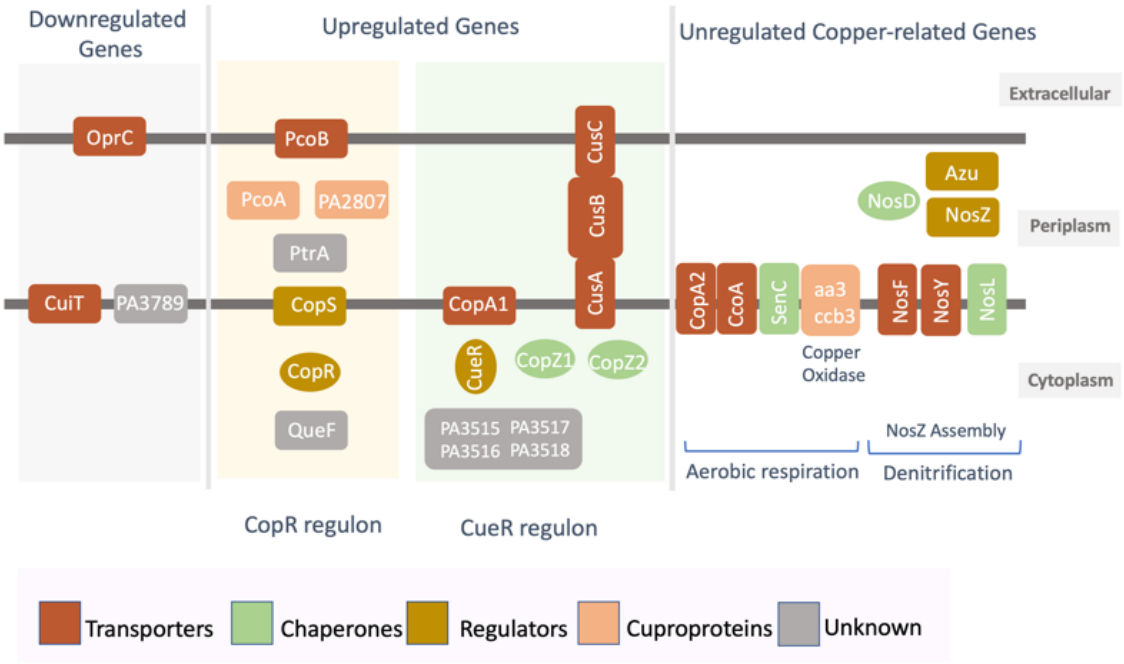

Figure 3 the transcriptomic analysis of P. aeruginosa under copper stress condition (37) The potential candidates of copper-importing transporters are shown in the section of downregulated genes, the CopR and CueR regulons are shown in the middle, and the proteins responsible for metalating cuproproteins are shown in the section of unregulated copper-related genes.

A transcriptome analysis revealed expression level changes of the genes in *P. aeruginosa* under copper stress condition (Figure 3) (37). The upregulated genes include the genes in the CueR and CopR regulons and the biological functions of maintaining copper homeostasis by some of these genes has been described (37). In the CueR regulon, CopA1 can transport Cu^+ from cytoplasm to periplasm and provide copper tolerance to the bacterium (38). CopZ1 can transfer the Cu^+ to the sensor protein CueR and to initiate the transcription of copper-handling proteins CopZ1, CopZ2, and CopA1. Expression of CopZ2 increases significantly than CopZ1 and the CopZ2 provides a fast response to excess copper by sequestering copper (13). In the CopR regulon, a recent study revealed the mechanism of controlling periplasmic copper homeostasis by the two-component system CopR/CopS (39). When periplasmic copper level is below the activation threshold of CopS, the sensor has a Cu-dependent phosphatase activity to maintain the non-phosphorylated state of CopR, while under the copper binding of sensor CopS, the CopR will be phosphorylated and activate the transcription of its regulon (39).

Three genes, CuiT, OprC, and PA3789, are strongly downregulated in *P. aeruginosa* under copper stress condition (37). The strongly downregulated expression of these genes suggests the role of copper importers. However, this function has not be experimentally characterized.

1.4 Copper importing mechanisms

Research on copper-importing transporters has reieved little attentions and only three membrane proteins, namely CcoA, NosA and OprC, in bacteria have been proposed to import Cu^+ into the cell envelop (40-42). Cytoplasmic membrane CcoA, a Major Facilitator Family (MFS) member, imports Cu^+ to facilitate the assembly of cbb3-Cox biogenesis (40,43). Outer membrane NosA is required for the insertion of copper in N_2O reductase, while functions of the other transporter OprC are channel-forming and copper binding (41,42).

Three genes, CuiT, OprC and PA3789, have been strongly downregulated (more than 70%) under copper stress condition in *P. aeruginosa* (37) and this work focuses on the cytoplasmic membrane protein-CuiT. qPCR analysis has confirmed that the expression level has been decreased to around 60% under copper stress condition (Figure 4A) (37). The CuiT homology model derived from *E. coli* YajR (3WDO) structure (44) revealed two putative Cu^{+} binding sites clusters formed by highly conserved His/Met/Cys residues (Figure 4B). The evidence from gene expression and structure perspectives suggests that CuiT is likely a copper-importing transporter. The copper-importing phenotype of CuiT must be determined to pave the way for studying the copper binding and transfer mechanisms.

