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Periplasmic Copper Homeostasis in *Pseudomonas aeruginosa*

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by

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Approved:

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Abstract

Cu^{+}-ATPases are imperative integral membrane proteins that utilize the hydrolysis of ATP to transport Cu^{+} ions into the periplasm. Although much is known of the mechanism of these proteins, the studies of the interactions with molecular chaperone proteins are still incomplete. The goal of this Major Qualifying Project (MQP) was to better understand copper partitioning in the periplasm and identify periplasmic partners of Cu-ATPases in the bacteria *Pseudomonas aeruginosa.* Protocols for copper determination in subcellular protein fractions, copper sensitivity, cytochrome c oxidase and Cu^{2+} reductase activity assays were optimized. Assays involving mutant ΔcopA1 and ΔcopA2 strains demonstrated evidence of altered copper homeostasis, however showed no clear results on Cu^{2+} reductase activity or interactions with periplasmic copper chaperones. Part of these data were reported in the Metallomics (2013) (2): 144-151.

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Introduction

It is well known that many heavy metals are essential for life as they serve multiple catalytic processes from cellular respiration to gene transcription (1). The homeostasis of these metals is critical to maintaining life; too much or too little of a metal could be fatal. To maintain this homeostasis organisms have developed metal transporters to control the distribution of metals to different compartments in the cells.

P-type ATPases are a part of a large family of integral membrane proteins that utilize the energy of ATP hydrolysis to pump certain molecules in or out of the cell. The coupling of ATP hydrolysis with conformational change of transmembrane (TM) segments of the protein allows for subsequent translocation of the molecule (2). Unlike other ATPases, P-type ATPases form a phosphorylated intermediate during the transport of their ion substrate. By translocating ions across the membrane P-type ATPases maintain critical electro-chemical gradients. Many ATPases, like the Na^+/K^+-ATPase and H^+-ATPase, sustain the membrane potential in eukaryotic cells and fuel central cellular processes like secondary transport of metabolites and the electrical excitation in neurons (3).

The P-type ATPases are separated into subfamilies on the basis of substrate specificity (Figure 1). These subfamilies include P_{1A}-ATPases, which transport K^+ ions, P_{1B}-ATPases translocate heavy metals, P_2-ATPases include Ca^{2+}, Na^+/K^+, and H^+/K^+ pumps, P_3-ATPases pump H^+, P_4-ATPases transport phospholipids, and lastly

the P_5-ATPases that have an unknown substrate (8). Transition metals are elements in the d-block of the periodic table that have an incomplete d sub-shell and cannot be free in a cell because of their tendency to form free radicals. Since transition metals are not free in the cell the ATPases that transport them do not operate by interacting with free metal substrates as alkali and alkali earth metal transporters do. These differences between transition metals and alkali metals points towards differences in their transporters, structure and translocation mechanism (2).

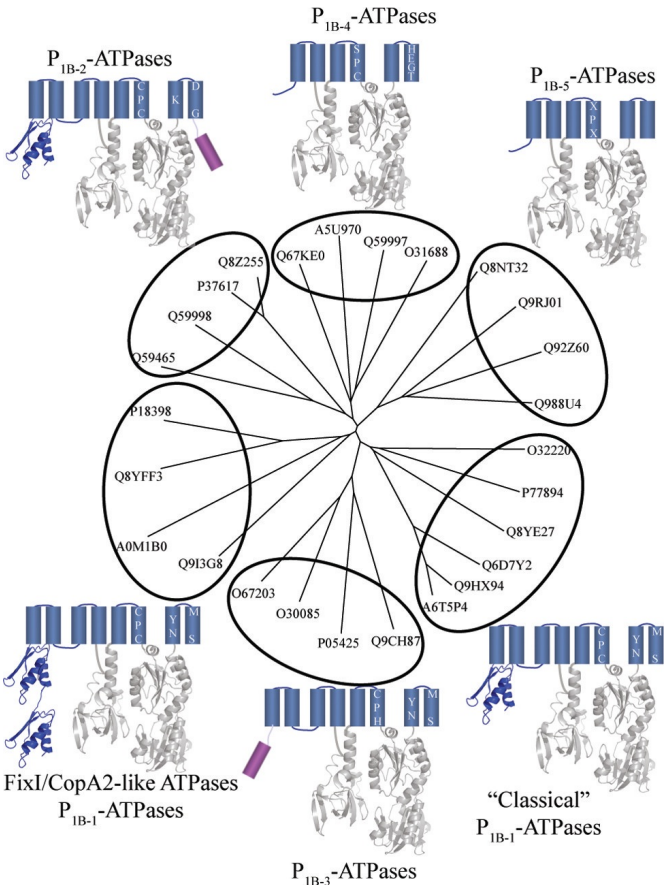

Figure 1: Multiple different P-type ATPases from different organisms organized into their subfamilies including the P-type ATPase general structures (1).

Structure of $P_{1B\text{-}Type}$ ATPases

P_{1B}-ATPases are polytopic membrane proteins that channel metals and serve critical functions in all living organisms with more prevalence in bacteria and archaea. All P-type ATPases exhibit two soluble cytoplasmic domains, an ATP-binding domain (ATP-BD) and an actuator domain (A domain) (8). They also contain a central core comprised of six to eight TM helices containing metal translocation sites and domains involved in ATP binding, energy transduction, and metal controlled regulation (Figure 2) (1).

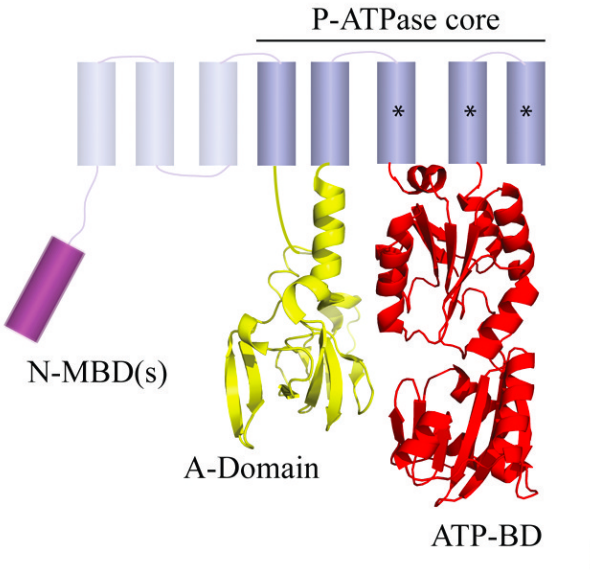

Figure 2: P_{1B}-type ATPase general structure highlighting the TM segments with N-termini metal binding domains (N-MBD), A Domain, and ATP binding domain (ATP-BD). TM segments numbered M1-M8 left to right. (1)

Along with these general P-type ATPases features, most P_{1B}-ATPases also contain two additional TM helices at the N-terminal side (M2 and M3 segments in Figure 2) of the core domain and many soluble metal-binding domains along the N- and C-

termini. The hallmark of the P_{1B}-ATPase subgroup is the conserved amino acids responsible for metal ion binding located in the transmembrane segments M4, M5, and M6 (1). The ATP-BD and the A domain have been characterized separately in Cu^+ ATPases. It was shown that the ATP-BD contains two subdomains, the N and P domains. The P domain includes an invariant DKTGT motif, of which the aspartic acid residue is phosphorylated during the catalytic cycle (5). The A domain contains a conserved rigid GE sequence that is required for dephoshorylation of the enzyme and also likely interacts with the DKTGT motif in the P domain. The A domain contains ten β strands with helices at the N- and C-termini leading into the membrane domains. These domains and other structural features can be seen in the P_{1B}-ATPase CopA, from bacteria *Legionella pneumophila* (Figure 3, with the two Cu^+ binding sites shown in red) (2). By locking this protein in its E2 catalytic conformation (explained in detail in the next section) the location of the transmembrane metal binding sites (TM-MBS) was confirmed and the metal path through the protein to the periplasm by the first two TM segments (MA and MB) was also revealed (1).

