Chapter 1. Introduction

1.1 Bacterial resistance to the toxicity of metals

Life is a game of survival, and the fittest survives. In order to adapt the environmental changes, bacteria are capable to continuously adjust themselves to be fitted into their changing surroundings. For example, *Staphylococcus aureus* harboring penicillinase plasmid were found to carry additional genetic determinants of resistance to a series of heavy metal ions as well as penicillin and, in some cases, erythromycin (Novick, 1963; Novick and Roth, 1968). Similarly, plasmid-mediated resistance to metal ions, such as antimonite, arsenic, arsenate, cadmium, cobalt, copper, lead, silver and mercury was frequently found in other prokaryotes (Rensing *et al.*, 1999). In these cases, cells utilize transport system to exclude the incoming metals from their cytoplasm in order to confer the resistance.

1.2 Cadmium resistance

Cadmium is a highly toxic heavy metal. Once cadmium enters the cell, it may binds to the –SH group of proteins and enzymes, and leads to their inactivation. If cadmium inactivates to those proteins involved in the respiration, cell death becomes inevitable (Weiss *et al.*, 1978; Tynecka *et al.*, 1981). There are many bacterial plasmids encode resistance systems to cadmium have been reported to date. For example, Nies *et al.* isolated a 9.1-kb *EcoR*I fragment, which confers resistance to cadmium, zinc and cobalt salts in sensitive stains, from 238-kb plasmid, pMOL30 found in *Alcaligenes eutrophus* CH34 (Nies *et al.*, 1987). Later, Nies *et al.* has also reported that the genes contribute to these three metals resistance was *czc*

operon, which encodes five polypeptides: CzcR and CzcD are required for regulation of *czc* expression , while CzcA, CzcB and CzcC are essential for full resistance for all the three metals cations (Nies and Silver, 1989; Nies *et al.*, 1989; Nies, 1992). The three peptides form a CzcABC membrane-bound efflux complex utilizing proton as the energy source (Nies, 1995). Some cadmium resistance systems were found in *Staphylococci*. In *S. aureus* strains, cadmium resistance is conferred by two separated plasmid genes, called *cadA* and *cadB* (Novick and Roth 1968; Smith and Novick, 1972; Weiss *et al.*, 1978). The *cadA* and *cadB* operons represents the two known mechanisms of plasmid-mediated cadmium resistance in *S. aureus*. The *cadA* gene product is a 727-amino acid protein, which shows sequence similarity to the P-type ATPase Nucifora *et al.* (1989). In fact, it has been well established that CadA is an energy-dependent cadmium efflux ATPase, which utilizes ATP as the energy source to actively pump out cadmium form cells (Tsai *et al.*, 1992). On the other hand, the cadmium resistance by *cadB* gene is a less defined and found on an incompatibility group II plasmid, pII147 (Smith and Novick, 1972; Perry and Silver, 1982). However, *S. aureus* harbors pII147 does not accumulate cadmium intracellularly. It has been suggested that CadB does not promote cation efflux but may provide protection to the cell by binding cadmium on the membrane (Perry and Silver, 1982).

Recently, Chaouni *et al.* (1996) reported a third cadmium resistance element found in plasmid pLUG10 from *S. lugdunensis*. It encodes two genes, *cadB*-like cadmium resistance gene and a regulatory gene, *cadX*, which results in a high-level resistance to cadmium. Another cadmium resistance gene, designed *cadD*, has been identified from the *S. aureus* plasmid pRW001 (Crupper *et al.*, 1999). A high degree of

sequence similarity was found between and the *cadB*-like gene from *S. lugdunensis*, but no significant similarity was found between *cadA* and *cadB*. The expression of *cadD* in *S. aureus* and *Bacillus subtilis* resulted in a low-level resistance to cadmium when compared it with that of *cadA* and *cadB* (Crupper *et al.*, 1999).

1.3 CadA, the cadmium-efflux ATPase

As described in the previous section, the *cadA* gene was identified on a penicillinase plasmid pI258, along with others determinants for the resistance to heavy metal ions (Novick and Roth, 1968). In the early study by Chopra (1975) has showed that protein synthesis in cell-free extracts from resistance or sensitive bacteria was equally susceptible to inhibition by Cd^{2+}, but spheoplasts from resistance bacteria retained their resistance. Based on these findings, a Cd^{2+} résistance due to a change in the orientation of the membrane protein or phospholipids and leading to shielding of the –SH groups has been proposed. However, Tynecka *et al.* (Tynecka *et al.*, 1981) showed that Cd^{2+} enters into cells via the energy-dependent Mn^{2+} transport system in *S. aureus*, and same result was observed by Weiss *et al.* (1978) who has suggested that a resistance mechanism dependent upon a blockage of energy-dependent Cd^{2+} transport. Also, Weiss *et al.* (1978) demonstrated that Cd^{2+} inhibit the uptake of Mn^{2+} and accelerate the loss of intracellular of Mn^{2+} in the susceptible cells, but no effect was found on Mn^{2+} transport in resistant *S. aureus*. On the other hand, Tynecka *et al.* (1981) found that the net efflux of Cd^{2+} was blocked by 2,4-dinitrophenol, *N,N,*-dicylohexylcarbodiimide (DCCD) and the incubation at 4^{o}C. Indicating that the resistance is in an energy-dependent fashion. Furthermore, they also found that valinomycin had no effect on the reduced Cd^{2+} uptake by the resistance strain and

suggesting that a $Cd^{2+}/2H^{+}$ antiporter for cadmium resistance (Tynecka *et al.*, 1981).

1.3.1 The *cad* operon

1.3.1.1 The *cad*A

However, another model was proposed soon after the open reading frame (ORF) of the cadmium resistant determinant was cloned and sequenced by Nucifora *et al.* (1989). A 3.5 kb *Bgl*II-*Xba*I DNA fragment containing cadmium resistance determinant form pI258 plasmid contains two ORFs. The larger ORF with a predicted polypeptide of 727 amino acids, is sufficient to confer resistance when expressed in a sensitive *Bacillus subtilis* strain. This ORF was designed as *cad*A. By alignment of the predicted *cad*A amino acid sequence, it shows high homologous to the called E_1E_2-ATPase or P-type ATPase (Nucifora *et al.*, 1989; Silver *et al.*, 1989; Silver and Walderhaug, 1992). To date, there is hundreds of enzymes of this class been found (Axelsen and Palmgren, 1998), for example, K^{+}-ATPase of *E. coli* (Hesse *et al.*, 1984; Walderhaung *et al.*, 1987), the Mg^{2+}-ATPase of *Salmonella typhimurium* (Snavely, *et al.*, 1991) H^{+}-ATPase of *saccharomyces cerevisiae* and *Neurospora crassa* (Hager *et al.*, 1986; Addison 1986; Serrano *et al.*, 1986) and the Ca^{2+}-ATPase form sacroplasmic reticulum (MacLennan *et al.*, 1985; Shull *et al.*, 1985; Brandl *et al.*, 1986)

1.3.1.2 The *cad*C

The smaller ORF with a 3' end of the gene overlaps 8 bp to *cad*A encodes for a short polypeptide of 122 amino acids (Nucifora *et al.*, 1989).

