摘要

在金黃葡萄球菌質體 pI258 上的重金屬鎘抗性基因，早於 1992 年已被證實具有轉運鎘的作用，之後由於它的結構特殊性，因此將此種抗鎘幫浦、與其他類似的酵素，共同歸類為 CPx-ATPases 的酵素家族。然而，目前對於其排除鎘的酵素機制仍不清楚。因此本論文藉著測量 CadA-ATPase 水解 ATP 的酵素活性，針對具有高度保留特性的胺基酸序列如:Cys-X-X-Cys、及 Cys-Pro-Cys 進行探討，並且期盼能建立起一套完整的酵素活性監測方法，以因應未來進一步純化蛋白的工作所需。雖然對於胺基酸序列 Cys-X-X-Cys，在 CPx-ATPases 所扮演的角色，目前尚無定論。但一般相信，它可能會結合金屬離子，接著利用分解 ATP 所產生的能量，透過 CPC 所形成的通道，將金屬離子排出細胞外。因此含有 CadA 的細菌，便可存活在高濃度鎘的環境之中。在本研究中，藉由基因工程技術將原有 Cys-X-X-Cys、及 Cys-Pro-Cys 上的 Cysteine 胺基酸，改變為 Serine、或 Glycine，所產生的突變基因，轉移至 pKJ100 表達系統中，並在 cadmium-sensitive 的大腸桿菌 RW3110 中表達。當這些大腸桿菌，在生長時分別將重金屬鎘、鋅、以及鉛加入培養基中，發現除了部分 Cys-X-X-Cys 的突變株，保有些許重金屬抗性之外，其他的生長狀況都比野生株 CadA 還差。然而透過 Enzyme coupling assay，來分析這些突變種蛋白，在水解 ATP 的酵素活性的差異，結果卻發現這些突變蛋白並不具備水解 ATP 酵素活性。同時為了得到高純度的 CadA 蛋白，我們嘗試利用界面活性劑 Triton X-100 來純化，卻因為 Enzyme coupling assay 的干擾作用，而無法得到良好的再現性。除此之外，經由 *in vitro* translation 所合成的 CadA 蛋白也同樣對於 ATPase assay 有干擾作用。由於大腸桿菌細胞膜中含有許多不同於 CadA 的 ATPase，利用剔除 F_0F_1-ATPase、Kdp-ATPase、ZntA 的大腸桿菌突變株 BF2000，來進一步探討可能扮演直接轉運金屬離子的 Cys-Pro-Cys。在抗鎘的表現分析之中，我們發現 ATP-binding domain 的突變以及所有的 Cys-Pro-Cys 突變都失去抗性。除此之外，在微量的鎘存在之下 CadA 的酵素活性能約略增加，

但更高濃度的鎘卻無法得到更強的酵素活性。實驗中我們也發現 P-ATPase 的抑制劑，如 Vanadate 似乎不能影響 CadA 的酵素活性。最後，以 Phosphate precipitation method 與 GST-CadA 蛋白表達系統，建立一套較為可行的酵素活性監測方法，以便做為未來純化 CadA 蛋白時的監測所需。

Abstract

The *cadA* gene encoded cadmium resistance found in staphylococcal plasmid pI258 has been characterized previously as a cadmium-efflux P-ATPase. Recently, based on its unique structural features, the CadA cadmium resistance ATPase, alone with other similar membrane efflux ATPases were further classified as a member of CPx-ATPases, these features include a conserved CPx motif and a CxxC motif. However, without direct evidence, the exact role of CxxC and CPx motif in cadmium translocation mechanism remain undefined. In this thesis study, the two conserved unique motifs were studied their role in cadmium resistances as well as ATP hydrolysis. To determine the role of cysteine residues within these motifs, eight cysteine mutants were prepared and expressed them in a cadmium-sensitive *E. coli* strain RW3110. Surprisingly, some cysteine mutants of CxxC motif seem less sensitive to heavy metal than those cysteine mutants in CPC motif. On the other hand, ATPase assay using enzyme coupling assay was established to monitored the ATP hydrolytic activity of those cysteine mutants. All cysteine mutants have shown various and reduced activity compared to wild type *CadA*, especially the activities from some mutants of CxxC motif were less active than those of CPC motif. Suggesting that the C26, and possibly the C23 residue may participate in other role in CadA enzyme cycle, instead of directly involving in ATP-dependent metal translocation process. Meanwhile, to eliminate the possible interference in membrane vesicles, Triton X-100-solubilized or *in vitro* translational CadA were prepared for ATPase assays in this study. However, both of these methods

did not generate a convinced result. More recently, an *E. coli* strain with triple mutation (*unc*$^{-}$, *kdp*$^{-}$, *zntA*$^{-}$) and alone with a new CadA expression system were included in this study. Using this latest CadA expression system, we found that β-mercaptoethanol was able to slightly increase the ATPase activity of CadA. Even though the activity of CadA achieved in this study was less than other P-ATPases, our data did create a new avenue toward the better understandings of CadA enzyme mechanism and provide a new direction for future protein studies of CadA and other similar CPx-ATPases.

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Chapter 1. Introduction

1.1 Bacterial resistance to toxic metals

Probably the ancient lives evolved from hazardous environments, the archaean microorganisms have to develop resistant mechanisms to cope with (Rensing *et al.*, 1999). The origins for these resistant mechanisms might be raised from various sources, and several theories have been proposed to describe the resistances (Lyon 1987; Speer 1992). Even though a variety of different mechanisms were found for the resistant phenomena, including those of membrane impermeability, detoxification, and active extrusion. However, only those resistant systems involved in the energy-dependent efflux pumps, encoded by either acquired plasmid or by bacterial chromosomal genes, were extensively studied in the past several decades (Silver, 1996). Once when the resistances were established, and through the horizontal gene transfer, the resistant determinants were spread among all species to confer resistance to those poisonous substances (Salzberg *et al.*, 2001). Among them, resistance to those of heavy metals including cadmium, lead and other components found in earth are frequently observed.

註解: You should be able to find a reference from this web site: **(http://www.bact.wisc.edu/Bact330/lecturebactres**

1.2 Cadmium, one of the toxic components found in the environment

Cadmium, one of the highly toxic heavy metals, is commonly found in batteries, pipes and paint pigment. In mammals, the toxicity of cadmium is believed to damage especially the nerves tissues (Krigman *et*

al., 1985), and mental disorders are frequently observed. Some malignancies are also believed to be associated with the exposures of this metal including the prostate cancer and probably many others (Nakamura *et al.*, 2002; Hossain *et al.*, 2002). As a transitional metal with a highly polarizing power, Cd(II) tends to form a strong linkage with sulfur ligand such as the sulfhydryl group of cysteine residues in most enzymes and subsequently disrupt their functions (Rensing *et al.*, 1999). Since cadmium and its compounds are very easily to reach in our daily lives, therefore, it becomes one of the most likely toxic metals to affect our community. In order to survive under the selection pressure, bacteria have to develop mechanism(s) to deal with this poisonous metal.