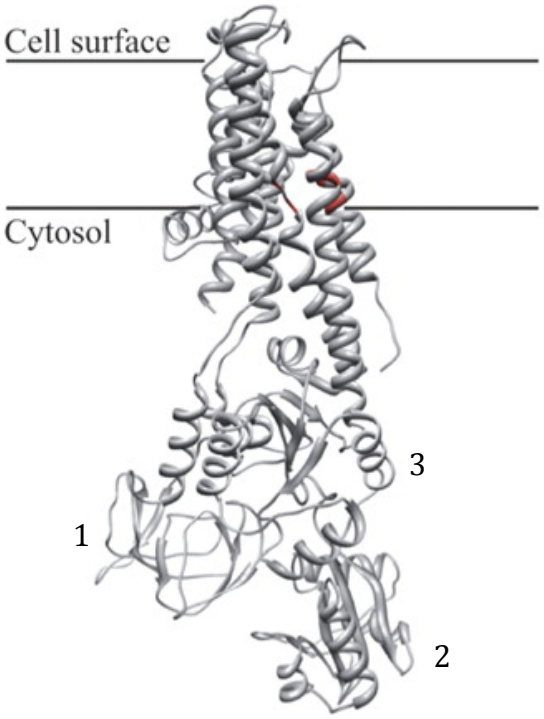

Figure 3: CopA protein structure from bacteria *Legionella pneumophila.* Amino acids forming the two transmembrane copper binding sites are shown in red. A-domain (1) and the N (2) and P domains of the ATP-BD are also indicated (ref 2).

Metal transport over the cell membrane involves complete coordination by each TM segment especially those flanking the ATP-BD. The Cu^+ ATPases bind and transport two Cu^+ ions per one ATP hydrolyzed. Although the MBD's are not required for ATPase activity they do play a regulatory role by reducing metal ion interaction with the ATP-BD (1). Binding site one is comprised of two cysteine residues of M4 and a Tyrosine from M5, while binding site two is constituted by an Asparagine of M5 and a Methionine and Serine of M6 (1). Typical N termini MDB are around 60-70 amino acids long with a βαββαβ ferredoxin-like fold, a shared characteristic of soluble Cu^+ chaperones like CopZ. Along with ion binding domains the P_{1B}-type ATPases have

positively charged residues, which provide complementary electrostatic docking regions for chaperone proteins that deliver the ions to the channel or receive the ions after their translocation (2).

Transport Mechanism of P_{1B}-Type ATPases

The specificity of the P-type ATPases for their substrate ion depends on the highly conserved amino acids in the three metal binding TM segments. These segments flank the catalytic cytosolic loop where the binding, hydrolysis, and consequently couple transport to ATP hydrolysis (2). The catalytic action of P-type ATPases is characterized by the E1/E2 Post-Albers catalytic cycle (Figure 4). In this mechanism, determined by studies in *Archaeoglobus fulgidus*, (2) E1 and E2 represent the two enzyme conformations during ion transport with separate affinities for the metal ion. In the E1 form the enzyme binds nucleotides at the ATP binding domain and the metal substrate, with high affinity, at the TM transport sites from the cytoplasmic side. Although both nucleotides and the metal substrate can bind independently, the coupled simultaneous binding of each is essential for driving the phosphorylation of the aspartic acid residue in the invariant DKTGT sequence and subsequent enzyme turnover (8) Since Cu^+ P_{1B}-type ATPases transport two Cu^+ ions per ATP hydrolysis, full occupancy of transport sites is also required for catalysis.

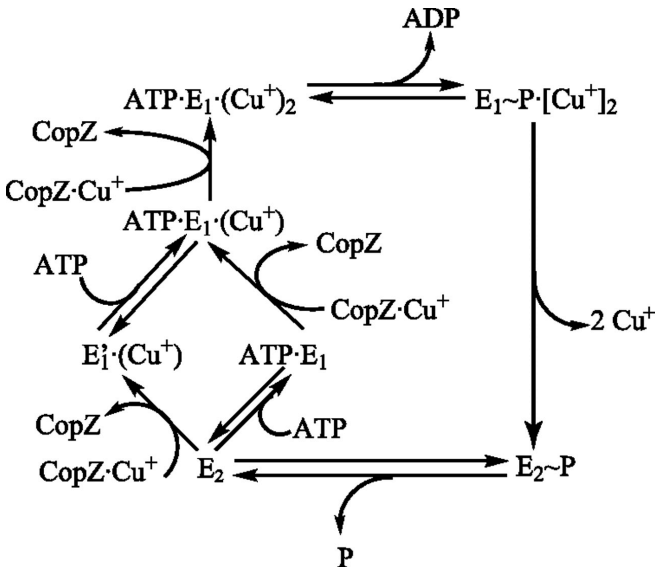

Figure 4: E1/E2 catalytic and transport mechanism of Cu^+ ATPases (6)

The phosphorylation of the aspartic acid residue in the enzyme creates conformational change causing the enzyme to release the Cu^+ ions into the periplasmic region of the cell. This action is the enzyme's transformation to its phosphorylated E2 conformation. It is important to note that P_{1B}-type ATPases have a relatively slow turnover rate due to the fact that transition metals are not free/hydrated in the cell. This creates a need for metal chaperones, like CopZ, to transport the ions to and from the integral ATPases. In this mechanism, CopZ plays an important role of transporting Cu^+ ions to the E1 form of the enzyme. The metal ions do not access the transport sites of the enzyme freely but bound to the chaperone molecule. The electronegative site of CopZ is attracted to the electropositive site on the ATPase causing docking of the chaperone on the ATPase and subsequently the initiation of metal release from CopZ to the ATPase by ligand exchange (5).

The small diamond in the pathway in the figure demonstrates that either the ATP or Cu^+ ion can bind to the E1 enzyme independently, however not until both are bound at the same time does the pathway continue. The hydrolysis of the ATP molecule catalytically drives the movement of the Cu^+ ions over the membrane to the periplasm. As indicated, the phosphorylation of the aspartic acid in the enzyme initiates the release of the Cu^+ ions into the periplasm, and it is hypothesized that the presence of another chaperone molecule speeds up the slow transport rate of the P_{1B}-type ATPase (2). The metal transfer from CopZ to the TM-MBs of the ATPase is essential to the copper homeostasis, allowing the transfer of cytoplasmic Cu^+ ions out of the cell while preventing release of the free ion (8).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is a rod shape, gram-negative bacteria belonging to the family Pseudomonadaceae. *P. aeruginosa* is a free-living bacteria residing mostly in soil and water. This bacterium, usually a plant pathogen, is also recognized as an opportunistic pathogen, meaning it exploits a break in the host's defenses to initiate an infection. The bacterium almost never infects healthy human tissue, but can infect nearly any compromised tissue. This most often happens in a nosocomial setting, where a host's immune system is already weakened. According to the Center for Disease Control (CDC), *P. aeruginosa* now accounts for 10.1 percent of all hospital-acquired infections (9).