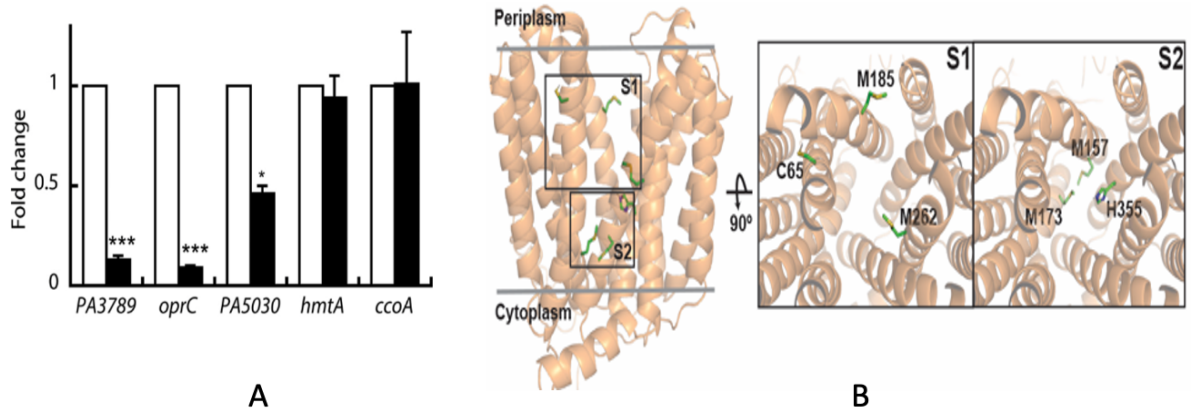

Figure 4 Gene expression and structure of CuiT
Figure A shows the qPCR analysis of CuiT (PA5030) confirming the gene downregulation under copper stress condition (37). Figure B shows the homologous model derived from YajR (3WDO) in *E. coli*, the two putative copper binding sites are marked as S1 and S2, and the two binding sites are composed of highly conserved His/Met/Cys residues (Data from former lab members).

1.5 P_{1B}-type ATPases in bacteria

P-type ATPase functions as ion- and lipid-pumps across impermeable membrane bilayers (45). The superfamily of P-type ATPase can be divided into five subfamilies based on their distinct substrates: the K^{+} transporter P_{1A}-type ATPase, the heavy metal pumps P_{1B}-type ATPase, the Ca^{2+},

Na^+/K^+, and H^+/K^+ transporter P_2-type ATPase, the H^+ pumps P_3-tyepe ATPase, the lipid pumps P_4-type ATPase, and the P_5-type ATPase is designated to the unknown substrates (45,46). Among the subfamilies of ATPases, P_{1B}-type ATPase is omnipresent in all life forms and is found to be the most common P-type ATPase in bacteria (45). P_{1B}-type ATPases serve two purposes: one is to provide micronutrient metals to specific enzymes, while the other is to efflux excess heavy metals in cells (45). Various P_{1B}-type ATPases have been well characterized, such as CopA (11,38,47,48), ZntA (49,50), HMA2 (51), CtpA (52-54) and CadA (55).

Cu^+ ATPases translocate Cu^+ across the membrane through the transmembrane metal binding sites (TM-MBSs) and the structures of the TM-MBSs have been well characterized (14,46). The Cu^+ ATPases can bind two Cu^+ in trigonal planar coordination (14,56). One binding site is composed of two Cys in M4 and a Tyr in M5, and the other binding site was constituted by an Asn in M5, and Glu and Ser in M6 (10). One interesting feature is the existence of N-terminus region of Cu^+ ATPases (10). The N-terminus region normally constitutes around 60 amino acids with a βαββαβ ferredoxin-like pattern (10). It has been proved that the N-terminus region receives Cu^+ from cytoplasm chaperone CopZ but there was no further Cu^+ movement from the N-terminus region to the TM-MBSs (22,57).

The Cu^+ transporting mechanism of Cu^+ ATPases follows the description of the classical Albers-Post cycle (10). Briefly, the inward confirmation E1 of the Cu^+ ATPase binds to two Cu^+ ions; then coupling with the enzyme phosphorylation the E1-Cu^+ confirmation converts to outward E2-Cu^+ confirmation; the Cu^+ ions then will be released to the periplasm or extracellular environment; the E2 confirmation then will be changed to inward confirmation by dephosphorylation (10).

1.6 Cu^+ ATPase in *P. aeruginosa*

In *P. aeruginosa*, two Cu^+ ATPases, CopA1 and CopA2, have been found (38). These two ATPases are highly homologous, share the same membrane topology, own the same TM-MBSs, and same metal binding sites in N-terminus region (38). A previous study revealed different biological functions of the ATPases, CopA1 and CopA2, in *P. aeruginosa (38)*. Mutation of CopA1 significantly compromised the growth under copper stress conditions, while the mutation of CopA2 did not change the growth phenotype comparing to the WT strain (38). Mutation of CopA2 led to weakened activity of the cytochrome C enzyme (38). The transport assay using everted vesicles proved that both CopA1 and CopA2 can transfer Cu^+ to the periplasmic space, but with significantly different velocities (38). The evidence suggests that CopA1 and CopA2 serves different biological purposes under copper stress condition. However, other questions still need to be addressed, i.e., how do the ATPases interact with cytosolic and periplasmic chaperones to maintain copper homeostasis? Prior to solve the further questions in a biochemistry view, the two ATPases must be heterogenous expressed and purified. The purification of membrane proteins is normally laborious and difficult, and therefore, expression optimization step is always performed to ensure a high expression level of target proteins.

1.7 Research questions

This work addresses two questions of copper homeostasis in *P. aeruginosa*. The first one is to investigate the phenotype of the putative copper importer CuiT under copper stress condition, and the second goal focuses on optimizing the expression levels of CopA1 and CopA2 in *E. coli* host.

2. Materials and Methods

2.1 Bacterial strains, plasmids, and growth conditions.

P. aeruginosa PAO1 served as WT strain. Mutant strain CuiT (PA5030) was obtained from the *P. aeruginosa* PAO1 transposon mutant library (University of Washington, Seattle, WA). Complemented CuiT_comp strain was obtained by Dr. Lorna Novoa-Aponte, a former lab member. All *P. aeruginosa* strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with 25 μg/ml Irgasan.

2.2 Growth curve experiment

P. aeruginosa stains were cultured in LB medium containing 25 μg/ml Irgasan at 37 °C 180 rpm for overnight. Adjusting the optical density (OD600) of the *P. aeruginosa* strains to the same level using the same Irgasan-containing LB medium, following by adding indicated 0 mM, 1 mM, 2 mM, 3 mM, 4 mM of $CuSO_4$ to prepare the bacterial mixtures. 200 μl of the bacterial mixtures were used to monitor the cell growth for 12 hours by Epoch 2 microplate spectrophotometer (BioTek) and the growth conditions are at 37 °C with continuous shaking.