1.3. Introduction to bacterial cadmium resistance

To date, many plasmid-born cadmium resistant determinants were discovered in bacteria. For example, a G(-) soil bacterium, *Alcaligenes eutrophus*, has been reported to harbor a large plasmid encodes a cadmium-resistance determinant called *czc* operon (Nies *et al.*, 1989). The *czc* operon was further characterized and five distinct genes were found, called CzcABCD and CzcR. CzcA, CzcB and CzcC confer resistance to cadmium, zinc and cobalt, whereas CzcR and CzcD are believed to be the regulators for CzcABC structural gene expressions (Nies *et al.*, 1989). Cadmium resistant systems were also discovered in G(+) *Staphylococci spp.* as well. Clinically isolated *Staphylococcus aureus* contained penicillinase-plasmid, pI258, which also carried resistant genes to some heavy metals, including cadmium, lead, mercury,

and many others. Two genes in pI258 plasmid were found associated with cadmium resistant determinant, called *cadA* and *cadC*, and more recently these two genes were put into a so-called CadA operon. Other than the CadA operon in pI258, there is another distinct gene, called *cadB*, was also found in *S. aureus* to confer cadmium resistance. Even though CadA and CadB are both cadmium resistant genes in *S. aureus*, however the *cadA* gene determinant might contribute to the major cadmium resistance in this bug while *cadB* might perform a mild resistance to cadmium (observation in our lab). Especially, the evidence have shown that CadA operon is very similar to the CzcABC complex both structurally and functionally, and an efflux pump of CadA utilizing ATP as energy source was also demonstrated (Tsai *et al.,* 1992). On the other hand, *cadB* gene seems to play a different role using a less defined mechanism. The CadB operon encodes *cadB* and *cadX* genes reside on an incompatibility group II plasmid pII147 (Smith and Novic, 1972; Perry and Silver, 1982). Instead of pumping out toxic metal, *cadB* is speculated to bind cadmium in the membrane to prevent its entry to cell (Perry and Silver, 1982), while *cadX* is a positive regulator with 40% sequence homology similar to *cadC*, another gene in CadA operon. However, the exact role for *cadX* is yet to be decided.

Interestingly, a third cadmium resistance element obtained on the plasmid pLUG10 of *S. lugdunensis* was recently reported (Chaouni *et al.*, 1996). The plasmid responsible for high cadmium resistance and two genes similar to *cadB* and *cadX* were identified. Additionally, the *cadX*-like gene was shown 40% homology to the predicted *cadC* gene

product from *S. aureus* plasmid pI258. Most recently, a novel cadmium resistance determinant was also found in the plasmid pRW001 of *S. aureus*, called *cadD*, is shown highly homologous to *cadB*-like gene using DNA analysis. Unlike its counterpart, *cadA* and *cadB*, the function of *cadD* gene product is not essential for cadmium resistance, but probably for other unrelated purposes (Crupper *et al.*, 1999).

1.4 The structure of *cad* operon

As mentioned above, the CadA operon in *staphylococcal* plasmid pI258 was discovered in 1989, and a 3.5-kb *Bgl*II-*Xba*I DNA fragment from pI258 was cloned and expressed in *Bacillus subtilis*. Bacteria carried these two genes were found full resistance against toxic cadmium (Nocifora *et al.*, 1989). As mentioned above, the DNA analysis of this fragment lead to identify two open reading frames (ORFs) within this operon, called *cadA* and *cadC* (Nocifora *et al.*, 1989). Nevertheless, these two genes unequally contribute to the resistance. The *cadA* gene obviously seems to be much important than *cadC* in turns of cadmium resistance. As it was found that the full complementation of cadmium resistance was observed only in the cell transformed with both *cadA* and *cadC* gene, but not with *cadC* alone (Yoon and Silver, 1991). On the other hand, *B. subtilis* harboring only *cadA* gene displayed a partial cadmium resistance. Suggesting that *cadA* is the structural gene for the resistant phenotype, but not the *cadC* gene. The deduced amino acid of *cadA* gene sequences suggested that the *cadA* gene product, the CadA protein, is a member of P-type ATPase family, previously called the

E_1E_2-ATPases, which major functions are to translocate cations across the cell membrane (Nucifora *et al.,* 1989).

The second ORF of the 3.5-kb *Bgl*II-*Xba*I DNA fragment from pI258, on the other hand, encodes a predicted 122 polypeptide called CadC. Even though the function of this small protein was not determined when it was first discovered, it was later identified as a DNA-binding transcriptional regulatory protein by *in vitro* analysis of the purified CadC (Endo *et al.*, 1995). The role as a regulator for CadC in CadA operon was further confirmed by gel shift and DNase I-footprinting experiment, in which CadC binds specifically to the *cad* operator/promoter. Meanwhile, CadC was also found as a member of a newly concluded metal-binding repressor protein family together with *arsR* (arsenic resistance repressor) and *smtB* (cyanobacterial metallothionein repressor)(Silver and Phung, 1996). Recently, the role of CadC was further confirmed by cotransformation with reporter gene under the control of *cadA*-operator/promotor in *E. coli* (Rensing *et al.*, 1998). Thus, without a doubt, the regulatory function of CadC to modulate the expression of CadA in CadA operon should be established.

1.5. The role of CadA as an energy-dependent extruder

The unique phenomenon for extruding cadmium by CadA has been demonstrated (Yoon and Silver, 1991). However, previous studies had found that when the protein synthesis in cell-free extracts from susceptible and resistant bacteria, both of them were subjected to the

inhibition by cadmium. However, the spheroplasts extracted from resistant strains restored their resistance to cadmium. Suggesting that the cadmium resistance was through the directional change of the membrane or phospholipid of resistant strain (Chopra, 1975). Later, using a competition experiment, Tynecka *et al.* demonstrated that cadmium entry bacteria through a Mn^{2+} transport system (1981). It has also confirmed by Weiss *et al*. (1978) that uptake of Mn^{2+} is inhibited by the presence of Cd^{2+}. On the other hand, Tynecka *et al*. (1981) discovered that the extrusion of cadmium is blocked by 2,4-dinitrophenol, N,N,-dicylohexylcarbodiimide (DCCD) and by incubation at 4^{o}C. Suggesting that the cadmium resistance is involved in an energy-dependent fashion. Likewise, they found that valinomycin did not reduce the uptake of cadmium by resistant strain and thus a $Cd^{2+}/2H^{+}$ antiporter was then proposed (Tynecka and Silver, 1981).

註解: I am not sure what this sentence to you? But I have no idea.

註解: You need to re-organize the sentences before your getting into another topic for CadA.