This gene was named *cadC* (Yoon and Silver, 1991). Deletion study analysis showed that either *cadA* or *cadC* alone is not enough to confer a full cadmium and zinc resistance in *B. subtilis* (Yoon and Silver 1991). However, when expressing *cadA* and *cadC in trans* in *S. aureus*, the full complementation of cadmium resistance was observed when compared it with only *cadA* or *cadC* gene alone. They suggested that the two gene products are necessary to provide a full cadmium resistance (Yoon and Silver, 1991). However, recent studies of gel-shift assay, purified CadC protein bound to the proposed *cadA* operator/promoter DNA, and, Cd^{2+}, Bi^{3+} and Pb^{2+} caused the release of CadC from DNA in gel retardation assay (Endo and Silver, 1995). Furthermore, the DNase I footprinting measurements showed that CadC protein specifically associated with and protected a region of operator/promoter of *cadA* gene, including its start site (Endo and Silver, 1995). These results suggested that *cadC* is a regulatory element in cad operon. A regulatory role for *cadC* gene was further confirmed by co-transformed this gene with a report gene under controlled by the *cadA*-operator/promoter in *E. coli* (Rensing *et al.*, 1998). Thus, it is now well accepted that role of *cadC* is a regulator for *cadA* expression in the operon.

1.4 The metal transport P-type ATPase

1.4.1 P-type ATPase

P-type ATPase are polytopic membrane proteins which major functions are cations transportation from downhill to uphill. ATPase of the "P" class are defined as those which form a covalent phosphorylated intermediate as a part of the reaction cycle (Perdersen and Carafoli, 1987a, and Perdersen and Carafoli, 1987b). The best known members of P-type

ATPase family, include the eukaryotic plasma membrane Na^+, K^+-ATPase and the Ca^{2+}-ATPase from sarcoplasmic reticulum (MacLennan *et al.*, 1992; Andersen and Vilsen, 1992; Jorgensen *et al.*, 1998). Recently, two novel types of P-ATPase have been found: lipid flippase (Silver *et al.*, 1989) and the P-type ATPase involved in the transport of heavy metal ions (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996).

As mentioned, the hallmark of all P-type ATPase is a phosphorylated asparate (here is how is the name from), which is formed transiently when the energy from ATP hydrolyzation is used to translocate the substrate. The transport cycle of P-type ATPase can be divided into two enzyme states: E_1 and E_2. (Fig. 1.1)The E_1 state has a higher affinity for the substrate, whereas in the E_2 state, the affinity is lower. Cation binding activates the ATPase which then catalyzes the transfer of the γ–phosphate of ATP to a conserved aspartyl residue which makes the enzyme itself turns into the E_1-P state. The high-energy state spontaneously converts into the low-energy state E_2-P state. The E_1-P to E_2-P conversion is the key event in the reaction cycle. This conversion makes the movement of the substrate to the other side of the cell membrane. After hydrolysis of the aspatyl phosphate intermediate, the enzyme returns back to the E_1 state. The conserved motifs found in P-type ATPase include: the DKTGT sequence (the kinase domain), the

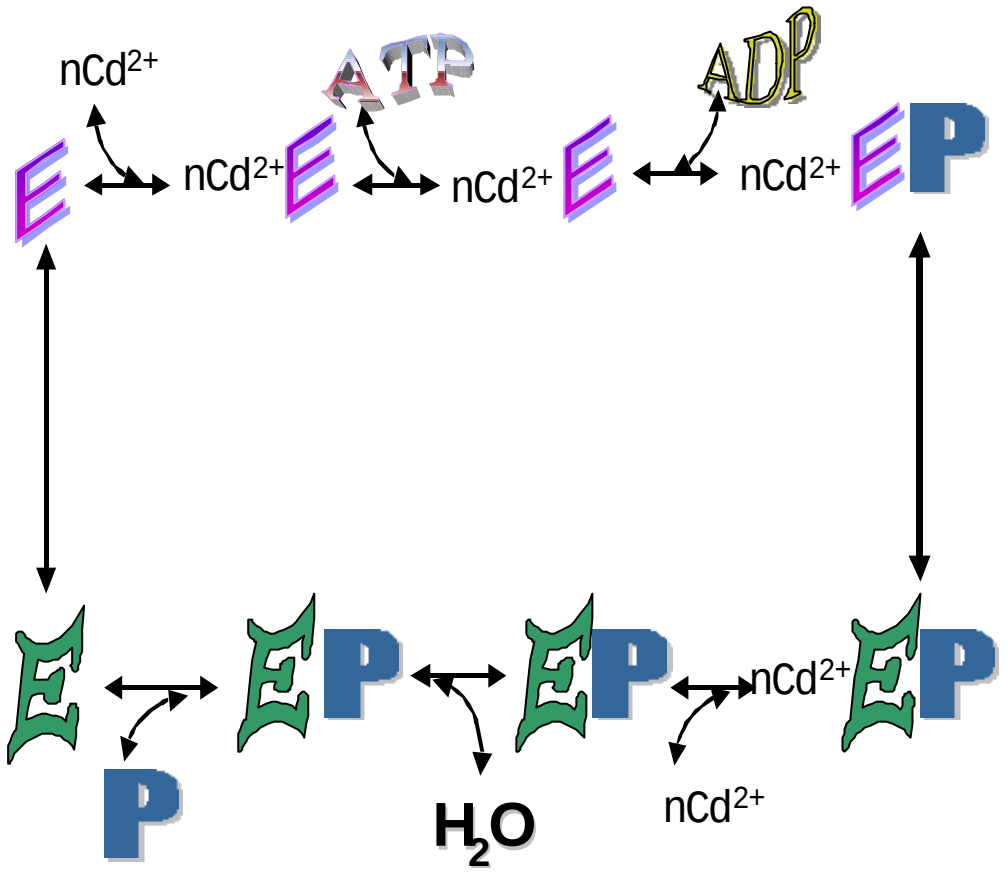

Fig. 1.1 A postulated model for CadA cadmium transport. This model was drawn based on the sarcoplasmic reticulum Ca^{2+}-ATPase. The stoichiometry of CadA enzyme is not known yet.