2.3 Spot test

P. aeruginosa stains were cultured in LB medium containing 25 μg/ml Irgasan at 37 °C 180 rpm for overnight. LB plates were prepared using the same Irgasan-containing LB medium and the indicated 0 mM and 1 mM of $CuSO_4$ were added. Adjusting the OD600 of the *P. aeruginosa* strains to the same level using the same Irgasan-containing LB medium. A series of bacterial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions) were prepared. 10^{-1} dilution was prepared by adding 200 μl of bacterial culture into 5 ml of Irgasan-containing LB medium; while the other dilutions were prepared in the denoted mathematical indications, for example 10^{-2} dilution was

prepared by adding 100 µl of 10^{-1} dilution into 900 µl of Irgasan-containing LB. One drop (10 µl) of each dilution was put on the surface of the LB plates and then incubated for 3 days at room temperature.

2.4 Copper uptake

Overnight cultures of *P. aeruginosa* stains were cultured in LB medium containing 25 µg/ml Irgasan at 37 °C 180 rpm for overnight. Then the overnight cultures were used to prepare mid-logged phase cells in antibiotic-free LB medium. LB medium supplemented with the indicated 0 mM, 2 mM, 4 mM, and 8 mM of CuSO4 were vortexed in Eppendorf tubes. *P. aeruginosa* strains were added into the premixed Eppendorf tubes to begin the Copper uptake reaction, and the reaction was stopped by adding 1 mM Bathocuproinedisulfonic acid disodium salt (BCS) and 1 mM DTT. The mixtures of copper uptake were centrifuged at 14000 rpm for 1 min and the pellets were washed twice by 150 mM NaCl to remove unabsorbed copper. Mineralization was achieved by adding 200 µl HNO_3, 100 ul H_2O_2, and 200 µl H_2O. The Cu uptake was determined by Atomic Absorption Spectroscopy (AAS) as described before (37). The protein concentration was measured by Bradford assay (58). The ug Cu/ mg Protein were calculated to indicate the Copper uptake level of each strain.

2.5 Codon optimization

Expression of *P. aeruginosa* CopA1 and CopA2, lacking the N-terminal metal binding domain was optimized. The sequences of ΔN-CopA1 and ΔN-CopA2 were obtained from the genome database of *P. aeruginosa* (https://www.pseudomonas.com/). To adapt the sequence to *E. coli* codon bias, the sequences were optimized using online tool (https://www.genscript.com/gensmart-free-gene-codon-optimization.html). The TEV and His-tag sites were attached to the end of the

sequences for the convenience of protein purification. The optimized sequences were cloned to the pET-30b(+) vector and the cloning sites are NdeI (CATATG-) and Xhol (CTCGAG-).

2.6 Bacterial transformation

The gene-carrying pET-30b(+) vectors were transformed into the BL21 (DE3) *E. coli* host. Briefly, adjust the concentrations of the vectors to 100 ng/ µl using deionized water; then mix 2 µl of the vectors with 60 µl of the BL21 (DE3) *E. coli* competent cells and incubate on ice for 20 min; subsequently the mixtures were applied with heat shock at 42 °C for 45 seconds; then 600 µl of LB medium was added to allow the *E. coli* host to rapidly duplicate; finally the cells were placed onto LB plates supplemented with 30 ug/ml of Kanamycin for overnight incubation. The single colonies were picked to culture.

2.7 Restriction digest

Restriction digest was performed to verify whether the sequences were cloned into the pET-30b(+) vectors. Xbal and Xhol enzymes (New England Biolabs, MA) were used to digest the vectors. The reactions were performed in the CutSmart buffers (New England Biolabs, MA) at 37 °C for 1 hour. The digested products were analyzed by Agarose gel.

2.8 Determination of small-scale protein expression.

The expression of codon-optimized ΔN-CopA1 and ΔN-CopA2 were compared to the previous ΔN-CopA1 and ΔN-CopA2 *E. coli* strains in the lab. The codon-optimized ΔN-CopA1 and ΔN-CopA2 were in pET-30b(+) vectors, while the previous ΔN-CopA1 and ΔN-CopA2 were in the pBAD vectors. CopZ1 *E. coli* strain was used as a positive control.

Precultures of codon-optimized ΔN-CopA1 and ΔN-CopA2, previous ΔN-CopA1 and ΔN-CopA2, and CopZ1 were cultured overnight in 5 ml of LB supplemented with either 30 ug/ml of

Kanamycin or 25 ug/ml of Ampicillin. Then 250 µl of precultures were added into 10 ml of LB supplemented with corresponding antibiotics and were incubated at 37 °C for 2 hours. 1 mM of Isopropylthio-β-galactoside (IPTG) was used to induce the expression of codon-optimized ΔN-CopA1 and ΔN-CopA2 and CopZ1, while 0.5% of Arabinose was used to induce the previous ΔN-CopA1 and ΔN-CopA2. The expression periods were three hours for all the proteins. 2 ml of the expressed cultures were pelleted by centrifugation and subsequently the pellets were resuspended in the buffer (25 mM Tris-HCl pH 8, 100 mM sucrose, and 150 mM NaCl) for sonication. 10 µl of each sonicated cultures were used for visualization in DotBlot. Nitrocellulose membrane was used to attach the proteins, 5% milk was used to block the unspecified proteins, and the membrane was immunostained with rabbit anti-His-tag polyclonal antibody and goat anti-rabbit IgG horseradish peroxidase-conjugated polyclonal antibody (GenScript, NJ).

Different concentrations of IPTG (400 µM and 1 mM) and incubation hours (3 hours and 5 hours) were applied to the codon-optimized ΔN-CopA1 and ΔN-CopA2 to acquire maximum expression based on the recommendations from the manufacturers of the competent cells. Autoinduction method was also applied to explore the best efficient expression conditions. The reagents used in Autoinduction are as follows: 20X Phosphate solution (1M Na_2HPO_4, 1M KH_2PO_4, 0.5M $(NH_4)_2SO_4$), 50X 50505 solution (25 % Glycerol, 10% of α-Lactose monohydrate, 2.5 % D-Glucose), 1000X metals solution (20 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $ZnSO_4$, 5 mM $CoCl_2$, 5 mM $CuCl_2$, 5 mM NiCl2, 2 mM $NaMoO_4$, 2 mM H_3BO_3), 500X $MgCl_2$ solution (1 mM $MgCl_2$). The incubation time for the Autoinduction was 20 hours.