1.6. The CadA, the metal translocating P-type ATPases

1.6.1. CadA belongs to the P-type ATPase superfamily

As mentioned above, the deduced *cadA* gene was found similar to a class of enzyme called P-type ATPases (Silver *et al.*, 1989). Based on its sequence similarity to other ATPases, the resistance mediated by CadA should be performed through a primary transporter, like other P-type ATPases, but not like the secondary transporter proposed by Tynecka *et al.* (Tynecka and Silver, 1981). *In vitro* transport assay has confirmed that CadA is able to catalyze ATP-dependent ^{109}Cd(II) in everted membrane

vesicles prepared from *B. subtilis* harboring the CadA operon (Tsai *et al.*, 1992). In their experiments, Tsai *et al.* found another crucial piece of evidence that CadA only utilize ATP, but no other different energy sources including NADH and phenazine methosulfate plus ascorbate, which generate proton motive force (*pmf*) (Tsai *et al.*, 1992). Indicating that CadA is indeed an ATPase. A mild inhibition was demonstrated when cadmium transport assay in the presence of bafilomycin, a moderate inhibitor for P-ATPase, also supported that CadA is a member of P-type ATPases (Tsai *et al.*, 1992)

1.6.2 P-type ATPase superfamily

Previously, all known P-ATPases were found their functions to maintain suitable ion concentrations in cells. Nowadays, more and more P-ATPases have been discovered, and their roles in ion homeostasis ranging form human to bacteria were also demonstrated (Solioz and Vulpe, 1996). In essence, all P-ATPases share several unique features distinct them from other membrane transporters. For instance, mostly they are single polypeptides, ranging from 70 to 200 kDa, with a famous example of Na^+/K^+-ATPase found in animal cells, and inactivated in the presence of micromolar of vanadate. The last but not least, the prefix “P” given is owing to a phosphorylated intermediate formed during the enzymatic cycle (Pedersen and Carafoli, 1987). So far, only few ATPases have been studied extensively including: plasma membrane Na^+/K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase.

The most striking feature sharing by all P-type ATPases is the transient existence of acylphosphate aspartate when the high-energy phosphate hydrolyzed from ATP. An invariant aspartic acid residue (D) located within a stretch of conserved sequence DKTGT, is phosphorylated at the very beginning when ATP binds to the ATP-binding domain.

1.6.3 CadA is a member of CPx-type ATPases

Based on their substrate specificities, P-type ATPases has been further divided into 5 phylogenetic groups (Axelsen and Palmgren, 1998). Apart from other 4 branches, CadA is classified in the subgroup of CPx-type ATPase (Solioz and Vulpe, 1996), which also called P1-type ATPase (Lusenkoand Kaplan, 1995) or heavy metal P-ATPase. The CPx-type ATPase have several unique features differed them from other P-type ATPases, which include 1-6 proposed metal-binding sites with conserved sequences known as GXXCXXC or (M/H)XXMDH(S/G)XM located at the N-terminal end of these proteins, 8 transmembrane segments and a proline residue flanked by two cysteine/histidine/serine residues known as CPH, CPS or SPC , the so-called CPx motif, and buried within one of the transmembrane segment (Gatti *et al.*, 2000), and a conserved HP dipeptide 34-43 amino acids to carboxyl-terminal to CPX motif (Solioz and Vulpe, 1996). So far, only few CPx-ATPases have been determined their transporting substrates including CadA transports cadmium in *S. aureus*, CopB trnaports copper in *Enterococcus hirae* and ZntA transport zinc and lead in *E. coli* were demonstrated (Tsai *et al.*,

1992; Silver and Odermatt, 1995; Beard *et al.*, 1997; Rensing *et al.*, 1997).

1.6.4. Topological structures and conserved regions of P-type ATPases

Typically, P-type ATPases were derived from a large group of transporters whether they are using ATP or *pmf* as fuel to drive movement of different substances (Driessen *et al*., 2000). Therefore, it is very likely to find core structures or conserved domains from their evolutionary parents, such as the well-known ABC (ATP-binding cassette) transporters, according to the deduced sequence analysis. For example, ABC proteins composed of two homologous cytoplasmic domains, two extramembrane loops and three pairs of transmembrane segments (Gatti *et al.*, 2000). Due to the insufficient data from crystal structure, however, many studies manage to detour the problem and disclose the topology model of these proteins using biochemical approaches. In our recent work (Tsai *et al.*, 2002), the topology of CadA shows a structural homology to its closely related cousin, the CadA of *Helicobacter*. There are 8 transmembrane segments, 3 cytoplasmic loops and 4 periplasmic loops (the CadA topology model shown as figure below). Along with its structural characteristics, there are several motifs necessary for efflux function were also identified in this CadA topology model, including the kinase domain (DKTGT), ATP-binding domain (GDGXNDXP) and phosphatase domain (TGES/A)(Solioz and Vulpe, 1996; Tsai *et al.*, 2002).

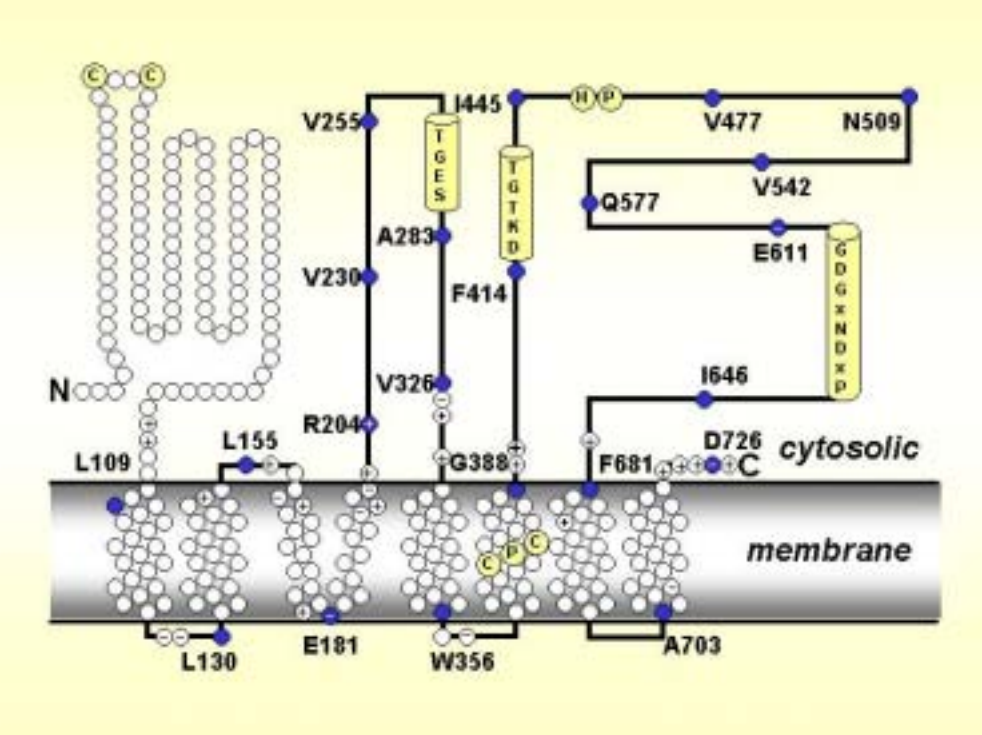


Fig. 1. Topology structure and conserved motifs of CadA protein (Adapted from Tsai *et al.*, 2002).