ATP binding domain (MXGDGXNDXP), and the phosphatase domain (TGES/A). The invariant aspartic acid (D) residue in the conserved sequence DKTGT is first phosphated by ATP, which is bound to the ATP-binding domain in the protein (Solioz and Vulpe, 1996). In addition to the formation of the phosphorylated intermediate, other features of P-type ATPase includes: a single, large, catalytic monomer of 70-200 kDa, and inhibition by micromolar concentrations of vanadate (Perdersen and Carafoli, 1987a, and Perdersen and Carafoli, 1987b) (Fig. 1.2 and Fig. 1.3)

1.4.2 CadA is the member of P-type ATPase

Based on the sequence alignment with the known amino acid sequence of transporters, CadA was found to be a member of the P-type ATPase (Nucifora, *et al.*, 1989; Silver *et al.*, 1989, Silver and Walderhaug, 1992). The first experimental evidence for the P-type ATPase of CadA was provided by Tsai *et al.* (1992). Using everted membrane vesicles prepared from *B. subtilis* having *cadA* gene and different energy sources in the cadmium transport assay, they showed that the everted membrane vesicles prepared from cells with *cad*A gene had a 2.5 to 5-fold increase of cadmium uptake (Tsai *et al.*, 1992). The cadmium transport was driven only in the presence of ATP but not NADH or phenazine methosulfate plus sodium ascorbate, which generate a proton motive force (Tsai *et al.*, 1992). Also, the effects of inhibitors on the cadmium transport were tested. No inhibition was observed when inhibitors for H^+ antiporters, such as nigericin, FCCP and DCCD were added in the transport reaction. Bafilomycin A_1 showed inhibition on the

cadmium transport at the micromolar range and similar to other P-type ATPase (Tsai *et al.*, 1992). Later, in 1993, Tsai and Lynn further characterized this protein through the observation of phosphorylated intermediate (Tsai and Linet, 1993) and showed that the phosphoenzyme formation is cadmium–dependent.

1.4.3 The heavy metal transport P-type ATPase

CPx-type ATPase classified by Soliz and Vulpe (1996) or the so-called P_1-type ATPase named from Lutsenko and Kaplan (1995) is the subtype of P-type ATPase. The ATPase is due to the substrate they transport, which was identified in the metal homeostasis (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996; Solioz and Odermatt, 1995; Beard *et al.*, 1997). In addition to the typical features of P-type ATPase, some striking features are found in P_1-type ATPase: 1-6 GMTCXXC motif(s) or (M/H)XXMDH(S/G)XM, at the N-terminus of the molecule which is metal-binding domains (Lutsenko and Kaplan, 1995), and the CPx (x represents C or H) motif in the sixth transmembrane region that might be part of the cation channel, and a conserved histidine-proline (HP) sequence 34 to 35 residues after the CPx motif (Solioz and Vulpe, 1996)(Fig. 1.4). There are some CPx-ATPases have been cloned and identified, however, only few have been demonstrated to transport metals: CadA transport cadmium ion (Tsai *et al.*, 1992), CopB, found in *Enterococcus hirae*, to transport copper and silver ions (Silver and Odermatt, 1995), ZntA, which is encoded in the chromosome of *E .coli.* (the homologue of CadA) (Beard *et al.*, 1997; Rensing *et al.*, 1997), and CopA, which involves in the cooper homeostasis in *E.coli.* (Rensing *et al.*, 2000). (Fig. 1.5, Fig. 1.6 and Fig. 1.7).

Recently, two human genes in this subgroup, ATP7A and ATP7B, encodes for the copper-transporting ATPase have been identified (Mercer *et al.*,1993; Vulpe *et al.*, 1993; Chelly *et al.*, 1993; Tanzi *et al.*, 1993; Yamaguchi *et al.*,1993; Bull *et al.*, 1993). Mutations in either genes lead to copper homeostasis disorders: Menkes disease in case of ATP7A, and Wilson disease in case of ATP7B. The Menkes protein (MNK) transports copper in the gut and the other tissue but not in the liver, while the ATPase (WND) mutated in Wilson disease is a part of the copper export pathway from the liver. Therefore, it is a systematic lack of copper in Menkes disease, leads to the lack of important enzymatic activity, while, in Wilson disease, there is excess of copper in the liver and brain. Recent immunohistochemical studies have showed that the WND ATPase is localized to the *trans*-Golgi network under steady state (Hung *et al.*, 1997; Schaefer *et al.*, 1999). Under elevated copper levels, the ATPase undergoes a reversible, copper-mediated relocalization to both the plasma membrane and a cytoplasmic vesicular compartment, which is localized toward the hepatocyte canalicular membrane. The unique feature of MNK and WND is that there are 6 metal binding site in their N-terminal region. Actually, only two metal binding sites found in yeast *Saccharomyces cerevisiae*, Ccc2p (encoded by the *CCC2* gene), the homologue of MNK and WND (Yuan *et al.*, 1995), and three in nematodes *Caenorhabditis elegans* (Koch *et al.*, 1997)