3 Results

3.1 The phenotypes of the putative copper importer-CuiT

3.1.1 ΔCuiT strain does not have copper tolerance alterations when compared with both WT and ΔCuiT_comp strains in growth curve experiment.

As a putative copper-importing transporter, deletion of CuiT in *P. aeruginosa* would block or decrease the routes of copper entry. Less cellular copper in ΔCuiT strain would confer more copper tolerance than WT and ΔCuiT_comp strains under copper stress condition. The growth curve experiment was applied to investigate the copper-importing role of the CuiT. The OD600 value that suggesting bacterial cell abundance is used here to indicate the growth condition of the strains.

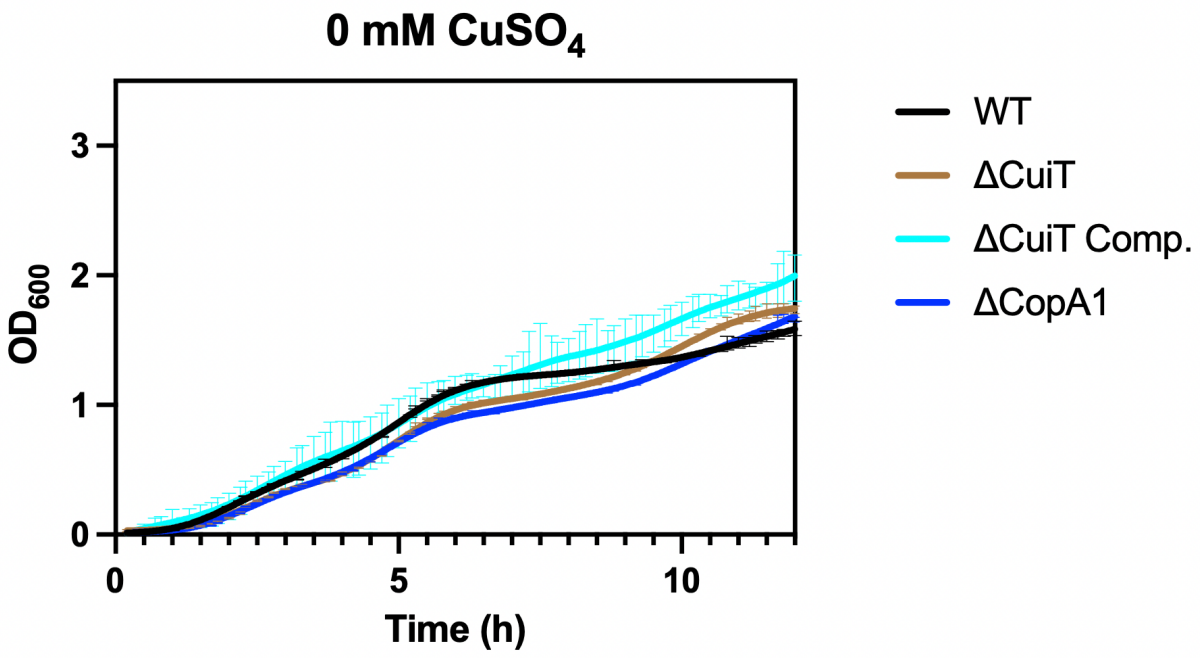

Figure 5 The growth curve result of WT, ΔCuiT and ΔCuiT_comp under 0 mM of $CuSO_4$

Previous studies demonstrated that ΔCopA1 strain showed significant decrease in growth under copper stress condition (13,38). Therefore, ΔCopA1 strain was used as a positive control. Complemented ΔCuiT_comp strain was used to investigate whether the phenotype, if any, was caused only by the deletion of CuiT. Under 0 mM of $CuSO_4$, all the strains (WT, ΔCuiT, and ΔCuiT_comp) show similar growth trend (Figure 5). This suggests that WT, ΔCuiT, and ΔCuiT_comp have the same baseline in terms of growth without copper stimulation.

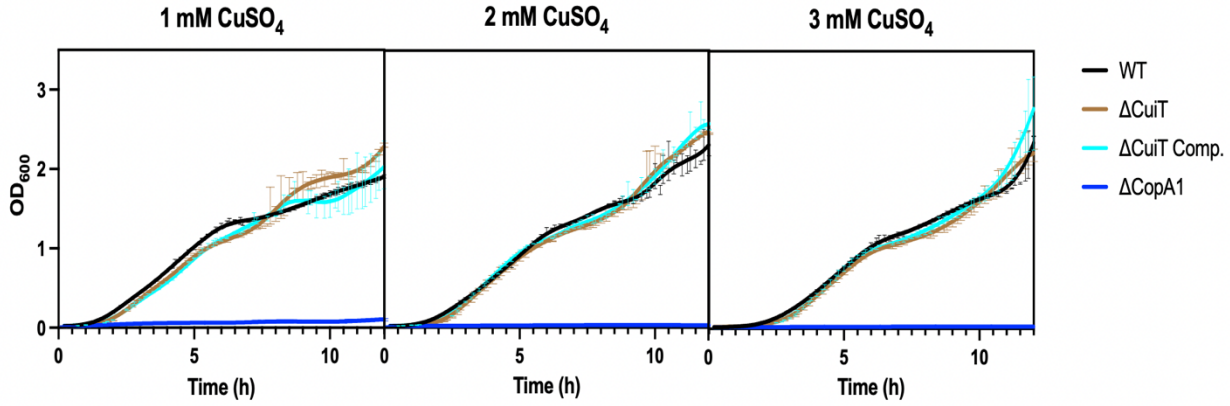

Figure 6 The growth curve result of WT, ΔCuiT and ΔCuiT_comp under 1-3 mM of $CuSO_4$

ΔCopA1 strain stops the growth when concentration of $CuSO_4$ goes up to 1 mM and keeps pause on growth under both 2 mM-4 mM of $CuSO_4$ (Figure 6 and 7). The compromised growth of ΔCopA1 is consistent with the previous studies. All the strains show delayed growing points when concentrations of $CuSO_4$ increase: 1 hour under 1 mM of $CuSO_4$, 1.5 hours under 2 mM of $CuSO_4$, and 2 hours under 3 mM of $CuSO_4$ (Figure 6). However, the WT, ΔCuiT, and ΔCuiT_comp exhibit no significant difference for growing trend during the 12 hours of incubation (Figure 6). When concentration of $CuSO_4$ continue to increase, WT, ΔCuiT, and ΔCuiT_comp stop the growing under 4 mM of CuSO4 (Data not shown here).