P. aeruginosa contain two well-characterized Cu^+ ATPase homologues CopA1 and CopA2, expressed in response to different Cu^+ stresses. CopA1 is expressed due to

high copper content in the cell and when deleted creates copper sensitivity. CopA1 has demonstrated a lower affinity for Cu^+ ions and a higher ion transfer rate. CopA2 is co-expressed with cytochrome c oxidase subunits and when mutated shows reduced oxidase activity. CopA2 has the opposite characteristics to CopA1, a higher affinity for Cu^+ and a lower turnover rate. Mutations in both CopA1 and CopA2 lowered virulence in a plant infection assays indicating the importance of these ATPases in the biology of *P. aeruginosa*. Multiple periplasmic chaperones are likely to play a role in copper transfer from Cu^+ ATPases to target molecules (6).

There is a high desire to understand the movement of metals through an organism to help comprehend metal homeostasis. Much is known regarding the mechanism of the transport of copper from the cytosol to the ATPases and then into the periplasm in *P. aeruginosa*. However, once the Cu^+ ion has been transported the interactions necessary to transport the Cu^+ ions from the periplasm to the extracellular space or to be involved in periplasmic mechanisms is not well known. This study was conducted to gain more knowledge about the partitioning of copper in the periplasm and the periplasmic proteins that interact with the Cu^+ ion after the ATPase has translocated it across the membrane (Figure 5).

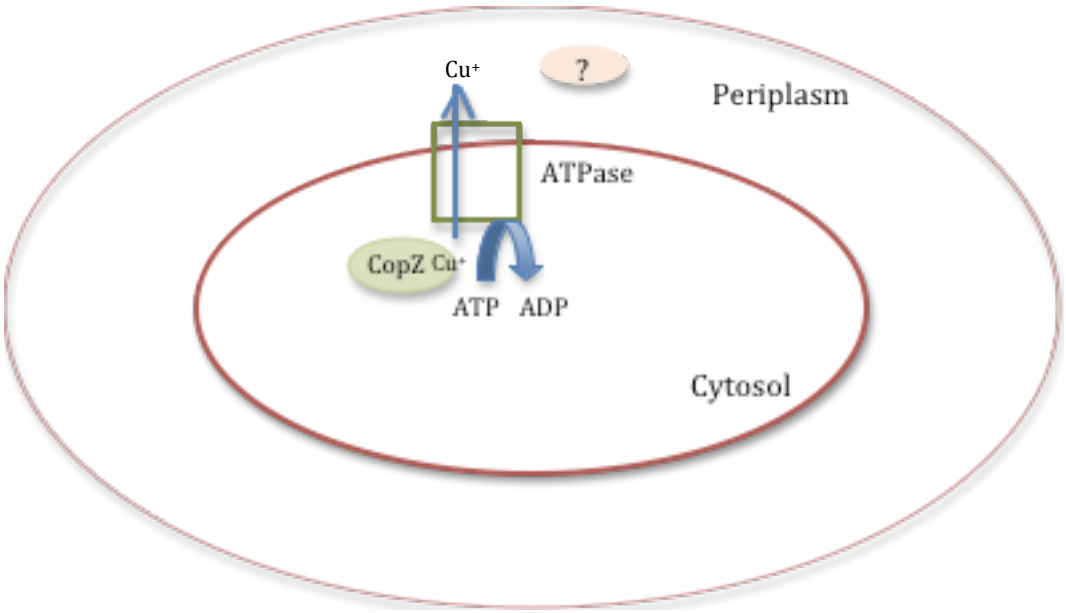

Figure 5: Diagram of Cu ion transport from the cytosol into the periplasm with CopZ chaperone and unknown periplasmic chaperone(s).

Several periplasmic proteins hypothesized to have an interaction with the Cu^+ ions and their ATPases were chosen to study (Table 1). Previous mass spectrometry studies of Cu^+ and Zn^+ identified periplasmic proteins that could possibly play a role in the homeostasis of Cu^+ in the periplasm (7). A few other periplasmic proteins were chosen based on their amino acid sequence and structure predicted to interact with Cu^+. PA0222, PA0223, PA1074, PA4922, PA5217, and PA5489 were all chosen for study due to their identification in mass spec analyzes. PA2807, PA3785, and PA3868 were chosen because of their sequences that could possibly make them good candidates for possible interaction with Cu^+.

Table 1: Periplasmic and cytoplasmic proteins chosen for study

Gene	Protein	Reason chosen for study
PA0222	Hypothetical Protein	Identified in mass spectrometry analysis of Cu^+ spot Possible candidate to work with CopA1 in Cu^+ efflux system
PA0223	Probable dihydrodipicolinate synthetase	Contiguous protein to PA0222 Possible candidate to work with CopA1 in Cu^+ efflux system
PA1074	Branched-chain amino acid transport protein BraC	Identified in mass spec in WT and CopA1 periplasmic protein samples Branched amino acid metabolism hampered by Cu^+
PA2807	Hypothetical Protein	Induced after a Cu^+ shock in *P. aeruginosa* Protein orthologs bind Cu^+ in periplasm
PA3785	Hypothetical Protein	Candidate to bind Cu^+ in periplasm because of its sequence, containing methionine
PA3868	Hypothetical Protein	Candidate due to its amino acid sequence CSWDQFC
PA4922	Azurin Precursor	Observed in mass spec Cu^+ spot of WT, ΔcopA1, and ΔcopA2
PA5217	Probable binding protein component of ABC iron transporter	Identified by mass spec in WT Zn^+ spot
PA5489	thiol:disulfide interchange protein DsbA	Identified by mass spec Contains CXXC motif

To study these proteins it is important to isolate them and characterize their activities and interactions with CopA1 and CopA2. Cultures of wild type, PAO1, and mutant, Δ copA1, Δ copA2, Δ PA4922, Δ PA3868, Δ PA0222, Δ PA1074, Δ PA2807, Δ PA5217, Δ PA3785, Δ PA5489, and Δ PA0223 *P. aeruginosa* cells had to be grown. From the cultures of PAO1, Δ copA1, and Δ copA2 cytosolic, periplasmic,

membrane, and secreted protein fractions could be isolated, purified, and concentrated. Copper sensitivity assays, Cu^{+2} reductase activity assays, and cytochrome c oxidase assays were then preformed to determine which periplasmic chaperone's activity most likely corresponds to the integral Cu^{+} ATPases.