1.4.4 The two conserved motifs in P_1-ATPase: the GMTCXXC motif(s) and the CPx motif

1.4.4.1 GMTCXXC motif, the metal binding motif

As described, one of the unique features of P_1-ATPase is that there

are 1-6 metal binding motif(s) on the N terminal of the protein, prior to the first transmembrane reagion. In CadA, ZntA, and CopA form *E. hirae*, there is only one metal binding motif on their N terminal end, two motifs in CopA from *E. coli*, and six motifs in Menkes and Wilson disease protein. The two cysteines are thought to be the metal binding sites in this motif are arranged in the following fashion: GMTCXXC. Silver *et al.* (Silver *et al.*, 1989) first proposed that the involvement of this sequence in heavy metal binding. He pointed it out that the presence of such a conserved sequence in CadA, in the periplasmic mercury-binding protein MerP and in three different MerA mercury reductase. Using mutagenesis technique, it has demonstrated that Hg(II) binds to this motif by Sahlman and Skärfstad (1993). The NMR structural differences between the reduced and mercury-bound forms of MerP shows that the bound forms of MerP is bico-ordinate with Hg(II) by the cysteines side chain ligands (Steele and Opella, 1997). When the first N-terminal 52 amino-acids peptide of CopA from *Helicobacter pylori* was overxpressed, purified and subjected to electrospray mass spectra analysis, it showed that this motif binds only specifically to Cu^{2+} (Bayle *et al.*, 1998). Based on a series of experiments, including direct measurements of copper incorporated *in vivo*, and results of metal-chelate chromatography for the purified protein and the fluorescent labeling of the cysteines in the presence of different heavy metals, Lutsenko *et al.* demonstrated that both N-terminal domains of the Wilson and Menkes disease protein readily and selectively bind copper *in vivo* and *in vitro* (Lutsenko *et al.*, 1997). Their results indicate that there are 5-6 copper molecules bound per N-terminal domain molecule, suggesting that each of the sequence repeats is likely to be involved in copper binding. Voskoboinik *et al* (1999) reported the detail functional analysis in mammalian cells of Menkes protein and its mutants with various meta-binding sites by site-directed mutagenesis. They

showed that mutations in metal binding site 4-6 or all six metal binding sites results in a copper accumulating phenotype, and this phenomenon coincided with the inability of the same mutant protein to traffic through the plasma membrane in response to copper (Strausak *et al.*, 1999).

1.4.4.2 The CPx motif

The other unique feature of this subgroup of ATP-driven ATPase is the CPx motif in the sixth transmembrane region (Solioz and Vulpe, 1996). However, research about this motif is relatively few. Vilsen *et al.* (1989) mutated the proline^{308}, in the membrane region of Ca^{2+}-ATPase, to glycine and alanine, the affinity for Ca^{2+} is reduced and suggested that it could be part of Ca^{2+} binding site. This region was proposed to form an ion transduction domain or ion channel through the membrane. By contrast, the proline is flanked by a cysteine to the amino terminus and either a cysteine, histidine or serine to the carboxy terminus. If the Cys-Pol-Cys sequence is changed to Cs-Pro-Ala in *Cenorhabditis elegans* putative copper ATPase (CUA-1), this mutant could not rescue the yeast △*ccc2* mutant, suggesting that the carboxyl terminal cysteine residue in the conserved Cys-Pro-Cys motif is essential for copper transport (Yoshimizu *et al.*, 1998).

1.5 Rationale and experimental approach

The main purpose of this thesis study is to characterize the role of the four cysteines in CadA protein, that is, Cys23, Cys26 on the metal binding motif, and Cys371, Cys373 on the CPx transmembrane region. I hope this study will provide more information about these four cysteines on CadA function. The approaches will be taken are shown as following:

1. Mutagenesis will be used to mutate each cysteine to serine and glycine, respectively.
2. Measure the ability of these mutants to grow in metal medium.
3. Uptake assay will be taken to know ^{109}Cd uptake in mutants.
4. Cadmium transport activity of these mutants in everted membrane vesicles.

Chapter 2. Methods and Materials

2.1 Materials

Ammonium persulfate (APS), acrylamide, bis-acrylamid, Commassie blue, EDTA, glucose, sodium hydroxide (NaOH) and all the buffers were purchased from Sigma Chemical Co. (St Louis. MO). Antibiotics, cadmium chloride ($CdCl_2$), phenol are the products of United States Biochemical (Cleveland, OH). All the restriction endonucleases and enzymes were purchased form New England Biolabs (Beverly, MA). Altered Site II *in vitro* Mutagenesis System was pursched form Promega (Madison, WI). Oligonucleotide primers in the mutagenesis reaction were pursched from Integrated DNA Technologies (Coralville, CA)

2.2 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in the following table. Plasmid pKJ3, which containing entire *cadA* gene, was used to express CadA in *E. coli*, is provided by Dr. Kan-Jen Tsai in the department of Medical Technology of Chung-Shan Medical and Dental College. Plasmid pKJ4 was constructed by insertion of the *XbaI* fragment which contains the 3′ end of *cadC* and the entire *cadA* gene form pKJ3 into pALTER-1 vector. This plasmid was used for the *in vitro* mutagenesis study.

Table 1. Strains and plasmids

Strain		
Strain	Genotype	Source
W3110	K12F$^-$ IN(*rrnD-rrnE*)	Dr. Rensing *et al.* (1998)
RW3110	W3110 *zntA::km*	Dr. Rensing *et al.* (1998)
JM109	*endA1 recA1 thi hadR17(rk$^-$, mk$^+$) relA1 supE44 l$^-$ △(lac- proAB) (*F' *traD36 proA$^+$B$^+$ laci*qZ△M*15)*	Promega
ES301 *mutS*	*Lacz53 mutS*::Tn5 *thyA36 rha-5 metB1 deoC* IN*(rrnD-rrnE)*	Promega
Plasmid		
Plasmid	Description	
pET-11a	expression vector for cadA gene	Promega
pALTER-1	a chimeric phagemid containing an M13 origin; used for the mutagenesis study	Promega
pKJ3	the 2.6 kb XbaI fragment of cadA gene in pET-11a vector	Dr. Tsai
pKJ4	the 2.6 kb *Xba*I fragment of cadA gene in pALTER-1 vector	Dr. Tsai

2.3 DNA manipulation

DNA were prepared by the procedures described by Sambrook *et al*. (1989). Two to five mls of bacterial cells were used for the DNA preparation. After overnight culture with appropriate antibiotics (100 μg/ml of ampicillin or 10 μg/ml of tetracyclin), cells were collected by centrifugation. The pellet was resuspended in glucose buffer (25 Mm Tris [pH 8.0], 50 mM glucose, 10 mM EDTA) with the addition of lysozyme (1 mg/ml) for 10 minutes at room temperature followed by addition of alkaline lysis by 2 volumn of 0.2 M NaOH plus 1% SDS on ice. After 10 minutes, neutralization buffer (3 M potassium acetate and 2 M glacial acetic acid) added into the reaction mixture for 10 minutes on ice. After centrifugation for 10 minutes, 0.1 mg/ml ribonclease A was added to the supernatant and incubated for 15 minutes at room temperature. Protein was extracted by phenol extraction procedure. DNA was precipitated by addition of 95% ethanol in the presence of 0.1 volume of 3 M sodium acetate followed by centrifugation at 4^{o}C. The pellets were washed with 70% ethanol, then air dried and resuspend in TE buffer.