The results of Growth curve experiment suggest that the WT, ΔCuiT, and ΔCuiT_comp strains don't have significant difference on growing under copper stress condition for 12 hours of incubation.

3.1.2 ΔCuiT strain does not have copper tolerance phenotype with both WT and ΔCuiT_comp strains by Spot test.

The copper tolerance phenotype of CuiT was investigated using another common method: Spot test. Comparing the Growth curve experiment, the Spot test provides more visual evidence showing the growing conditions of the strains. Drops (10 μl) from a series of dilutions of the strains were placed on the LB medium surface containing 0 and 1 mM of $CuSO_4$, and the growing of the strains will be pictured. ΔCopA1 strain was used as positive control. On day 1, ΔCuiT strain shows a compromised growth comparing to ΔCuiT and ΔCuiT_comp strains under 0 mM of $CuSO_4$ (Figure 7, Left, Line 2). After three days of incubation, the ΔCuiT strain still shows slightly compromised growth than other two strains (Figure 7, Left, Line 5). This suggests that WT, ΔCuiT and ΔCuiT_comp strains grow differently without the copper stimulation.

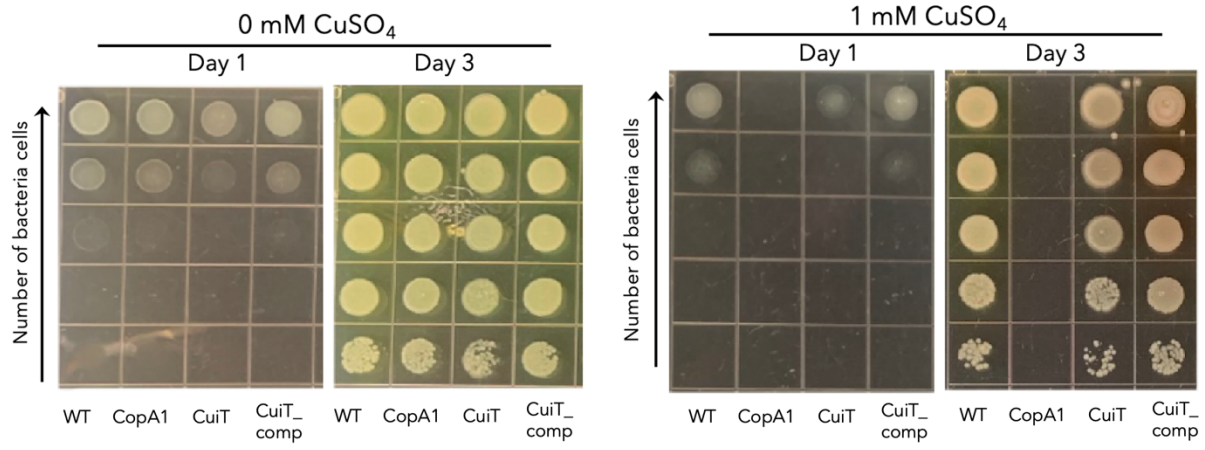

Figure 7 The growth conditions of the WT, ΔCuiT and ΔCuiT_comp under 0 and 1 mM of $CuSO_4$

Under the stimulation of 1 mM of $CuSO_4$, WT, ΔCuiT and ΔCuiT_comp strains exhibit compromised growth on both Day 1 and Day 3 comparing to the result without copper stimulation. Although the ΔCuiT show declined growth than WT and ΔCuiT_comp strains under 1 mM of $CuSO_4$, this cannot prove that the growth difference between the ΔCuiT strain and WT and ΔCuiT_comp strains due to the weaker growth of ΔCuiT strain without copper stimulation. The results in Spot test suggest that there are no clear growth differences between WT, ΔCuiT and ΔCuiT_comp strains under copper stress condition for three days of incubation.

3.1.3 ΔCuiT strain accumulates less copper than WT and ΔCuiT_comp strains.

Copper uptake experiment was applied to further explore the copper-importing role of CuiT. Measuring copper uptake could provide direct evidence showing copper quantity in bacterial cells of the strains. The mid-log phased cells of the strains were mixed with different concentrations of $CuSO_4$ for different incubation times, and then ratio of μg Cu/mg protein was used to indicate the copper level in each strain.