1.6.5 The CXXC and CPC motifs in CadA:

In order to transport metal ions across membrane barrier, it is necessary for transporters to interact with their substrates at certain level. For CPx-type ATPases, there are putative 1-6 metal binding motif(s) localized on the N-termini of the protein before the first transmembrane segment (as shown in the CadA demonstrated topology above). The unique sequence of the metal-binding motif is conserved among CPx-ATPases, including CadA in *S. aureus*, CopA and ZntA from *E. hirae* or Menkes/Wilson disease proteins, which have one and six repeats

respectively. The hypothesis of binding metal ion associated with the sequence GMTCXXC (Silver *et al.*, 1989) has been adapted from the studies of MerP (mercury-binding protein) with NMR analysis (Sahlman and Skarfstad, 1993). Once the sequence had been modified by mutagenesis, variations have been observed between the mercury-bound and unbound MerP. The binding to the mercury as a substrate in MerP was resulted from the interaction between mercury and cysteine side chain in a way of bi-coordination. Moreover, similar results were demonstrated from the studies of CopA (Bayle *et al.*, 1998) and Menkes/Wilson protein (Lutsenko *et al.*, 1997) that copper binds specifically and selectively to the double-cysteine motif. Together with *in vivo* experiment, which confirmed the accumulation of copper ions was due to the mutations of metal-binding site while the wild-type can successfully transport copper (Strusak *et al.*, 1999). Therefore, the role of GMTCXXC as a metal ion anchor should be established.

On the other hand, another unique feature of CadA protein is its CPC motif buried in the 6^{th} transmembrane segment (as shown in the CadA demonstrated topology above). In fact, the newly classified CP_X-ATPases are named after the discovery of invariant residues-CPC or CPH (Solioz and Vulpe, 1996). Identified CP_X motif was speculated as part of cation transduction pathway, according to the studies of Ca^{2+}-ATPase in sacroplasmic reticulum. When the $proline^{308}$ of the Ca^{2+}-ATPase was mutated, and the affinity of Ca^{2+} is declined implying the proline residue is essential for metal binding (Vilsen *et al.*, 1989). Therefore, it is very likely that the CP_X motif might participate in the heavy metal

transduction pathway.

1.6.6. The mechanism of heavy metal transduction of P-ATPase

Only few crystal structures of membrane transporters had been revealed, due to the nature of membrane proteins which are difficult to be crystalized, especially when they associated with complex phospholipids. Currently, there are insufficient data to clarify the molecular mechanism of P-ATPases, and the CPx-ATPase as well. Therefore, it is then reasonable seeking other homologues in the superfamily of cation transporters to disclose the cation-translocating mechanisms underlying their enzyme activities. Much of these data were from kinetic and electrophysical evidence suggested a “gate model” to describe the uni-directional movement of cation across the membrane (Moller *et al.,* 1996). The gate model had depicted a general mechanism for P-ATPases (Fig. 2): Upon cation interacting with metal binding domain, it results in a conformational change to open its cytosolic gate. By induced fit mechanism, the bound cation is then occluded into membrane and the gate closed followed by the phosphorylation of ATP. The protein structure undergoes subsequently conformational change with opening the gate toward extracytosolic space allowed cation efflux (Tanford *et al.,* 1987). The “gate model” was reported to share similarity with previous “jaw-closing model” in Ca^{2+}-ATPase and many other homologues, such as Na^{+}/K^{+}-ATPase (Post *et al.,* 1972) and SERCA ATPase (Orlowski and Campeil, 1991). Not until atomic resolution model provide structural evidence, do we unveil the exact mechanism of heavy metal traffic across

biological membrane. Furthermore, how ATPs will be utilized to provide energy for the conformational change guiding for cation transporting remain unclear. Thus, in this thesis study, our major goal is to demonstrate the ATPase activity of the CPx-ATPase, and to determine the roles of those unique domains during ATP hydrolysis process using CadA as a model.

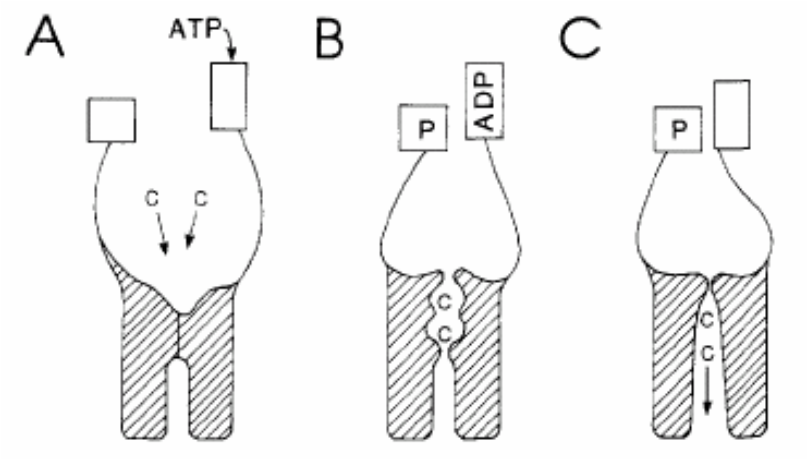


Fig. 2. Hypothetic “gate model” for translocation of cation (C).
(A) Cations attatch to intracellular metal-binding site. (B) translocation process initiated by phosphorylation of ATP. (C) conformational change lead to cations extrusion (Lauger, 1991; Tanford *et al.*, 1987).

1.7 Rationale and experimental approach

The main purpose of this thesis study is to determine the effects of two cysteines in CadA, namely, Cys371 and Cys373 of the CPx motif in transmembrane region, and their role in ATPase activity. Also, C-terminal deletion mutant (ATP-binding motif) of CadA will also be examined. In order to pursue these goals, several strategies will be taken as fallowing:

1. Generate Cysteine mutants and deletion mutants in the GST-CadA fusion protein.
2. Determine the survival of these mutants in toxic metal medium.
3. ATPase assay will be established and taken to measure the ATP hydrolytic activities of those mutated CadA.

Chapter 2. Materials and methods

2.1 Materials

All restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The *E. coli* S30 Extract system for circular DNA was purchased from Promega (Madison, WI). The chemicals and buffers used in this study were products from Sigma (St Louis, MO).

2.2 Bacterial strains and plasmids

The collection of *E. coli* strains and plasmids are listed below in Table 1. The plasmid pKJ100 containing cadA gene was constructed in our lab. While the pGST-cadA fusion plasmid, BF2000 and RW3110 strain were provided from Dr. Barry Rosen in Department of Biochemistry and Molecular Biology of Medical school of Wayne state university, U.S.A.