Materials and Methods

Cell Culture and P. aeruginosa Strains: PAO1, ΔcopA1, ΔcopA2 Δ PA4922, Δ PA3868, Δ PA0222, Δ PA1074, Δ PA2807, Δ PA5217, Δ PA3785, Δ PA5489, and Δ PA0223 insertional mutation strains were obtained from the Comprehensive *P. aeruginosa* Transposon Mutant Library at the University of Washington Genome Center. Cells were grown aerobically at 37° C, 200 rmp in LB medium supplemented with 1 μM Cu and 60 μg/ml as required. Complemented strains were obtained as previously described and grown in LB supplemented with 30 μg/ml gentamycin (6).

Preparation of Subcellular Fractions: Cells cultured in 125 ml of LB were harvested at early stationary phase by centrifugation at 4,000 *x g*, 4° C for 10 min. Proteins in the supernatant were concentrated to 5-10 mg/ml using 3 kDa-Centricon tubes and considered the secreted protein fractions. Pelleted cells were resuspended and washed with 30 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 0.3 M sorbitol buffer S and incubated 20 min at room temperature. A third wash was preformed with buffer S to remove remnant ETDA. Finally, cells were resuspended in ice-cold MilliQ H_20, incubated for 5 min and then centrifuged at 14,000 *x g* at 4° C for 30 min. Supernatant proteins were collected, centrifuged to remove possible remnant

cellular contamination, concentrated to 5-10 mg/ml using 3 kDa-Centricon filters and considered periplasmic protein fractions. The pelleted cells were again resuspended in 50 mM Tris-Cl pH 7.4 and passed through the French press at 2 p.s.i. to lyse/break cells. The membrane fraction was recovered after centrifugation at 100,000 *x g* for 1 hour at 4° C and resuspended in the same buffer. All fractions were analyzed immediately to minimize metal equilibration and potential metal transfer (6).

Copper Content Determination: Protein fractions were acid digested with concentrated nitric acid overnight at room temperature. Following digestion, samples were treated with 1.5% H_2O_2. The copper concentration in each sample was measured by furnace Atomic Absorbance Spectrometry (6).

Cu(II)-reductase Activity: PAO1, ΔcopA1, ΔcopA2 cultures were grown, both aerobically and anaerobically at 37° C overnight in minimal salt medium M9 with 0.1% tryptone and supplemented with either 0.5% glycerol or glucose as indicated. Cultures centrifuged at 4,000 *x g* for 15 min at 4° C and pellets resuspended in 15 ml citrate-phosphate buffer supplemented with corresponding 0.5 % glycerol or glucose. 15 ml samples split into two separate tubes of 7.5 ml supplemented with 0.2 mM $CuSO_4$ and 0.5 mM Bathocuproine disulfate (BCS). Samples were incubated at 37° C up to 120 min. Every 20 min from time zero aliquots were taken and centrifuged at 14,000 *x g* for one min and supernatant's OD was determined at $A_{480\text{nm}}$ (10).

Copper Sensitivity: LB medium cultures of PAO1, ΔcopA1, Δ PA4922, Δ PA3868, Δ PA0222, Δ PA1074, Δ PA2807, Δ PA5217, Δ PA3785, Δ PA5489, and Δ PA0223 cells were grown over night. At OD_{600} of 0.1 cultures were supplemented with 0 mM, 1 mM, 2 mM, and 4 mM of $CuSO_4$ and again grown overnight. Cells were measured at OD_{600} to determine the amount of cell growth in each concentration of $CuSO_4$ (4).

Preparation of Double Mutants: ΔPA4922, ΔPA3868, ΔPA0222, ΔPA1074, ΔPA2807, ΔPA5217, ΔPA3785, ΔPA5489, and ΔPA0223 mutant genes were cut and amplified by PCR then ligated into a pCHESI-Ωkm vector. The pCHESI-Ωkm vectors containing periplasmic mutant genes were transformed into competent DHSα *E. coli* cells. PAO1, ΔcopA1, ΔcopA2 *P. aeruginosa* strains and all periplasmic mutant genes expressed in *E. coli* stated above were cultured in 5 ml LB overnight. 100 µl of either ΔcopA1 or ΔcopA2 *P. aeruginosa* strain, kanamycin resistant pRK2013 helper, and one of the periplasmic mutant genes expressed in *E. coli* were placed in an eppendorf tube (24 combinations and tubes total), mixed, and centrifuged at room temperature at 6,000 *x g* for two min. Pellets were resuspended in one ml of LB and centrifuged again. Finally pellets were resuspended in 30 µl warm LB and placed on a single filter in a warm LB plate containing 30 µg/mL tetracyclin and 100 µg/mL kanamycin to select for the new *P. aeruginosa* double mutant strains. All eight plates, containing three filters each, were placed in the incubator overnight. Each single filter was placed in 200 µl warm LB and vortexed and grown in 5 ml LB overnight.

Cytochrome c oxidase Activity: 1 mL cultures of PAO1, ΔcopA1, ΔcopA2, all periplasmic mutants and all double mutants were pelleted from overnight 5 mL cultures were washed twice with 0.9% NaCl and resuspended in 1 mL buffer C. Steady-state reduction of TMPD in the presence of ascorbate, and O_2 was measured in 1/10 dilutions of the buffer C resuspensions to observe cytochrome c oxidase activity.

Results

The overall objective of this study was to gain more knowledge involving the partitioning of copper in the periplasm and the periplasmic proteins that interact with the Cu+ ions after the ATPases CopA1 and CopA2 translocates them across the membrane of the bacteria *P. aeruginosa.* To accomplish this objective PAO1, ΔcopA1, and ΔcopA2 bacterial strains were involved in several copper assays and also cultured for preparation of cytosolic, periplasmic, membrane, and secreted protein fractions. Other insertional protein mutants, PA4922, PA3868, PA0222, PA1074, PA2807, PA5217, PA3785, PA5489, and PA0223, were also cultured and tested for copper sensitivity assays and used to create double mutants with ΔcopA1 and ΔcopA2 mutant strains.

Cu^{2+} reductase activity

In *Escherichia coli* it was previously shown that electron flow through the electron transport chain promoted cupric ion reduction by NADH dehydrogenase-2 and

quinones. In order to test this activity in Cu-ATPases of *P. aeruginosa*, PAO1 WT, ΔcopA1, and ΔcopA2 mutants were grown, both aerobically and anaerobically, in minimal salt medium in the presence of $CuSO_4$ and supplemented with 0.5% glycerol as a reduced substrate. Since Bathocuproine disulfonate (BCS) forms a colored complex with Cu^+ ions the compound was added to the cultures at time 0 min and used to measure the amount of Cu^+ ions in solution over 120 mins. The presence of Cu^+ in the solution indicates that the bacterial strains are interacting with NADH and quiniones to reduce Cu^{2+} to Cu^+ in the system. For all strain types the appearance of Cu^+ in the system accumulated over the 120 min (Figure 6). Compared to the PAO1 WT strain, there was no significant decline in Cu^{2+} reductase activity in either the ΔcopA1 or ΔcopA2 mutant strains. The alteration of either CopA1 or CopA2 did not hinder the reduction of Cu^{+2} to Cu^+.