DNA for construction was digested with appropriate restriction enzyme(s), then purified from agarose gel after electrophress. DNA in the isolated agarose was purified following the instruction of gel extraction kit (Clonetech, CA). Ligation reaction was set up following Sambrook *et al*. (1989), and the transformation was carried out by electroporation.

2.4 Site-directed mutagenesis

2.4.1 Preparation of single stranded DNA

The mutants were constructed by the protocol manual of Alter-Site II Mutagenesis System (Promega; Madison, WI). To prepare single stranded *cadA* DNA, JM109 cells harboring pKJ4 plasmid were picked from fresh prepared M9 plate containing 10 μg/ml of tetracycline and overnight cultured in LB broth supplemented with 10 μg/ml of tetracycline. The cultured was transferred into fresh LB broth (1:50 dilution). After 30 minutes shaking at 37 °C, helper phage R408 was added at a multiplicity of infection of 10 to 20 (i.e, add 10-20 helper phage per cell) to infect the cell. Continue shaking for 6 hours with vigorous agitation at 37 °C. Harvest the supernatant by pelleting the cells at 12,000 × g for 15 minutes. Precipitation the phage by adding 0.25 volume of phage precipitation solution (3.75 M ammonium acetate [pH 7.5], 20% polyethylene glycol [MW 8,000]) to the supernatant, leave on the ice for 60 minutes, then centrifuge at 12,000 × g for 15 minutes. The pellet was suspended in 0.4 ml of TE buffer. The phage was lysed by adding 0.4 ml of chloroform: isoamyl alcohol (24:1), vortexed, then centrifuge 12,000 × g for 5 minutes to remove excess polyethylene glycol. The upper aqueous phase solution was transferred to a fresh tube and 0.4 ml of 25:24:1 (vol:vol:vol) phenol:chloroform:isoamyl acohol was added. The mixture was vortexed for 1 minute and centrifuged at 12,000 × g for 5 minutes. The upper aqueous phase solution was transferred to a fresh tube and 0.2 ml of 7.5 ammonium acetate plus 1.2 ml of 100% ethanol were added. The precipitation was mixed and left at -70 °C for 30 minutes. The DNA was pelleted by centrifuge at 12,000 × g for 15 minutes, washed with ice-cold 70% ethanol once and dried. The DNA pellet was suspended in 20 μl of H_2O. The final concentration of phagemid DNA was determined by the measurement of the absorbance at 260 and 280 nm.

2.4.2 Design of primers

The oligonucleotide primers for the mutagenesis reaction in this study were listed as following:

Primer name	Primer sequence	Codon change
$cadA_{1131}$ T→A	5′-AGGATTTACA**A**GTGCAAATTG-3′	C21S
$cadA_{1131}$ T→G	5′-AGGATTTACA**G**GTGCAAATTG-3′	C21G
$cadA_{1140}$ T→A	5′-ATGTGCAAAT**A**GTGCAGGAAA-3′	C23S
$cadA_{1140}$ T→G	5′-ATGTGCAAAT**G**GTGCAGGAAA-3′	C23G
$CadA_{2175}$ T→A	5′-TGTAGTTGGA**A**GTCCTTGTGC-3′	C371S
$CadA_{2175}$ T→G	5′-TGTAGTTGGA**G**GTCCTTGTGC-3′	C371G
$CadA_{2181}$ T→A	5′-TGGATGTCCT**A**GTGCATTAGT-3′	C373S
$CadA_{2181}$ T→G	5′-TGGATGTCCT**G**GTGCATTAGT-3′	C373G

The boldfaced letters indicates the changed nucleotides

2.4.3 Mutagenesis reaction

Approximately 100 pmole of the mutagenic primer was phosphorylated by incubation of 2.5 μl of 10× kinase buffer (500 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 10 mM ATP) and 5 units of T4 polyncleotide kinase in a 25 μl of reaction mixture at 37 °C for 30 minutes, then incubate the mixture at 70 °C for 10 minutes to inactive the kinase. For the in vitro mutagenesis, 0.05 pmole of single-stranded pKJ4 DNA was annealed with 0.25 pmole of ampicillin repair oligonucleotide and 1.25 pmole of phosphorylated mutagenic oligonucleotide in a 20 μl of reaction mixture in the prensence

of 2 μl of 10 ×annealing buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$ and 500 mM NaCl). Heat the annealing mixture to 70 °C for 5 minutes and then allow then to cool slowly to room temperature, then place the mixture on ice. Added 3 μl of 10× synthesis buffer (100 mM Tris-HCl [pH 7.5], 5 mM each dATP, dCTP, dGTP and dTTP, 10 mM ATP, 20 mM DDT), 1μl of T4 DNA polymerase and 1μl of T4 DNA ligase to the 20 μl annealing reaction mixture and bring the reaction to a final volume of 30 μl by H_2O. The reaction was incubated at 37°C for 90 minutes. The synthesis reaction mixture was then transformed into ES1301 *mutS* competent cells by electroporation . After incubation at 37 °C for 30 minutes, ampicillin (100 μg/ml) was added, shake the culture at 37 °C overnight. DNA was prepared, then transformed into JM109 competent cell by electroporation. The transformation mixture was plated on LB plates supplemented with 100 μg/ml of ampicillin.

2.4.4 DNA sequenceing

DNA sequencing was performed by using ABI PRISM™ BigDye Primer Cycle Sequencing Ready Reaction Kit and followed the steps described in the protocol accompanying in the kit. Briefly, asymmetric polymerase chain reaction (asymmetric PCR) was carried out in the reaction mixture containing plamsid (as the template), deoxynucleotides, fluorescein labeled dideoxynucleotides (that is ddATP, ddTTP, ddGTP and ddCTP), AmpliTaq DNA polmerasse and sequencing buffer (final concentration 1×). After the PCR reaction, 1/10 volume of 3M sodium acetate and 2.5 volume of 98% ethanol was added, and then chilled on ice for 10 minutes to precipitate the product before centrifugation. The pellet was dried in a vacuum centrifuge. The sample was then loaded and

electrophoresised in a 6% polyacrylamide sequencing gel. Signals representing for four nucleotides were detected by the sequencer detector.