2.3 Cadmium resistance assay

Cadmium resistance assays were performed as described (Tsai, 1992). Single colonies from strain RW3110 or BF2000 harboring *cadA*, *cadA* mutants and vector without *CadA* were inoculated into LB medium (BF2000 strain must supplement with potassium chloride at final concentration: 50mM). After incubating at 37^{o}C with 200 rpm shaking overnight, 50 times diluted aliquots of stationary cultures are inoculated into same medium supplemented with differential concentrations of

cadmium chloride. Cell growth was measured as optical density using a spectrophotomer at a wavelength of 600 nm after incubation at 37 ^{o}C, with 200 rpm shaking for 6 hours.

Table 1. Strains and plasmids used in this study

Strain		
BF2000	*TKR*2000 *zntA*::cm △*unc*	Dr. Rosen's lab
RW3110	W3110 *zntA*::km	Dr. Rensing *et al.*, (1998)
Plasmid		
pGEX-6P	Expression vector for *cadA* gene fused with GST protein at N-termini	Pharmacia
pGST-CadA	2.1 kb *BamHI/NotI* fragment of *cadA* in pGEX-6P	Dr. Rosen's lab
pKJ100	*NcoI-XbaI* fragment of entire *cadA* in pSE380	This lab
pSE380	Expression vector for *cadA* gene	Invitrogen
pKJ3	2.6 kb *XbaI* fragment containing the 3' end of *cadC* and complete *cadA* in pET-11a	This lab

2.4 Preparation of membrane vesicles

E. coli strain BF2000 harboring wildtype or mutant CadA were incubated overnight at 37 ^{o}C in 5ml LB medium supplemented with KCl (50mM) and Ampicillin (100 μ g/ml). The overnight culture was diluted 50-fold in same medium and induced with 0.4 mM IPTG for 2 hours when OD_{600} reach mid-log phase at 0.6-0.8. The cultures were then on ice for 10 minutes and harvested by centrifugation. The pellets were washed twice with 10 mM MOPS-KOH, pH7.0 and stored frozen until use.

The pellets were resuspended in Sucrose buffer (MOPS-KOH[pH7.0], 250 mM sucrose, 0.2 M KCl, 25 mM $MgSO_4$, 0.5 mM EDTA) at about 5 ml/g of wet cells. The membrane vesicles were prepared by passing the cell lysates through a french pressure cell at 10,000 p.s.i.. The reductant β-mercaptoethanol (10 mM), protease inhibitor PMSF(1 mM) and ATP(1 mM) were added immediately to the lysate. Cell debris and unbroken cells were pelleted by centrifugation at 10,000 x g. for 30 min, and the supernatant was ultracentrifuged at 100,000 x g for 90 min to isolate the membrane fraction. The membrane fraction was resuspended in Tris-HCl(pH7.0) at 10 mg/ml with DTT (1 mM) and PMSF (1 mM) and aliquots were stored in -80^{o}C until use. Protein concentration was determined by modified Lowry method using bovine serum albumin as standards (Bensadoun and Weinstein, 1976; Peterson, 1977).

2.5 *In vitro* transcription/translation

The plasmid pKJ100 and pSE380 were subjected to *in vitro* protein synthesis followed by the procedure from *E. coli S30* extract system for Circular DNA (Promega; Madison, WI). The DNA template (5 μg) was mixed with the reaction components (Amino acid mixture 5 μl, S30 premix 20 μl and S30 extract 15 μl), vortex gently and incubated the reaction at 37^{o}C for 1 or 2 hours. To stop the reaction, the mixtures were placed on ice for 5 min. The final reaction products were applied directly to ATPase assay.

2.6 ATPase assay (coupling method)

ATPase activity of *CadA* membrane vesicles were measured using the pyruvate kinase and lactate dehydrogenase coupling assay, which the regeneration of ATP is coupled to the oxidation of NADH (Sharma *et al*, 2000). Membrane vesicles (100 μg) are pre-incubated with reaction buffer mixture on ice for 10 min (Tris-HCl 100 mM[pH 7.0], KCl 50 mM, $MgCl_2$ 2.5 mM, Glycerol 10%, NADH 0.25 mM, phosphoenolpyruvate 1.25mM, pyruvate kinase 5units, Lactose dehydrogenase 5units) with or without cadmium. The mixtures were incubated at 37 ^{o}C before initiating the reaction by adding 5 mM ATP[pH 8.0]. The kinetic process was analyzed by a spectrophotometer at a wavelength of 340 nm.

2.7 ATPase assay (Phosphate precipitation method)

A rapid and sensitive method for quantitating phosphate releasing from CadA protein was employed. The reaciton buffer (25 mM Tris-HCl[pH7.0], 200 mM KCl, 250 mM Sucrose, 5 mM $MgCl_2$) was kept on ice with 100 μ g membrane with or without cadmium before incubation at 37 ^{o}C for 5 min. The reaction was triggered by adding 2mM ATP[pH8.0] and stopped by taking 20 μl of the reaction mixture mixed with 80 μl 0.2 M EDTA at desired time course. The mixture was diluted 5-fold with deionized water and the color developing was followed by adding 172 μl reagent A (28 mM ammonium molybdate, 2.1 M sulfuric acid) and 128 μl reagent B (0.76 mM Malachite green in 0.35% polyvinly alcohol). After incubating at room temperature for 20 min, the absorbance was measured by a spectrophotometer at a wavelength of 610nm.

2.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

To ensure the GST fusion proteins are produced, SDS-PAGE and Western blotting are performed according to standard procedures. Membrane fractions of fusion proteins are subjected to analysis in 7% SDS-PAGE as well as in Western blotting. After the electrophoresis, the fusion proteins were blotted onto a methanol-rinsed PVDF membrane (Bio-Rad) in blotting chamber at a current of 300 mA for 2 hours. Membrane was blocked with 5% non-fat milk in TBST buffer (10 mM

Tris-HCl[pH 7.5], 150 mM NaCl, 0.05% Tween 20) for 1 hour. The blots were then probed with mouse antibody against GST (Glutathione-S-transferase)(Pierce; Rockford, IL, USA) for 1 hour. Followed by 3 times of washes with the same buffer and reprobed the PVDF membrane with polyclonal anti-mouse antibody in TBST with 5% non fat milk. The membrane was finally washed 3 times as previous described and developed using BCIP-NBT substrate buffer (5-bromo-4-chloro-3-indolyphosphate-nitroblue tetrazolium in AP buffer) (Harlow, 1988).