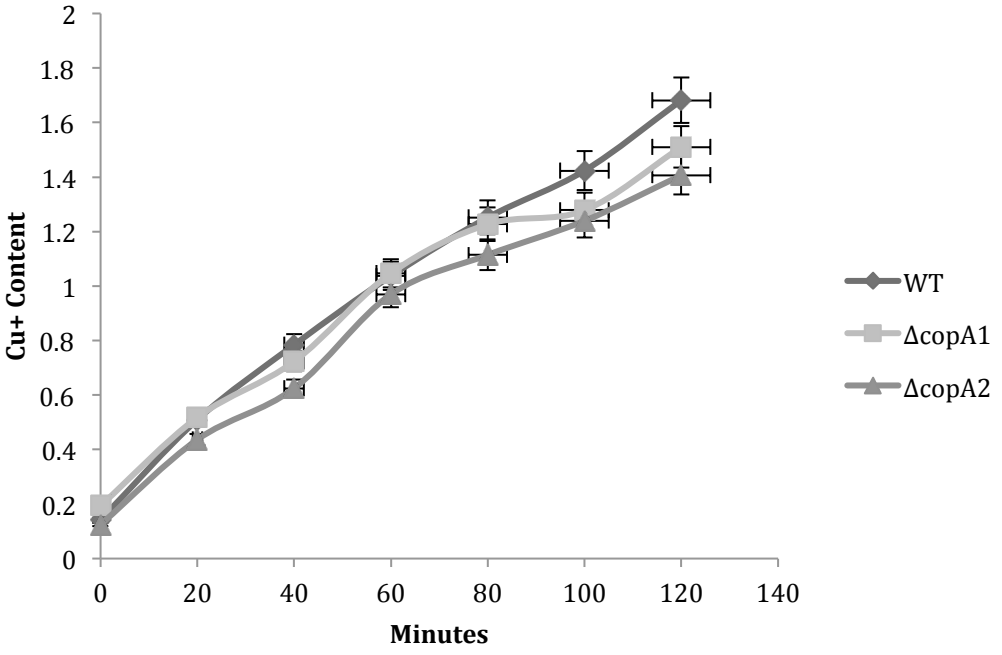

Figure 6: Cu^{2+} reductase activity by PAO1 WT, ΔcopA1, and ΔcopA2 mutant bacterial strains. Cultures grown aerobically overnight in minimal salt media with 0.2 mm CuSO4, 1% tryptone, and 0.5% glycerol. Reductase activity was measured at A_{480} with 0.5 mM BCS over 120 mins in five different experiments.

Reductase activity was also tested using whole cells grown two days anaerobically in Gaspak EZ pouch system (BD) with 12 mM nitrate. During anaerobic activity a respiratory electron transport chain is still used without oxygen as a final acceptor and in this case nitrogen. This experiment was conducted to see if either CopA1 or CopA2 are critical components in the anaerobic respiration. Samples were measured at OD_{600} for Cu^{+} content in solution (Figure 7). The results from the single study hint at a greater importance of CopA1 in Cu^{+} reductase activity in anaerobic conditions as evidenced by the decreased Cu^{2+} reductase activity in cells with mutated CopA1 but not mutated CopA2. Additional studies are necessary to confirm this observation and the importance of CopA1 in Cu^{+} reductase activity in anaerobic conditions.

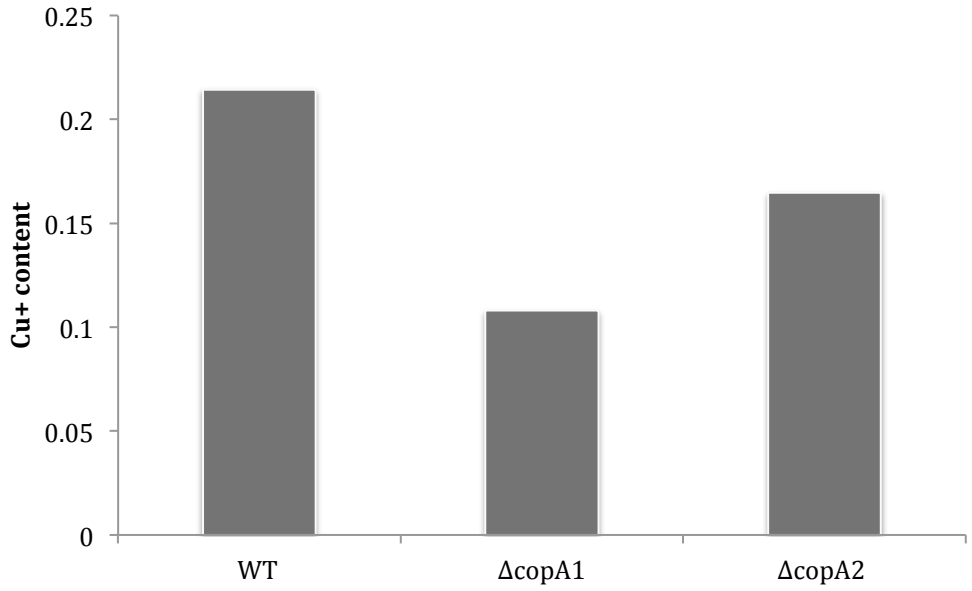

Figure 7: Cu^{2+} reductase activity by PAO1 WT, ΔcopA1, and ΔcopA2 mutant strains. Cultures were grown for two days anaerobically with 12 mM nitrate and measured at A_{600} in the spectrophotometer.

Cu^{+} Determination in Subcellular Protein Fractions

Since CopA1 and CopA2 both play a role in exporting cytoplasmic Cu^{+} to the periplasm, any manipulation to these proteins should alter copper homeostasis. Both CopA1 and CopA2 respond to the presence of intracellular copper with different transport rates and affinities adapted by their functional roles in copper homeostasis. Export of intracellular copper may also be affected by the presence of periplasmic chaperones. To identify these chaperones the protein bound copper in PAO1, ΔcopA1and ΔcopA2 mutant strains was measured in periplasmic, membrane and secreted proteins fractions using furnace atomic absorbance spectrometry (Figure 8). The protein fractions were isolated from each strain grown in the stationary in LB media and digested overnight with concentrated nitric acid and then treated with 1.5% H_2O_2. Over all three strains there was no change in protein bound copper in the secreted proteins. However, in membrane proteins of both the ΔcopA1 and ΔcopA2 mutants, the copper content was lower compared to the wild-type strain. Additionally, an increase in copper content of the cytosolic protein fraction in the ΔcopA1 mutant strain was observed. These findings correlate with the lack of functionality of the CopA1 mutant and the decrease in oxidase activity in the ΔcopA2 mutant. The increase in copper content of the Cu-ATPase's periplasmic protein fractions was unexpected and suggests significant changes in periplasmic copper homeostasis. Importantly, gene complementation rescued the copper levels in the periplasmic fractions of both mutants to levels equivalent to the wild type.