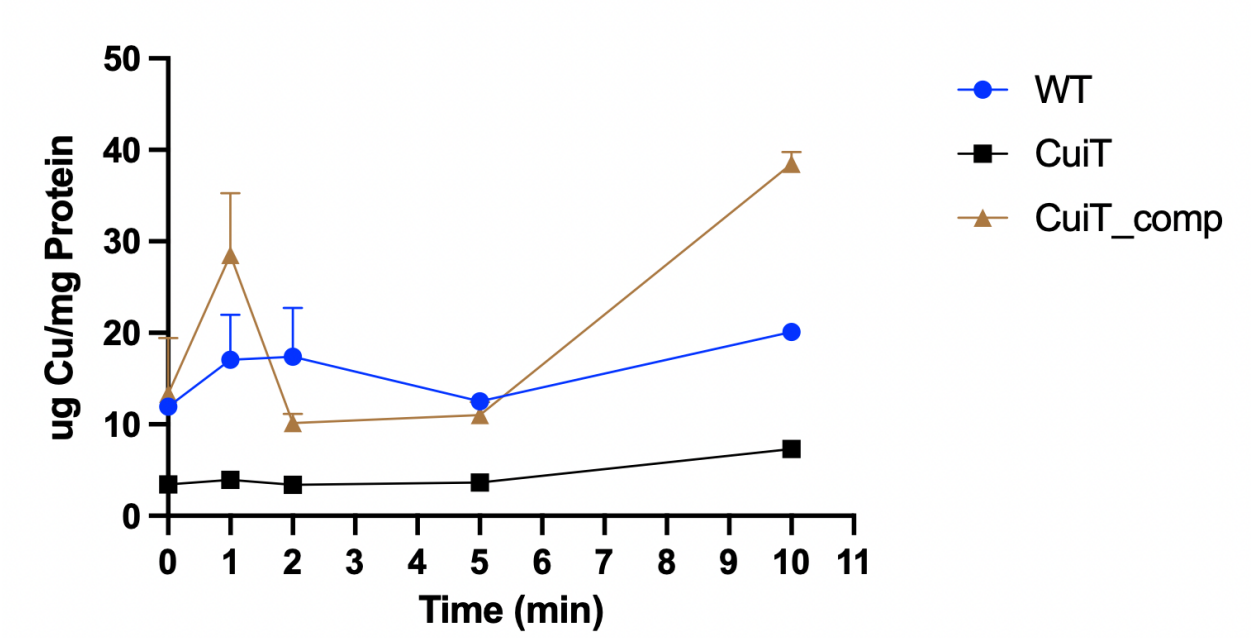

Figure 8 Cu uptake result of the WT, ΔCuiT and ΔCuiT_comp within 10 min of incubation
Each strain was mixed with 4 mM of $CuSO_4$, and the incubation time points were 0, 1, 2, 5, 10 min at 37 °C 180 rpm. The data are presented as Mean ± SEM.

Figure 8 clearly shows that ΔCuiT strain accumulated less copper in cells than WT at each time points and ΔCuiT_comp strain reversed the lower level of cellular copper in the ΔCuiT strain. This suggests that CuiT is functioning as a copper-importing transporter.

The copper uptake phenotype of CuiT under different concentrations of $CuSO_4$ was investigated as shown in Figure 9. Consistent with the result in Figure 8, ΔCuiT strain stored less copper in cells than WT strain, while the complemented strain-ΔCuiT_comp reversed the lower copper level to some degree. This also supports the copper-importing role of CuiT.

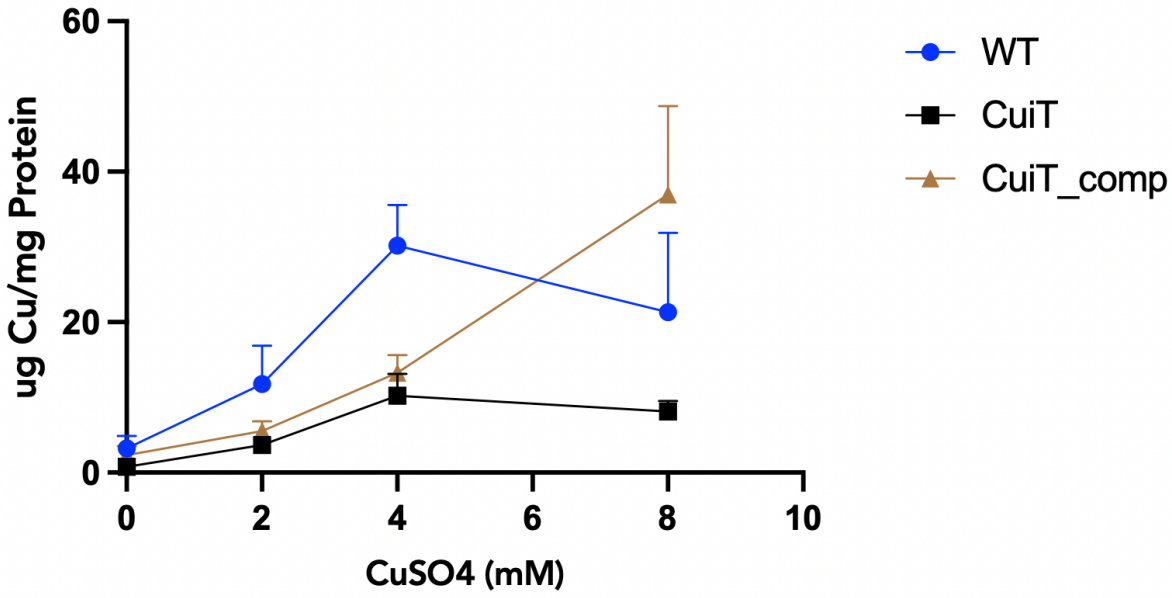

Figure 9 Cu uptake result of the WT, ΔCuiT and ΔCuiT_complemented strains under different concentrations of $CuSO_4$
Each strain was mixed with different concentrations of $CuSO_4$, and then subjected to 5 min of incubation at 37 °C at 180 rpm. The data are presented as Mean ± SEM.

3.2 Expression optimization of ΔN-CopA1 and ΔN-CopA2 in E. coli host

3.2.1 Clonning of codon-optimized sequences of ΔN-CopA1 and ΔN-CopA2 into the pET-30b(+) vectors.

To confirm the results of cloning of the codon-optimized sequences, the restriction digest was performed using two enzymes (Xbal and Xhol). Xhol is one of the ligation sites, while the Xbal locates on the backbone of the pET-30b(+) vector. When the vector was digested by either Xbal or Xhol, a single band with a molecular weight of the whole construct (7335 bp for ΔN-CopA1, 7329 bp for ΔN-CopA2) would be expected. When digested by both the two enzymes, two bands would be expected for both the ΔN-CopA1 and ΔN-CopA2 (~2100 bp and ~5000 bp). As shown

in Figure 10, the agarose gel shows the expected bands for both the ΔN-CopA1 and ΔN-CopA2 and this proves successful cloning of the codon-optimized sequences in the pET-30b(+) vectors.