Chapter 3. Results

3.1 Mutagenesis of pGST-cadA

As mentioned above, to disclose the enzymatic mechanism for CPx-ATPase requires more experiments. The CxxC proposed metal binding motif, the ATP-binding domain and CPC motif could be the necessary answers to draw the complicated system like CPx-ATPase. Previous works in our lab have created eight cysteine mutants in either CxxC or CPC motif by *in vitro* site-directed mutagenesis strategies (unpublished data). The cysteine residues at positions 23 and 26, and 371and 373 within the conserved CxxC and CPC motif were replaced by serine (conserved mutation) and glycine (non-conserved mutation), respectively. All mutations were cloned originally in pKJ4 (*cadA* gene in pALTER-1) and subcloned into pKJ100 and pGST-cadA, namely pKJ9-12 and pGST-105, pGST-106, pGST-107, and pGST-108. Or prospective DNA fragments corresponding to each cysteine mutation was used to replaced the wild-type *cadA* gene clone, the pKJ100, to create a series of plasmids called pKJ101-108 as listed in Table 2. On the other hand, ATP-binding domain (ABD) deletion was achieved by removing a 550-bp *EcoRI/HindIII* fragment containing ABD in pGST-cadA to generate pGST-dABD plasmid. Complete collection of CadA mutants used in this work are listed below in Table. 2.

3.2 Cadmium sensitivity of CadA mutants in RW3110 cells

The phenotype of cadmium resistance of these CPC motif mutants and ABD depletion mutants are determined by cell survivals in enrich medium in the presence of different concentrations of cadmium. All these mutants are expressed in *E. coli* strain, however, *E. coli* poses its own cadmium resistant determinant, the chromosomal *zntA* gene. To eliminate the ZntA, which lead to high resistance to znic and cadmium in *E. coli*, a *zntA* knockout *E. coli* strain W3110 was created by insertional inactivation with kanamycin gene and the resulted cadmium sensitive *E. coli* strain was called RW3110 (Rensing *et al.*, 1997). The RW3110 cells harboring wild-type and mutant *cadA* gene were grown no more than 8 hours to avoid stationary phase, and the turbidity of medium at OD_{600} was measured to monitor the survivals of the bacteria. Obviously, all CxxC and CPC defects in CadA lost their resistance to cadmium completely (Fig. 3.1 and 3.4), except for pKJ101 and pKJ103 (C23S and C26S substitution mutations) which displayed only partial cadmium resistance. Meanwhile, bacterial sensitivities to other heavy metals (lead and znic) were also determined in this study (Fig. 3.2, 3.3, 3.5 and 3.6). Simlarly to cadmium resistance, only those of pKJ101 and pKJ103 mutants displayed a partial resistance to zinc, but other mutants did not (Fig. 3.2). However, among these mutants, only the pKJ101 has shown a comparable resistance as wild-type CadA and pKJ104 has a partial resistance to lead were found (Fig. 3.3). Suggesting that mutations at different amino acids might cause different resistant phenotypes. Data shown in the Fig. 3.13 have further confirm the necessity of the cysteine residues in CPC motif

of CadA, as well as the ABD domain, to confer resistance to cadmium. However, whether or not these resistant differences are the reflections of different enzymatic activity of CadA required further determination.

3.3 ATPase assay of CadA mutants in RW3110 cells by coupling method

Since wild-type CadA in RW3110 exhibits an extraordinary cadmium resistance than mutants both in CxxC and CPC motif. However, there is no knowing that whether the mutations apart from ATP-binding domain will affect the enzyme integrity. Hence, it is necessary to ensure the CadA protein synthesis in RW3110 cell membrane. To confirm the presence of both wild-type and mutant CadA proteins in these clones, membrane protein preparations from those RW3110 cells harboring either wild-type or cysteine mutation *cadA* gene was subjected to electrophoresis analysis. As shown in Fig. 3.7, the prospective 79 kDa CadA proteins were visible in 10 % SDS-PAGE gels in both membrane proteins prepared from either wild-type or mutant *cadA* clone. In order to examine the enzymatic activity, the necessary ATPase activity of CPx-ATPase, of these cysteine mutants was measured using a coupling method as previous described (Hou *et al.*, 2001). Thus, a correlation between two most unique features of CPx-ATPase, the CxxC and CPx motif, and their role in ATP hydrolysis activity would be able to determine. It is not easy to give absolute answers consistently, however, most data suggest that mutation in these crucial regions will affect ATPase activity (Fig. 3.8). Since we do not have protocols for CadA

purification at this moment, and therefore it would be difficult to examine the ATPase activity using the crude extract from bacteria. Furthermore, it will be very difficult when we determine the enzymatic activity of CadA ATPase activity using an unpurified preparation. In order to overcome the problem, we decided to take the advantage of using the tag-fusion in our study. However, previous work in our lab has shown a his-tagged CadA displayed cadmium sensitivity (data not shown). Therefore, the alternative is to solubilize the CadA using detergents, such as Triton X-100, to achieve a partial purified CadA preparation for ATPase activity study. As shown in Fig. 3.9, the Trion X-100-solubilized CadA distinguished itself from other preparations. However, the solubilized CadA only present half catalytic ability when compared it to those data shown in Fig. 3.10. Unfortunately, using detergent-solubilized CadA did not generate a consistent ATPase result when using the coupling assay system for the activity determination. Meanwhile, the intention of CadA synthesis by *in vitro* transcription/translation method was performed in order to obtain purified CadA instead of using the traditional protein chemistry procedures. Preparations from *in vitro* transcription/translation were then subjected to coupling system to measure the ATPase activity. In the presence of typical P-type ATPase inhibitor, vanadate, in our ATPase assay, CadA ATPase activity was mildly inactivated (Fig. 3.11), and micro molar cadmium increased activity than CadA without cadmium ion.

註解: Put into discussion..

3.4 Cadmium sensitivity and ATPase activity of CadA mutants in BF2000 cells

To improve our ATPase assays as well as to eliminate the possible interference caused by other bacterial ATPase systems, a newly created *E. coli* strain, the BF2000 (unpublished data from Dr. Barry Rosen's lab), was used for the further ATPase assays. The BF2000 *E. coli* strain was created by insertional mutations of three genes which include the *unc*, F_1F_0-ATPase gene, the Kdp gene, which is one of the major ATPase regulating the bacterial osmolarity, and the ZntA gene, the *E. coli* version of CadA gene. Alone with BF2000, also a newly generated CadA clone, the pGST-CadA plasmid, obtained from Dr. Rosen's lab, was also applied for our ATPase assay. As we shown in Fig. 3.12, the BF2000 strain is less resistance to cadmium than RW3110 strain when both strains harbored the pGST-CadA plasmids. However, the resistance of pGST-CadA/BF2000 was higher than BF2000 strain harboring the parental vector (Fig. 3.12). When CPC and ABD (ATP-binding domain) mutant genes was cloned into the pGST-CadA expression system to examined for their abilities to resist cadmium, all these mutants were found sensitive to cadmium at a concentration less than 10 μM as shown in Fig. 3.13. Suggesting that GST-CadA fusion would be a probable system to test the ATPase activity, and the essences for the wild-type CPC and ABD will be needed for the full CadA activity, and most likely the ATPase acitivity.