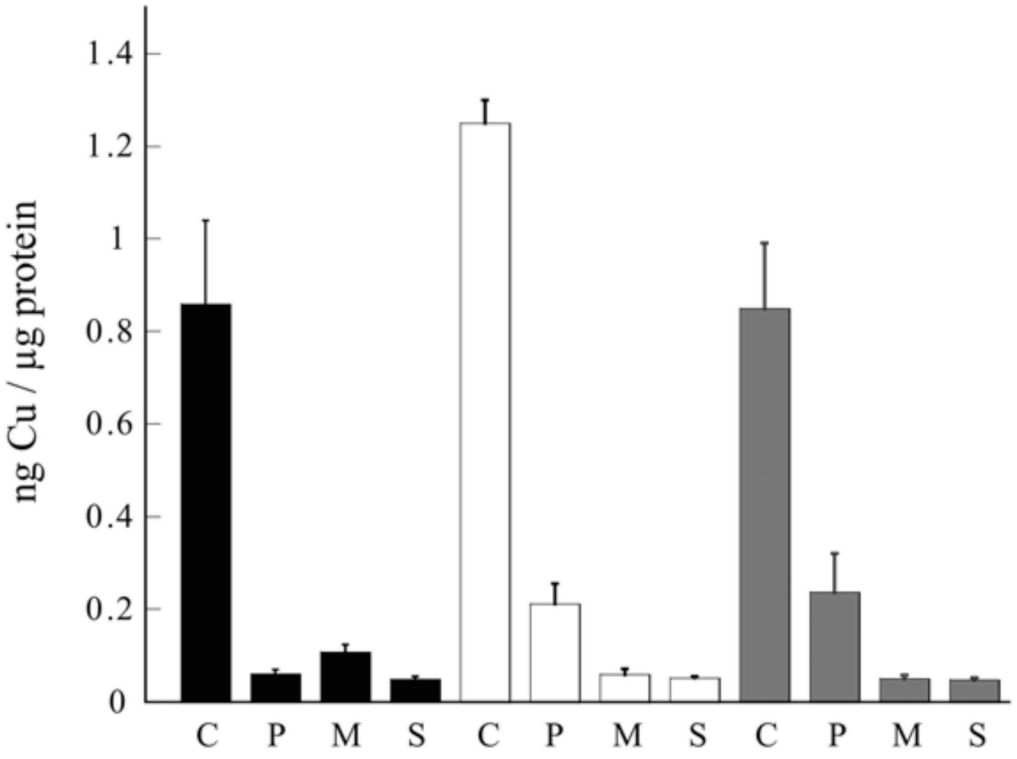

Figure 8: Cu content in periplasmic (P), membrane (M), and secreted (S) protein fractions of PAO1 WT (black), CopA1 (white) and CopA2 (gray). Cu was determined by furnace AAS and normalized to protein content in the sample (6).

Cu^+ Sensitivity

Since Cu-ATPases are critical for maintaining copper homeostasis in many bacteria, disruption of these proteins results in a sensitivity to copper, especially CopA1, which is induced with copper in the cell. To identifying certain protein-protein and copper-protein interactions in the periplasm after the Cu-ATPases have transported Cu^+ ions to the periplasm, multiple periplasmic protein mutants (Table 1) were tested for a sensitivity to copper. Each strain was grown overnight in LB media and then supplemented with either 0 mM, 1 mM, 2 mM, or 4 mM $CuSO_4$ and grown for another night. Cultures were then measured in the spectrophotometer at A_{600} (Figure 9). As expected the ΔcopA1 mutant displayed a copper sensitivity,

illustrating decreased growth in the presence of any concentration of copper. None of the protein mutant studies demonstrated any significant decreased growth at 1 mM Cu^+ like the ΔcopA1, but a few had varying growth at 4 mM Cu^+. Mutant strain ΔPA5217 demonstrated an increase in growth at 4 mM Cu^+ indicating a higher resistance to high Cu^+ levels.

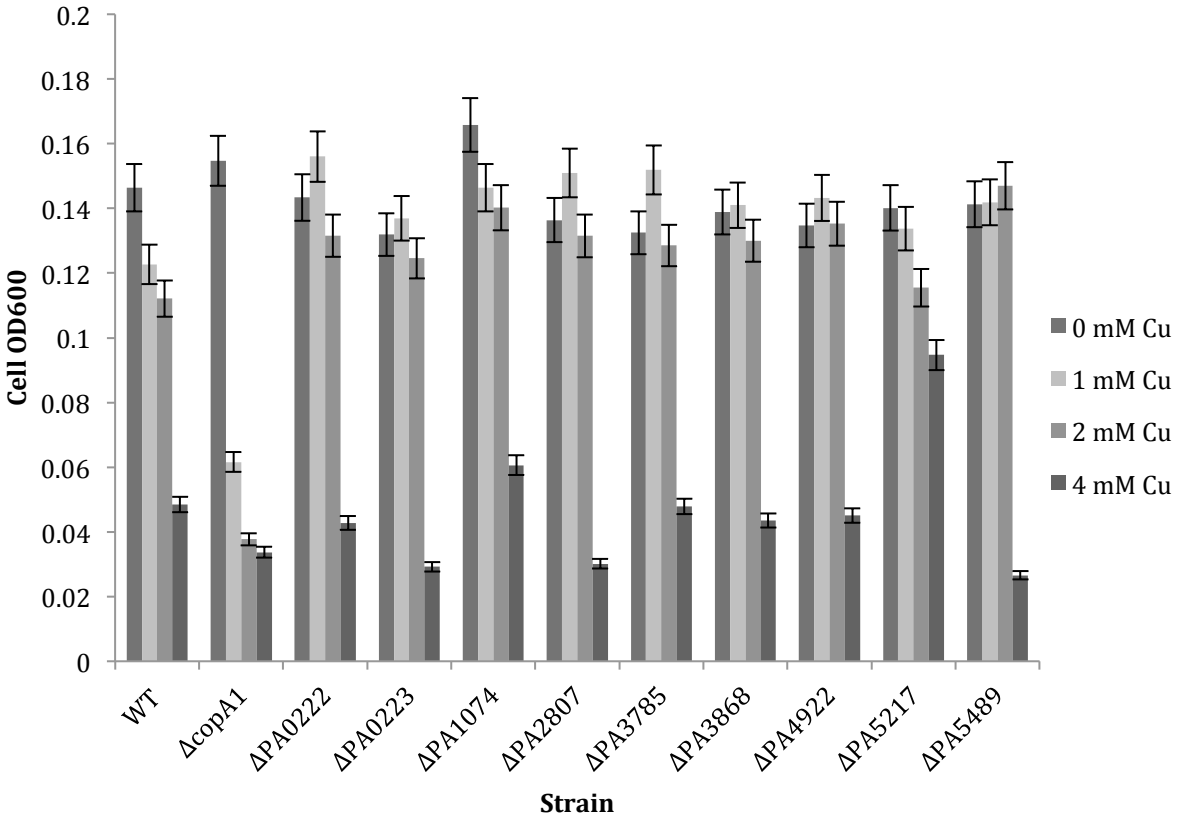

Figure 9: Copper sensitivity of periplasmic protein mutant strains. Grown overnight and supplemented with 0 mM, 1 mM, 2 mM or 4 mM CuSO4. OD measured to determine sensitivity.