2.5 Metal resistance assays

For determination of sensitivity of metal ions, a basal salts medium is prepared (Tris-HCl, 14.5g/L; NaCl, 4.68 g/L; KCl, 1.5 g/L; NH_4Cl, 1.0 g/L; glycerol, 5 g/L; Na_2SO_4, 0.043 g/L, $CaCl_2$, 0.03g/L). The pH was adjusted to 7.5 with conc. HCl. After autoclaving, $MgCl_2$ (1mM), glycerol 3-phosphate (0.5 mM), and casamino acid (0.05%) were added as described previously (Poole *et al.*, 1989). Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium supplemented with different concentration of $CdCl_2$, $PbCl_2$ or $ZnCl_2$ and incubate for 24 hr at 37 ^oC with shaking. Growth was monitored by measuring the absorbance at a wavelength of 600nm.

2.6 Preparation of everted membrane vesicles

Cells harbor wild type or mutant *cadA* were grow overnight at 37 ^oC in 20 ml of LB with shaking and diluted 50-fold in prewarmed medium. At an optical density of 0.6 at 600 nm the cultures were induced with sublethal of $CdCl_2$ for 2hours. Cells were harvested by centrifugation and washed twice with 10 mM MOPS (morpholinepropanesulfonic acid)- KOH, pH 7.0, 0.2M KCl, 25 mM $MgSO_4$. The washed cells were resuspended in sucrose buffer (MOPS-KOH [pH 7.0], 250 mM sucrose, 0.2M KCl, 25 mM $MgSO_4$, 0.5 mM EDTA) at about 5 ml/g of wet cells. The everted membrane were prepared by passage through a French pressure cell at 6,000 p.s.i.. The protease inhibitor PMSF was immediately added to the lysate. After

addition of DNase I (20 μg/ml), cells were incubated on ice for 30 min. Cell debris and unbroken cells were pelleted by centrifugation at 10,000× g for 30 min, the supernatant was centrifuged at 100,000×g for 60 min to isolate the membranes. The pelleted membranes vesicles were wash and resuspened in sucrose buffer, and aliquot were storeed n –70 ^{o}C until use. Protein concentration was measured by a modification of the Lowry assay using serial dilutions of bovine serum albumin as standards (Bensadoun and Weinstein, 1976; Peterson, 1977).

2.7 Whole cell uptake assay

Cells were grown overnight in basal salts medium at 37 ^{o}C with shaking at 200 rpm. Next morning, the culture were 50-fold diluted into the same medium for additional culture for 8 hours, then harvested by centrifugation, washed once with basal salt medium and resuspended in fresh medium to about 20 OD_{600} units. For assay, 50 μl of washed cells was diluted into 940 μl of basal salt medium. After incubation at 37 ^{o}C for 10 min, 10 of 0.02 mM of $CdCl_2$ (0.06 μCi ^{109}Cd) was added to the reaction. 50 μl of the reaction mixture was withdrawn and filtered thought a 0.2-μm-pore size membrane, wash with 5 ml of basal salt medium. The filters were dried and dissolved in 5 ml of scintillation cocktail and counted in a liquid scintillation counter.

2.8 Cadmium transport assay.

Cadmium transport assay were performed at room temperature as described by Tsai *et al.* (1992). Membrane protein (0.8-1.0 mg of membrane protein) were assay in the reaction mixture consisted of 20

mM Bis-Tris-Propane-HCl (pH 6.0), 200 m KCl, 250 m sucrose and $^{109}CdCl_2$ (0.4 μCi/ml) with or without 5 mM Na_2ATP in final volume of 1 ml. The reaction was initiated by addition of 5 mM $MgSO_4$. Aliquots were withdrawn after 10 minutes and filtered through nitrocellulose filters (.02 pore size, Whatman), and washed with 6 ml of 20 mM Bis-Tris-Propane-HCl (pH 6.0), 200 mM KCl, 250 mM sucrose, 10 mM $MgSO_4$ and 20 mM $CdCl_2$. The filters were dried, dissolved in 5 ml of scintillation cocktail, and counted in a liquid scintillation counter. The amount of binding in the absence of ATP was subtracted from all values.

Chapter 3. Results

3.1 *In vitro* Mutagenesis of the *cadA*

In order to clarify roles of these four cysteines in CadA finction, mutagenesis approach is taken to change the cysteine position 23, 26 (in the metal binding motif) and cysteine 371, 373 (the CPx motif in the transmembrane region) to serine (conserved mutation) and glycine (non-conserved mutation) respectively. The 2.6 kb of *Xba*I fragment containg *cadA* gene in pALTER-1 vector, pKJ4, was used to generate single stranded DNA (Fig. 3.1). *In vitro* mutagenesis was performed by using the primers listed chapter 2, and single stranded pKJ4 plasmid as the template. The mutants were sequenced and then subcloned into pET-11a vector for expression. These *cadA* mutants were named C23S, C23G (for cysteine residue changed to serine or glycine at amino acid position 23); C26S, C26G (for cysteine residue changed to serine or glycine at amino acid position 26); C371S, C371G (for cysteine residue changed to serine or glycine at amino acid position 371) and C373S, C373G (for cysteine residue changed to serine or glycine at amino acid position 373).

3.2 Metal ions resistance of *cadA* mutants

The ability of *cadA* mutants to grow in the presence of metal ions was assayed in strain RW3110 which *cadA* homolog in *E. coli.*, *zntA* was insertional inactiviated by homologous recombination of the kamamycin reistance gene and leads to hypersensitive to zinc (Rensing *et al.*, 1997). Cells with wild mutants *cadA* were cultured in the basal salt medium in order to avoid precipitation of metals ions with the complex ingredients

of other medium. After overnight culture with different concentrations of metal ions, turbidity at 600 nm was measured. As shown in Fig. 3.2a, and Fig. 3.2b, wild type CadA demonstrates better cadmium resistance then these cysteine mutants either in the metal binding motif or the CPx motif. Insteresting, CPx mutants are without the ability to grow in medium with zinc ion as *zntA* inactivated strain, however, metal binding sites mutants are still be able to grow to the same extend as wild type up to 20 μM, then the turbidity lowed down as to the *zntA*$^{-}$ control. (Fig. 3.3a, b). Rensing *et al.* (1998) have proven that CadA and ZntA are both Pb(II) pumps. The sensitivity to lead of *cadA* was also investigated. The different abilities of these two groups of mutants to the sensitivity of metal ions is more obviously when cells were cultured in medium with lead as shown in Fig. 3.4a, b. The metal binding site mutations, that is, C23S, C23G, C26S and C26G reduces the growth ability as half as the wild type, but, the ability of CPx mutants, namely C371S, C371G, C373S and C737G shows worse growth abilities than the cell without CadA higher than 10 μM of lead acetate.