On the other hand, in order to improve the detection of trace amount of phosphate hydrolyzed from ATP by CadA, a more sensitivity and rapid

method for ATPase activity (Cogan *et al.*, 1999) has been adapted to use since then. Using the improved method, the BF2000 strain harboring either pGST-CadA or parental vector were subjected for ATPase assays (Fig. 3.15). Furthermore, CPC mutants of *cadA* gene, pGST-105 to pGST-108, and pGST-dABD (ATPase domain deletion mutant), were also prepared and transformed them into the BF2000 strain. As shown in the Fig. 3.15, these mutated CadA showed lower ATPase activities than that of the wild-type CadA. The kinetic results were normalized according to western blot and shown in Fig. 3.14. Also shown in Fig. 3.16, the ATPase activity of pGST-cadA demostrated no significant difference in 10 μM cadmium but improved when β-mercaptoethanol was included.

Table 2. CadA mutants used in this work.

CadA Mutants	Mutation site	Source
pKJ101	C23S	pKJ100
pKJ102	C23G	pKJ100
pKJ103	C26S	pKJ100
pKJ104	C26G	pKJ100
pKJ105	C371S	pKJ100
pKJ106	C371G	pKJ100
pKJ107	C373S	pKJ100
pKJ108	C373G	pKJ100
pGST-105	C371S	pGST-cadA
pGST-106	C371G	pGST-cadA
pGST-107	C373S	pGST-cadA
pGST-108	C373G	pGST-cadA
pGST-dABD	Deletion of ATP-binding domain	pGST-cadA

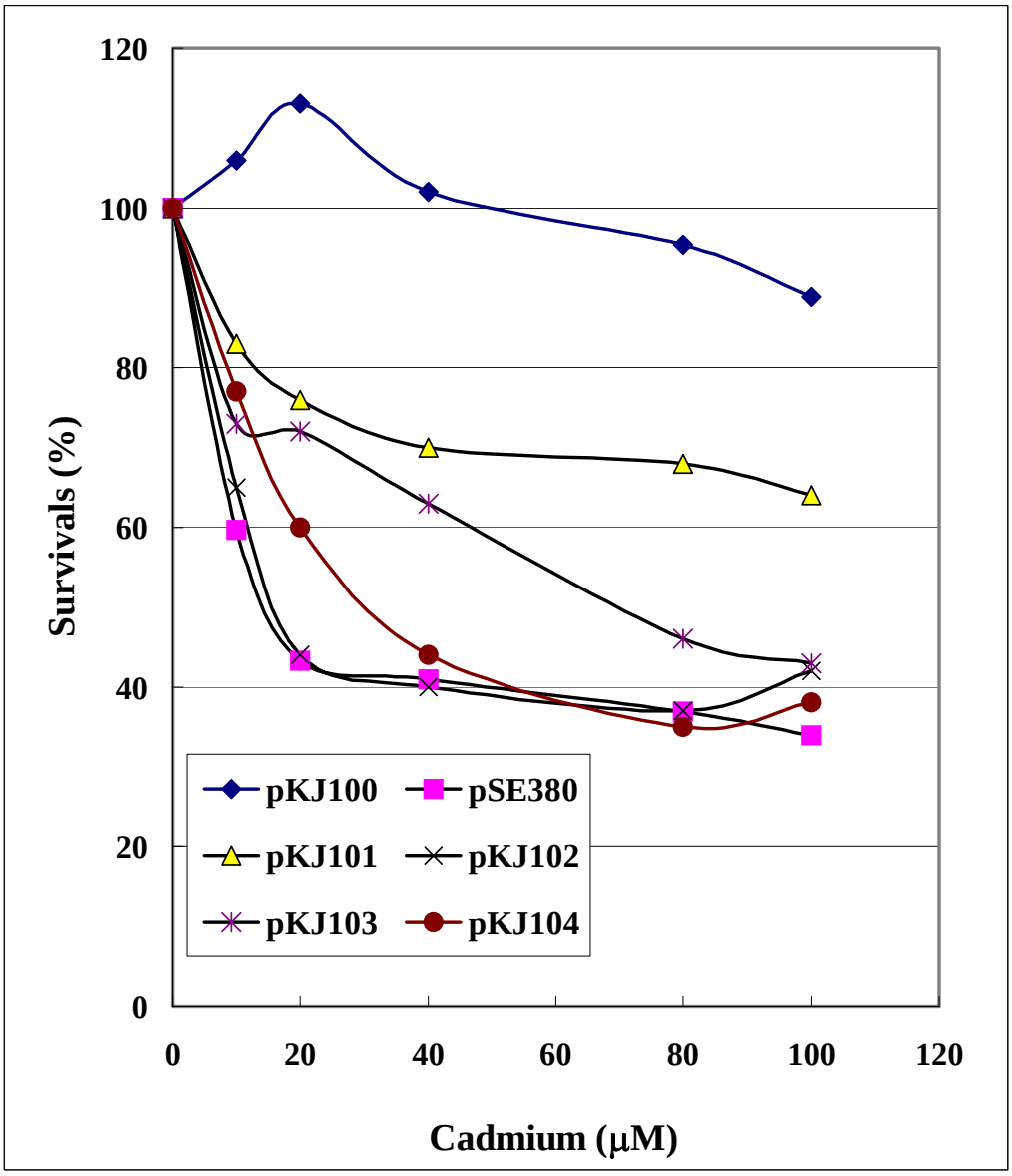


Fig. 3.1. Growth curves of CxxC mutants and wild-type CadA in LB in the presence of different concentrations of cadmium. Overnight cultures of RW3110 were diluted 50x in fresh LB and incubated at 37^{o}C for 6 hours with shaking at 200r.p.m. The survival rates of cultures were normalized using the reading from the cultures without cadmium and shown as percentage (%).