Cytochrome c oxidase Activity

Cytochrome c oxidase subunits contain essential copper centers that accept electrons from cytochrome c and transfer them to oxygen in order to form water at the end of electron transport chain. CopA2 is co-expressed with cytochrome c

oxidase subunits and when mutated shows reduced oxidase activity. It is hypothesized that these copper centers are transported from CopA2 to cytochrome c oxidase by other copper-binding chaperones. To further study the periplasmic interactions of Cu-ATPases, copper ions, and putative chaperones a study of cytochrome c oxidase activity was conducted. Double mutants of CopA2 and all periplasmic proteins were prepared to study the interactions between the cytochrome c oxidase co-expressed Cu-ATPase CopA2 and any proposed periplasmic Cu^{+} chaperones. All periplasmic protein mutants and ΔcopA2/periplasmic protein double mutants were grown overnight in LB and then a steady-state reduction of cytochrome c in the presence of oxidase, TMPD, ascorbate, and O_2 was measured in 1/10 dilutions of the buffer C resuspensions. The results of the first study demonstrated there was not a significant decrease in oxidase activity for any of the periplasmic mutants compared to the ΔcopA2 mutant (Figure 10). However, in the double mutant study the ΔcopA2/4922 H09 (azurin) mutant showed highly decreased oxidase activity comparable to the ΔcopA2 mutant. Most double mutants unexplainably displayed results comparable to the WT strain (Figure 11).

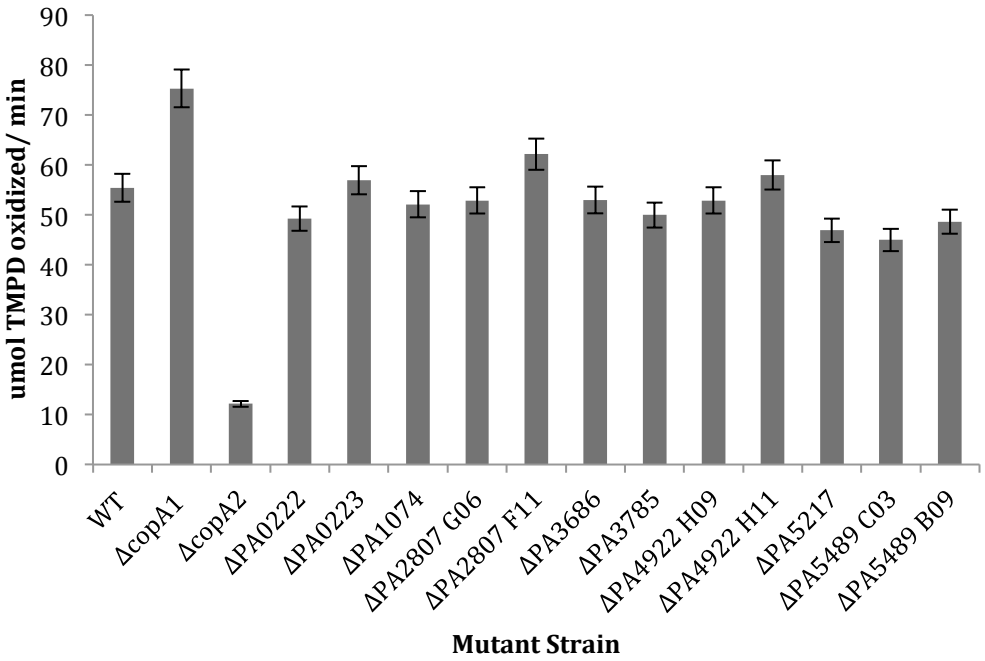

Figure 10: Cytochrome c oxidase activity assay preformed on all periplasmic mutant proteins. Steady-state reduction of TMPD was measured in the spectrophotometer at OD_{600} to illustrate cytochrome c oxidase activity.

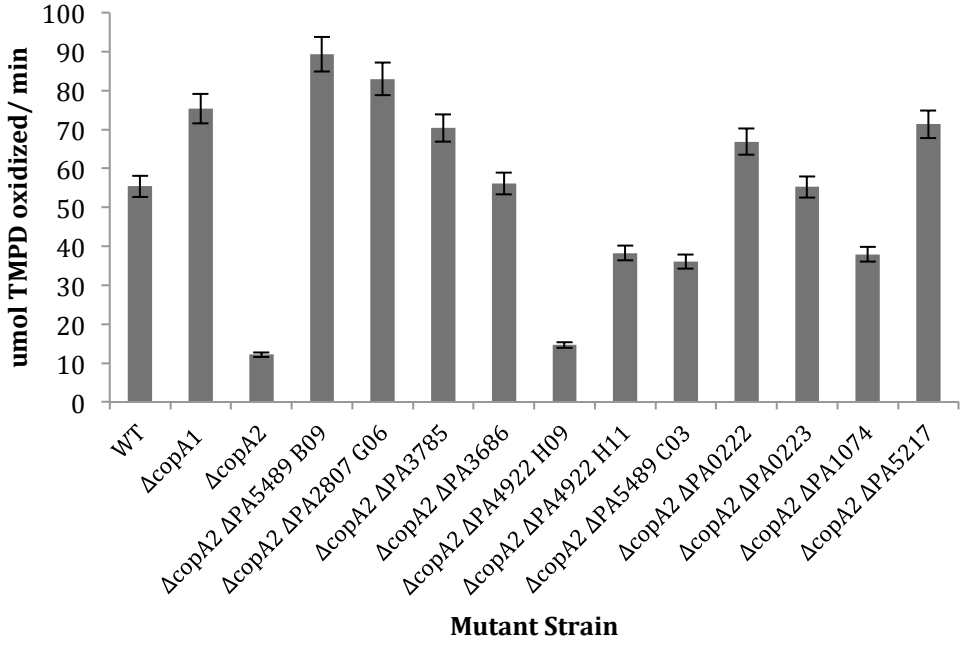

Figure 11: Cytochrome c oxidase activity assay preformed on all ΔcopA2/periplasmic double mutants. Steady-state reduction of TMPD was measured in the spectrophotometer at OD_{600} to illustrate cytochrome c oxidase activity.

Discussion

The overall goal of this study was to understand copper partitioning in the periplasm and investigate the role of periplasmic chaperones in copper homeostasis in *P. aeruginosa*. A series of studies were performed to investigate the role of CopA1 and CopA2 in Cu^{2+} reductase activity, copper partitioning through out the cell, cytochrome c oxidase activity, and the identification and interaction with periplasmic proteins. These studies provided some interesting results involving CopA1 and CopA2 and their interactions with putative periplasmic chaperones, which contributed to a larger study (appendix 1). Multiple periplasmic proteins were selected to study based on previous identification in mass spec analyzes or due to their structure, making them strong candidates for copper interaction. Experiments successfully done with other bacterial strains were conducted to isolate any periplasmic proteins that interact with the Cu-ATPases or Cu^{+} after its transport to the periplasm from the cytosol.

Cu^{2+} reductase activity has been previously shown in *E. coli* by the electron flow through the transport chain. As evidence by *E. coli's* ATPases it was hypothesized that ΔcopA1 and ΔcopA2 mutants of *P. aeruginosa* would demonstrate a decreased amount of Cu^{2+} reductase activity. However, ΔcopA1 and ΔcopA2 mutants demonstrated Cu^{2+} reductase activity comparable to the WT strain when grown aerobically (Figure 6). The strains were also grown anaerobically to see if reductase activity changed in different reparatory conditions with separate final acceptors (oxygen and nitrogen). The result of this experiment hinted that the ΔcopA1 mutant

demonstrated decreased anaerobic Cu^{2+} reductase activity, but since the methodology was not perfected and only one trial was preformed, more studies would need to be preformed to validate this result (Figure 7). Further trials and possible comparison by preforming this experiment with *E. coli* to refine the methods could conclude if ΔcopA1 did in fact shown a decreased amount of Cu^{2+} reductase activity.