3.3 Whole cell uptake assay with isotopic cadmium.

The activity of the mutated proteins were evaluated by the *in vivo* cadmium uptake assays. Cells with wild type *cadA*, mutants and vector control were incubated in basal salt medium with $^{109}CdCl_2$, aliquots were removed and filtered at time intervals. As shown in Fig. 3.5a, b cells harboring wild-type *cadA* gene displayed low ^{109}Cd uptake. On the other hand, all the mutants and the *zntA*-disrupted strain showed increased ^{109}Cd uptake. However, higher uptake was observed in the cells without *cadA* gene than these mutants.

3.4 Cadmium transport assays in everted membrane vesicles of *cadA* mutants.

The cadmium transport assay were performed *in vitro* in everted membrane vesicles prepared from cells harboring *cadA* mutants. Aliquots of reactions with or without ATP were withdrawn after 10 minutes and filtered through nitrocellulose filters. Cadmium bound to the vesicles in the prensence of ATP was subtracted with that without ATP. The results of assay were plotted as the percent of the wild type. As shown in Fig. 3.6 all the mutants exhibits no transport activity.

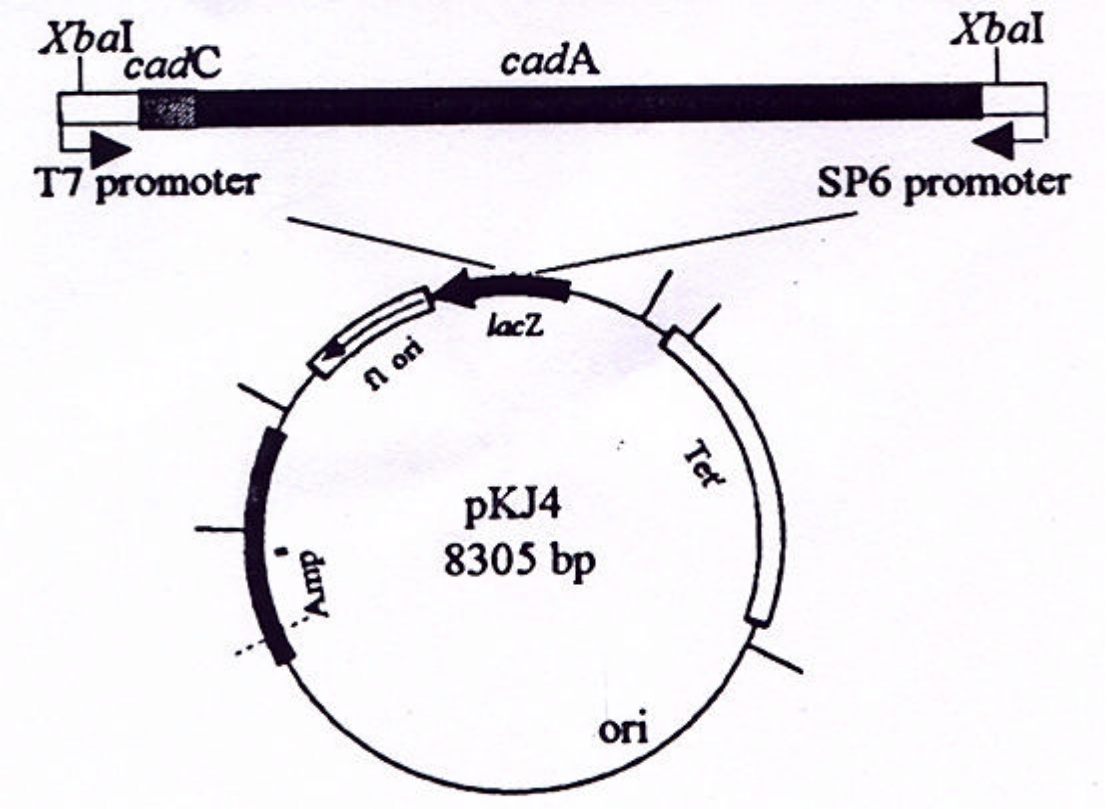

Fig. 3.1 pKJ4 plasmid used for single strand DNA preparation in in vitro mutagenesis (kindly provided by Dr. Tsai in the department of medical technology in Chung-Shan Medical and Dental college).

Fig. 3.2a Growth curve of CadA metal binding site mutants in medium with cadmium. Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium with different concentrations of $CdCl_2$, then incubated for additional 24 hr at 37 ^{o}C with shaking.

Fig. 3.2b Growth curve of CadA CPx motif mutants in medium with cadmium. Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium with different concentrations of $CdCl_2$, then incubated for additional 24 hr at 37 ^{o}C with shaking.

Fig. 3.3a Growth curve of CadA metal binding site mutants in medium with zinc. Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium with different concentrations of $ZnCl_2$, then incubated for additional 24 hr at 37 ^{o}C with shaking.

Fig. 3.3b Growth curve of CadA CPx motif mutants in medium with zinc. Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium with different concentrations of $ZnCl_2$, then incubated for additional 24 hr at 37 ^{o}C with shaking.

Fig. 3.4a Growth curve of CadA metal binding site mutants in medium with lead. Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium with different concentrations of PbAC, then incubated for additional 24 hr at 37 ^{o}C with shaking.

Fig. 3.4b Growth curve of CadA CPx motif mutants in medium with lead. Cells were grown overnight in liquid minimal medium, diluted 1:50 in the same medium with different concentrations of PbAC, then incubated for additional 24 hr at 37 ^{o}C with shaking.

Fig. 3.5a Cadmium uptake assay of the metal binding site mutants of CadA protein.

Fig. 3.5b Cadmium uptake assay of CPx mutants of CadA protein.

Fig. 3.6 Cadmium transport activity of metal binding sites mutants and CPx mutants in everted membrane vesicles.