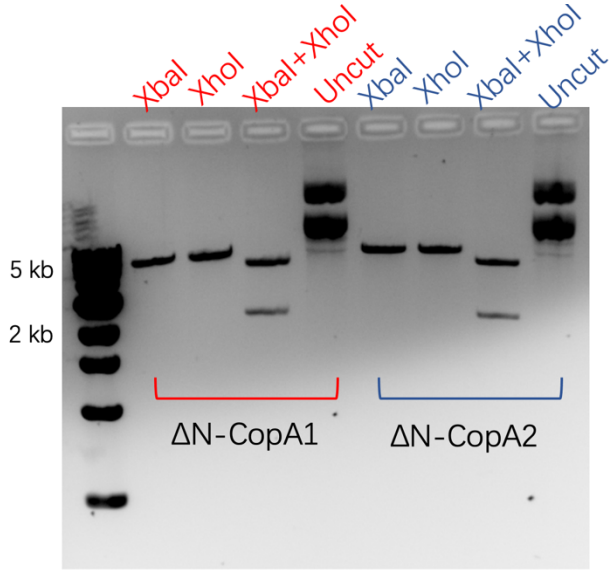

Figure 10 Restriction digest of the codon-optimized ΔN-CopA1 and ΔN-CopA2

3.2.2 The expression of ΔN-CopA1 and ΔN-CopA2 is improved after codon optimization.

P. aeruginosa CopA1 and CopA2, lacking the N-terminal metal binding domain was used in these studies. To investigate whether the expression of both ΔN-CopA1 and ΔN-CopA2 was improved by codon optimization, a small-scale protein expression was performed. CopZ1 is a soluble protein derived from *P. aeruginosa* with a good expression in *E. coli* host (13) and therefore, CopZ1 was used as a positive control. A series of dilutions of each expressed cultures were used to visualize the expression of the proteins on nitrocellulose membrane (Figure 11). The expression levels of both the optimized and previous ΔN-CopA1 and ΔN-CopA2 is significantly lower than the expression of CopZ1, however, the expression of the codon-optimized ΔN-CopA1 and ΔN-CopA2 is stronger than the previous ΔN-CopA1 and ΔN-CopA2 (Figure 11A). To have a clear comparison between the codon-optimized and previous ΔN-CopA1 and ΔN-CopA2, the interference from

CopZ1 needs to be removed and the result clearly shows that the expression of the codon-optimized ΔN-CopA1 and ΔN-CopA2 is significantly improved (Figure 11B).

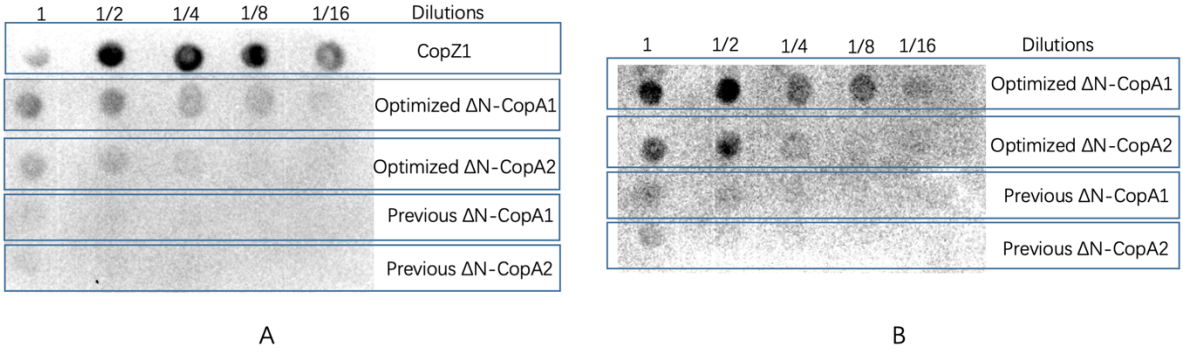

Figure 11 DotBlot results of the expression of ΔN-CopA1 and ΔN-CopA2

The target proteins were overexpressed in the *E. coli* host, and a series of dilutions of each strain were visualized on the nitrocellulose membrane. A: the comparison of expression levels between optimized ΔN-CopA1 and ΔN-CopA2 and the previous ones. B: the visualization of optimized ΔN-CopA1 and ΔN-CopA2 without interference from CopZ1.

3.2.3 The autoinduction is more suitable for expressing codon-optimized ΔN-CopA1, while traditional induction is suitable for expressing codon-optimized ΔN-CopA2.

To maximize the expression level of the codon-optimized ΔN-CopA1 and ΔN-CopA2, a further small-scale expression experiment was performed. Different induction methods, different concentrations of induction reagents, and different incubate time were tested. As shown in Figure 12, the signals of ΔN-CopA1 induced by both 400 μM and 1mM IPTG under autoinduction condition are significantly stronger than others, and this suggests that the autoinduction method is the most efficient way to express ΔN-CopA1. However, the stronger expression signal of ΔN-CopA2 falls into the condition of 1 mM IPTG for 5 hours traditional induction.

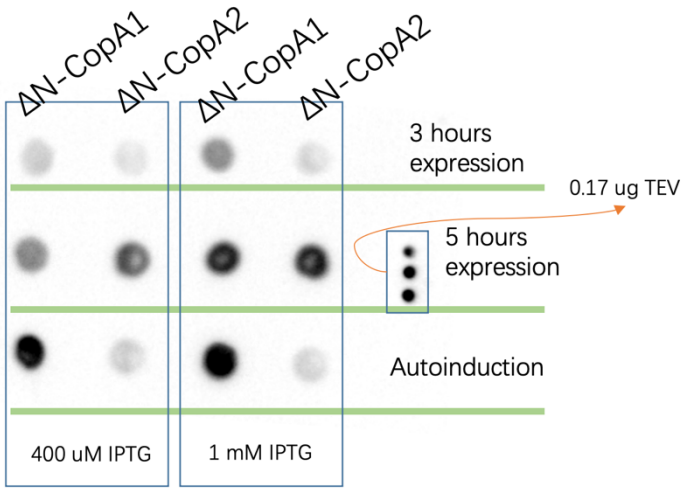

Figure 12 The DotBlot results of the expression optimization of ΔN-CopA1 and ΔN-CopA2
10 µl of each sonicated cultures were visualized on the nitrocellulose membrane by anti-His antibodies, and TEV was added as a positive control of His-tag protein. In traditional induction, the induction reagent was added after 3 hours or 5 hours of the cell growth; in autoinduction, the induction reagent was added in the beginning.