Copper presence in the periplasmic space is critical since it participates in aerobic respiration as well as required for redox stress proteins. Due to this, it was hypothesized that copper homeostasis and accumulation in subcellular fractions would shift in the Cu-ATPase mutant strains. Most notably it was expected that the amount of copper in the cytosol would increase with subsequent decrease of copper in the periplasm due to non-functioning Cu-ATPases. Alternatively, it was shown that there was a large increase in cooper levels in the periplasm sub fractions of the two Cu-ATPase mutants (Figure 8). In the ΔcopA1 mutant strain the copper content in the cytosol also increased. Both Cu-ATPase mutants demonstrated a decrease in copper levels in the membrane protein fractions. The increase in copper in the cytosol fractions of ΔcopA1 matches the hypothesis, however the rise in copper content in both mutant strain's periplasm is unexpected. Further studies of the cause of the increase in copper in the periplasm of the mutants revealed that there was also an increase in azurin production (Appendix 1).

CopA1 is expressed with excess amounts of copper in the cell and is essential for the homeostasis of copper. Any mutation that lacks in CopA1's functionality will cause

the cells to have a sensitivity to copper. In the search for putative periplasmic Cu^{+} chaperones a copper sensitivity assay involving all selected periplasmic protein mutants was performed. All of the periplasmic mutants had growth in the presence of 0 mM, 1m mM, 2 mM, and 4 mM $CuSO_4$ (Figure 9). None of the periplasmic protein mutants demonstrated a copper sensitivity comparable to that of the ΔcopA1 mutant. However, one periplasmic protein mutant, ΔPA5217, demonstrated a higher copper resistance than all other strains, having increased growth in the presence of 4 mM Cu^{+}. Further experiments involving ΔPA5217 could help characterize the mutant's copper resistance.

Since copper is critical in the electron transport chain of oxidative respiration and it is well known that CopA2 is co-expressed with cytochrome c oxidase, a cytochrome c oxidase activity assay was performed with all periplasmic protein mutants as well as all ΔcopA2/periplasmic double mutants. The experiment for any one single periplasmic protein mutant strain did not identify any difference between the mutants and the WT strain (Figure 10). This could be that the system was not dynamic enough to detect any subtle oxidase changes in the mutant strains. However, when the periplasmic strains were double mutated with ΔcopA2 an interesting result was shown (Figure 11). All the double CopA2/periplasmic protein mutants demonstrated an unexplainable regain in oxidase activity comparable to WT, except ΔcopA2/ΔPA4922. This double mutant strain, missing both the Cu-ATPase CopA2 and the PA4922 (azurin) periplasmic protein functionality, demonstrated very low oxidase activity similar to the single ΔcopA2 mutant. Preforming this study again with the CopA2/periplasmic protein double mutants as

well as CopA1/periplasmic protein double mutants is needed to verify the unexpected results of the CopA2 double mutants. Nevertheless, the lower oxidase activity of ΔcopA2/ΔPA4922 could suggest that the periplasmic azurin protein plays a role in oxidative stress.

Further studies could be conducted to try and narrow down the search for putative periplasmic chaperones. Since the Cu-ATPases have a relatively slow transport rate and possible chaperones in the periplasm may speed up the rate of transport, Cu^{+} transport experiments done with Cu-ATPases and the selected periplasmic mutant proteins could more closely suggest which periplasmic proteins interact with the ATPases themselves. When PA4922 was tested for copper sensitivity alone there was no difference compared to the WT, but when double mutated with ΔcopA2 there was a significant copper sensitivity. This shows that trying to isolate chaperones by studying one mutant protein at a time may be challenging due to other possible protein compensation. But pooled mutant protein studies (a strain including multiple periplasmic protein mutations) could be tested and further sub selection of positive mutant pools could be then be separated and retested to further identify periplasmic chaperones.

References

1. Arguello JM, Gonzalez-Guerrero M, Raimunda D. Bacterial transition metal P(1B)-ATPases: Transport mechanism and roles in virulence. *Biochemistry*. 2011; 50(46): 9940-9949. doi: 10.1021/bi201418k; 10.1021/bi201418k.

2. Arguello JM, Raimunda D, Gonzalez-Guerrero M. Metal transport across biomembranes: Emerging models for a distinct chemistry. *J Biol Chem*. 2012; 287(17): 13510-13517. doi: 10.1074/jbc.R111.319343; 10.1074/jbc.R111.319343.

3. Bublitz M, Morth JP, Nissen P. P-type ATPases at a glance. *J Cell Sci*. 2011; 124(Pt 15): 2515-2519. doi: 10.1242/jcs.088716; 10.1242/jcs.088716.

4. González-Guerrero M, Raimunda D, Cheng X, Argüello JM. Distinct functional roles of homologous cu+ efflux ATPases in pseudomonas aeruginosa. *Mol Microbiol*. 2010; 78(5): 1246-1258. doi: 10.1111/j.1365-2958.2010.07402.x.

5. Padilla-Benavides T, McCann CJ, Arguello JM. The mechanism of cu+ transport ATPases: INTERACTION WITH CU+ CHAPERONES AND THE ROLE OF TRANSIENT METAL-BINDING SITES. *J Biol Chem*. 2013; 288(1): 69-78. doi: 10.1074/jbc.M112.420810; 10.1074/jbc.M112.420810.

6. Raimunda D, Padilla-Benavides T, Vogt S, et al. Periplasmic response upon disruption of transmembrane cu transport in pseudomonas aeruginosa. *Metallomics*. 2013; 5(2): 144-151. doi: 10.1039/c2mt20191g; 10.1039/c2mt20191g.

7. Raimunda D, Khare T, Giometti C, Vogt S, Arguello JM, Finney L. Identifying metalloproteins through X-ray fluorescence mapping and mass spectrometry. *Metallomics*. (9): 921.

8. Rosenzweig AC, Arguello JM. Toward a molecular understanding of metal transport by P(1B)-type ATPases. *Curr Top Membr*. 2012; 69: 113-136. doi: 10.1016/B978-0-12-394390-3.00005-7; 10.1016/B978-0-12-394390-3.00005-7.

9. Todar K. Opportunistic infections caused by *pseudomonas aeruginosa*. In: *Todar's online textbook of bacteriology.* ; 2008.

10. Volentini S, Farías R, Rodríguez-Montelongo L, Raspisarda V. Cu(II)-reduction by *Escherichia coli* cells is dependent on respiratory chain components. *BioMetals*. 2011; 24(5): 827-835.

Appendix 1

Raimunda D, Padilla-Benavides T, Vogt S, et al. Periplasmic response upon disruption of transmembrane cu transport in pseudomonas aeruginosa. *Metallomics*. 2013; 5(2): 144-151. doi: 10.1039/c2mt20191g; 10.1039/c2mt20191g.

http://pubs.rsc.org/en/content/articlepdf/2013/mt/c2mt20191g