Chapter 4. Discussion

Function of the conserved metal binding motif, the usual N-terminal GMTCXXC sequence, and the CPx motif, found in the transmembrane region of P_1-type ATPase, have not been investigated biochemically, though there are some researches focus on the metal binding motif(s) of this subgroup P-type ATPases in high organisms, such as CCC2 in *S. cerevisiae*, Menkes and Wilson's protein (MEK, WND) (Chelly, *et al.*, 1993; Mercer, *et al.*, 1993; Vulpe *et al.*, 1993;). The major problem for the fuzzy is due to the difficulty of expressing these proteins in a large quantity for the study. In fact, there are only a few prokaryotic P_1-type ATPases have been cloned to date, for example, CopA in *E. coli* (Rensing *et al.*, 2000), which involved in the copper homeostasis, same as to CopA of *Enterococcus hirae* (Odermatt *et al.*, 1993), the Znic-transporting ATPase (ZntA), encoded by the ORF *o*732 of *E. coli* (Beard *et al.*, 1997; Rensing *et al.*, 1997), and the CadA cadmium resistant ATPase found in *S. aureus* (Nucifora *et al.*, 1989). However, the latter P_1-type ATPase also demonstrates resistance to other metals as well (Rensing et al., 1998). Among these metal-related ATPases, the unique cysteine-pairs are the most striking feature found in these ATPases. Presumably, the configurationally proximity of the thiol- groups in each cysteine-pair provide a possible binding packet for those cations (Gitschier *et al.*, 1998)

In order to disclose the functional roles of these four conserved cysteines universally found in these metal binding motifs and the CPx motifs, the cysteines at positions 23, 26, 371 and 373 of CadA protein were mutated to serine and glycine, respectively. In this thesis study, cysteine substituted mutants at these positions were prepared and their activities were measured. Firstly, these mutants were tested for their

abilities to grow in media in the presence of different metals at different concentrations. Our results have shown that all these mutants can not grow in the presence of cadmium (Fig. 3.2a, b), however, these two groups of mutants shows different abilities to grow in media supplement with either zinc or lead (Fig. 3.3a, b and Fig.3.4a, b). The CXXC mutants, including those of C23S, C23G, C26S and C26G are all grown better than those of CPx mutants, namely C371S, C371G, C373S and C373G, in media with low zinc concentrations, although all these mutants stop to grow at a higher zinc concentration. Surprisingly, the CXXC mutants reduce their abilities to grow in media with lead to half of the wild-type extend, however, CPx mutants are not. Suggesting that cysteines in CPx are much important for the resistance to lead and zinc. Presumably, the cysteines in CPx are required for the translocating metal ions. And the translocating activity might be various in different ions. On the other hand, the cysteine residues of CXXC might play some roles in the metal ion binding, even though the binding activity reduced in those CXXC mutants, however, metal ions still find their way to pass through the membrane.

Alternatively, the CXXC motif might function as the CadA regulator, in the presence of metal ions, the cysteine residues in CXXC bind to the metal ions initially, and subsequently result in the conformational change at the CPx domain and expose the cysteine residues for those metal ions to pass through. Therefore, even a substitution mutation in CXXC motif, however, the lead translocating activity does not completely abolish. The metal binding specificity might be different for various metals as well as the expression of the mutant CPx might lead to the instability of the protein, and subsequently resulted in the cell death. All these speculations will need more evidences to support.

Two explanations would be able to make for the observation: 1) Lead is not as toxic as cadmium and zinc in bacteria cells, and 2) Lead has a much better activator for CXXC motif to bind and, subsequently activates the CadA protein more efficiently. To support the hypothesis, we can examine the Fig. 3.6a, b and found that both wild type and cysteine mutants are more resistance to lead at a much higher concentration to cadmium and zinc. However, it is very difficult to clarify the resistance is really due to the active extrusion of the metal ions from cells. And thus, lead has a more actively translocating activity than those of zinc and cadmium.

To determine if cysteine substitution mutants of CadA do affect the actively transporting system, metal transport assays using a previous established cadmium transport system were performed in this study. In the cadmium whole uptake assay (Fig. 3.5a, b), we found that either CXXC CadA mutants or CPx CadA mutants take up more Cd^{2+} than that of the wild-type CadA as we have predicted. On the other hand, cadmium transport assays using everted membrane vesicles prepared from cells with either wild type or mutant plasmids have also demonstrated that Cd^{2+} transport activity is found very little to none in either CXXC and CPx mutants. Suggesting that these cysteine residues are all important for the CadA protein function.

Recently, several studies have tried to elucidate the role of metal binding motif(s) in other P_1-type ATPase. For expamle, *Lutsenko et al.*, (1997) overexpressed and purified the chimeric N-terminal six binding motifs of MEK and WND with maltose binding protein, they showed that each of the metal binding site binds per Cu(I) atom. In another *in vivo* experiment, the stoichiometry of copper binding is consistent with the *in vitro* results. Other acclaimed that the N-terminal cooper binding motifs of WND cooper binding domain are not functionally identical to the

C-terminal motifs by a serious of metal binding sites mutations (Forbes *et al.*, 1999). They also demonstrated that WND protein with only one cooper-binding motif is capable of fully complementing the deficiency of the *ccc2* mutant yeast, and suggested that the cooper-binding motif near the transmembrane domain of WND is more important for the cooper transporting activity than the other 5 motifs which was suggested to function for sensing the cytosolic copper concentration (Yuan *et al.*, 1997; Voskoboinik *et al.*, 1999).

CadA only contains a single binding motif is less than that of the copper translocating enzymes. The reasonable speculation is that copper is essential for the cell, for some metabolic reactions use, as the enzymatic cofactor, such as the cytochrome oxidase, superoxidase dismutase. However, in a higher concentration, the copper could cause certain oxidative reactions and kill cell. Therefore, it is important to monitor the cellular copper concentration in order to avoid deficiency or excess accumulating copper toxicity in the cell. Cadmium and lead are toxic to cells. Cells must have the ability to pump out these toxic metals as soon as they enter into cells. The CPx motif in P_1-type ATPase is thought to work as a transduction channel (Silver *et al.*, 1989) to manages the passing through of certain metal ions. After metal ions captured by the metal binding site, the overall conformation of the enzyme is changed resulting in the bound ions are then transferred to the membrane embedded CPx motif, and then pumps out from the cell. However, no direct evidences have been demonstrated that the cysteine(s) plays important role in metal transduction.

In this thesis study, we have elucidated for the first time that both of the cysteines in CPx are important for the CadA function. However, further immunological approach have to be performed to get the information about the protein expression levels in these mutants in order

to clarify if the abolished function raising from the lose of thio- groups provided by the cysteines or the protein instability after expression.

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