4. Discussion

The impermeability of the cell membrane lipid layer does not allow the entrance of ionic metals such as the micronutrient copper. The copper trafficking is mediated by various transporters on the membranes. CuiT, located at the cytoplasmic membrane, is a promising candidate for importing copper in *P. aeruginosa* based on preliminary analysis of gene expression and structure model (37). The gene expression experiment demonstrated that the bacteria strongly downregulated the expression of CuiT under copper stress condition, while the structure analysis based on the homologous model generated from *E. coli* YajR (3WDO) revealed two putative copper binding sites filled with highly conserved His/Met/Cys residues (37). The *P*. *aeruginosa* owns two distinct ATPases: CopA1 and CopA2 (37,38). The phenotype experiment suggested that deletion of CopA1 significantly compromised the bacteria growth, while the deletion of CopA2 didn't cause significant changes (38). The copper transport experiment by using everted vesicles demonstrated the copper transport ability of both ATPases (38). To further characterize the biochemical mechanism of the ATPases, the ATPases must be heterogeneously expressed and purified due to the low abundance in their natural environment (59). In this work, the phenotype experiments prove that deletion of CuiT in *P*. *aeruginosa* significantly decreases the copper accumulation in the cells than WT, and the complemented CuiT-deletion strain can reverse the low copper level; the optimum expression conditions are obtained using codon optimization.

4.1 There is no copper tolerance phenotype under long time of incubation

Although the evidence from gene expression and structure model suggests that CuiT is a putative copper-importing transporter, both the Growth curve experiment and Spot test could not reveal the copper tolerance ability from the growth phenotypes of ΔCuiT strain. The strains (WT, ΔCuiT,

and ΔCuiT_comp) did show delayed starting growing points under the copper stimulation in the Growth curve experiment, but no obvious differences were observed on their growing trends during the 12 hours of incubation. Similar in the Spot test, there is no significant growing differences between the three trains for three days of incubation.

One possible reason could be that the copper tolerance phenotype of CuiT was masked by the functions of other copper-handling proteins. A recent study showed that the copper-handling proteins started to express quickly after the copper stimulation and in 5 min the expression folds of CopZ2 and CopA1 increased to ~60 and ~190 folds, respectively (Figure 13) (13). CopZ2 is functioning as a copper sequester to minimize the copper toxicity and CopA1 is a copper efflux transporter with a high velocity (10,38). Therefore, it is likely that strongly upregulated chaperone and copper efflux transporter could eliminate the copper toxicity posed by excess extracellular copper under a relatively long incubation time used in these two methods.

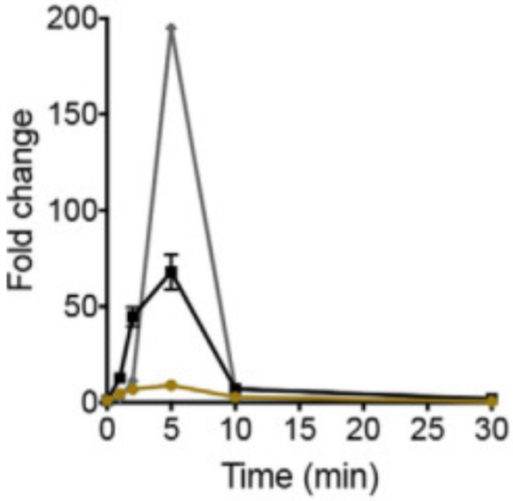

Figure 13 The expression levels of CopZ1, CopZ2, and CopA1 under copper stress condition (13).
CopZ1 (ochre circles), CopZ2 (black squares), and CopA1 (gray diamonds) upon 0.5 mm $CuSO_4$ treatment.

4.2 ΔCuiT strain accumulates less copper within 10 min of incubation

If the copper tolerance of CuiT was masked by the expression of copper-handling proteins, it is reasonable that the copper tolerance phenotype could be observed in a period of short incubation time. However, visible phenotypes in the Growth curve experiment and the Spot test need far more time than a few minutes required for expressing copper-handling proteins. Therefore, the copper uptake experiment was used to investigate the copper-importing phenotypes of CuiT within 10 min of incubation. The results, as expected, show that ΔCuiT strain accumulates less copper than WT at each time points within 10 min, while ΔCuiT_comp strain reversed the phenotype. This is consistent with the idea that the expression of copper-handling proteins would interfere the copper tolerance phenotype of CuiT in *P. aeruginosa*.

4.3 The optimum expressing conditions for both ATPases are obtained.

In this work, N-terminal truncated versions of ΔN-CopA1 and ΔN-CopA2 were used because the N-terminus is capable of binding copper but is unrelated to transfer copper across the membrane (57). The sequences of ΔN-CopA1 and ΔN-CopA2 were optimized for expressing in the *E. coli* host. This step is because most of the amino acids are encoded by synonymous codons and different organisms (*E. coli* and *P. aeruginosa*) normally have their own preferred codons for the same amino acid (60). Autoinduction requires little attention after the first inoculation comparing to the traditional IPTG induction and the lower temperature used in autoinduction also can relieve the overloaded translation in *E. coli* host (61). Therefore, the autoinduction for expressing the ATPases was tested here. However, the results show that the autoinduction method is only effective for expressing the ΔN-CopA1.

4.4 Summary

Although there are no observable phenotypes in both the Growth curve and Spot test experiments, the copper uptake experiment proves that ΔCuiT strain absorbs less copper than WT and ΔCuiT_comp strains within 10 min of incubation. This Cu uptake phenotype demonstrates that CuiT is a copper-importing transporter in *P. aeruginosa*. The copper homeostasis mechanism mediated by CuiT needs to be further elucidated by investigating the kinetic properties, the roles of the two copper binding sites, and the copper exchange with other copper-handling proteins.

Codon optimization of both ΔN-CopA1 and ΔN-CopA2 improved the expression level in *E. coli* host. Although the ΔN-CopA1 and ΔN-CopA1 prefer different induction methods, the expression levels of both ATPases achieve similar expression levels under their respective optimum conditions. The optimum expression condition for ΔN-CopA1 is autoinduction using 400 μM or 1mM IPTG and 20 hours incubation, while ΔN-CopA2 prefers traditional IPTG induction using 1 mM IPTG and 5 hours incubation.

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