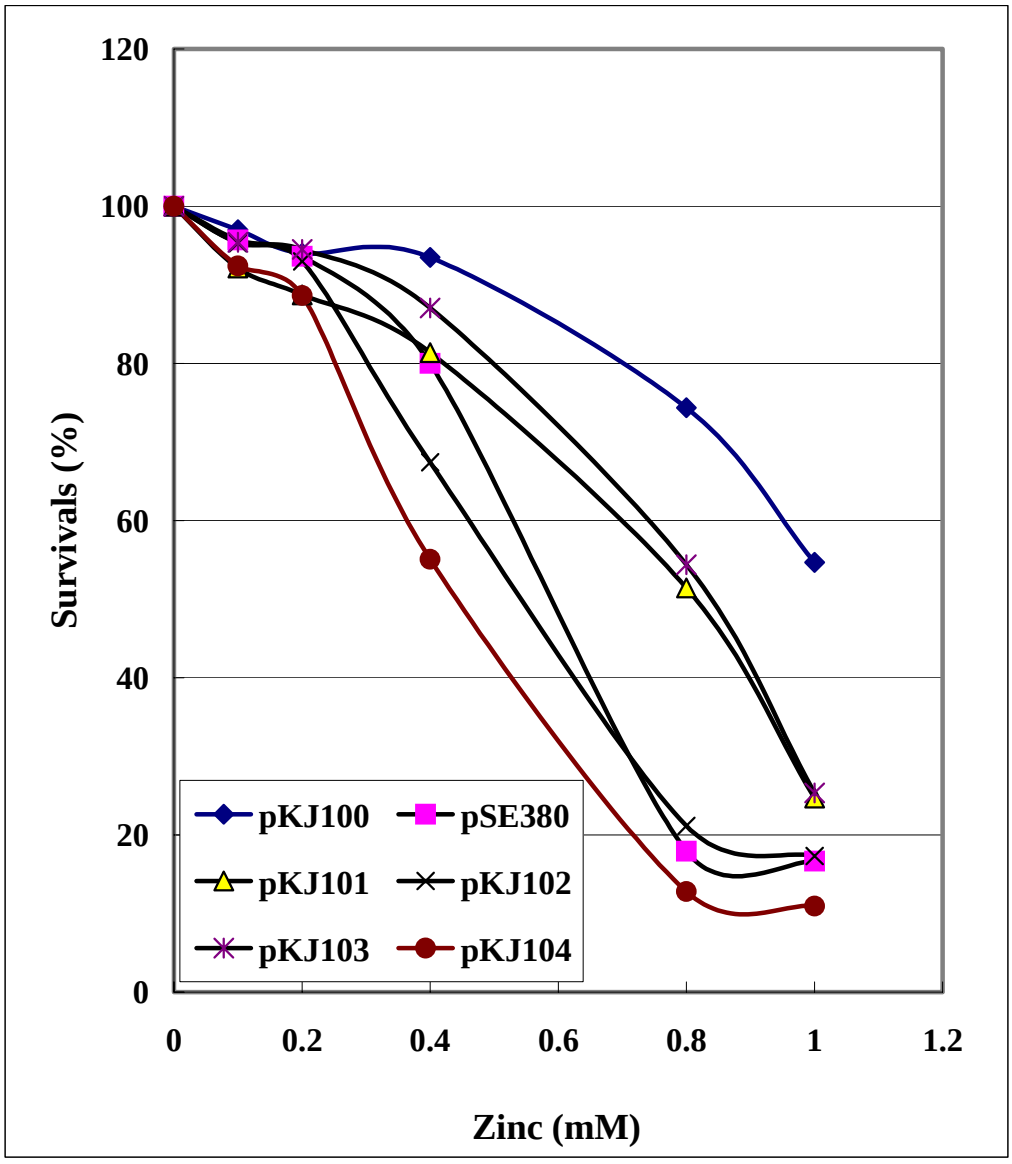


Fig. 3.2. Growth curves of CxxC mutants and wild-type CadA in LB in the presence of different concentrations of zinc. Overnight cultures of RW3110 were diluted 50x in fresh LB and incubated at 37^{o}C for 6 hours with shaking at 200r.p.m. The survival rates of cultures were normalized using the reading from the cultures without znic and shown as percentage (%).

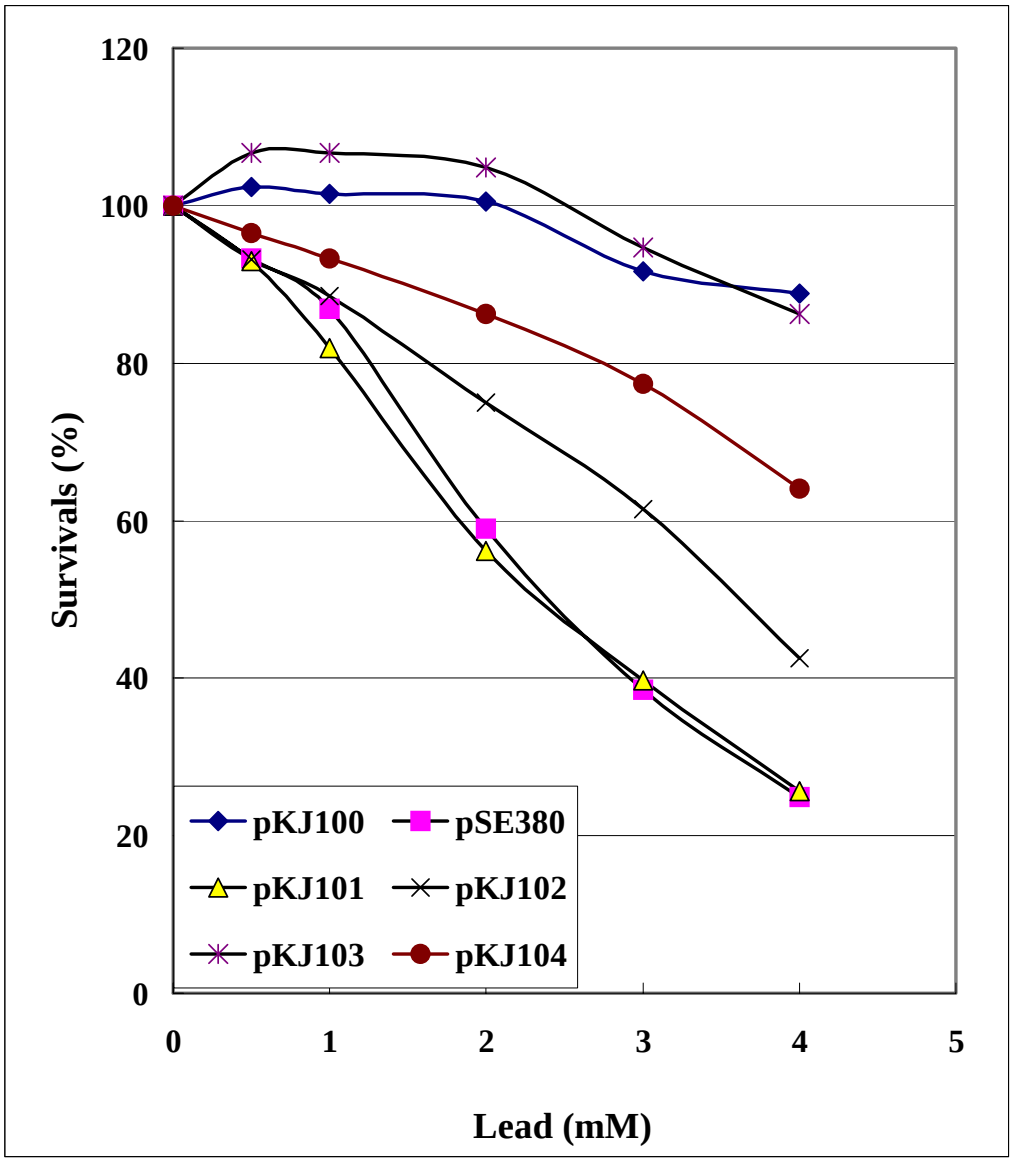


Fig. 3.3. Growth curves of CxxC mutants and wild-type CadA in LB in the presence of different concentrations of lead. Overnight cultures of RW3110 were diluted 50x in fresh LB and incubated at 37°C for 6 hours with shaking at 200r.p.m. The survival rates of cultures were normalized using the reading from the cultures without lead and shown as percentage (%).

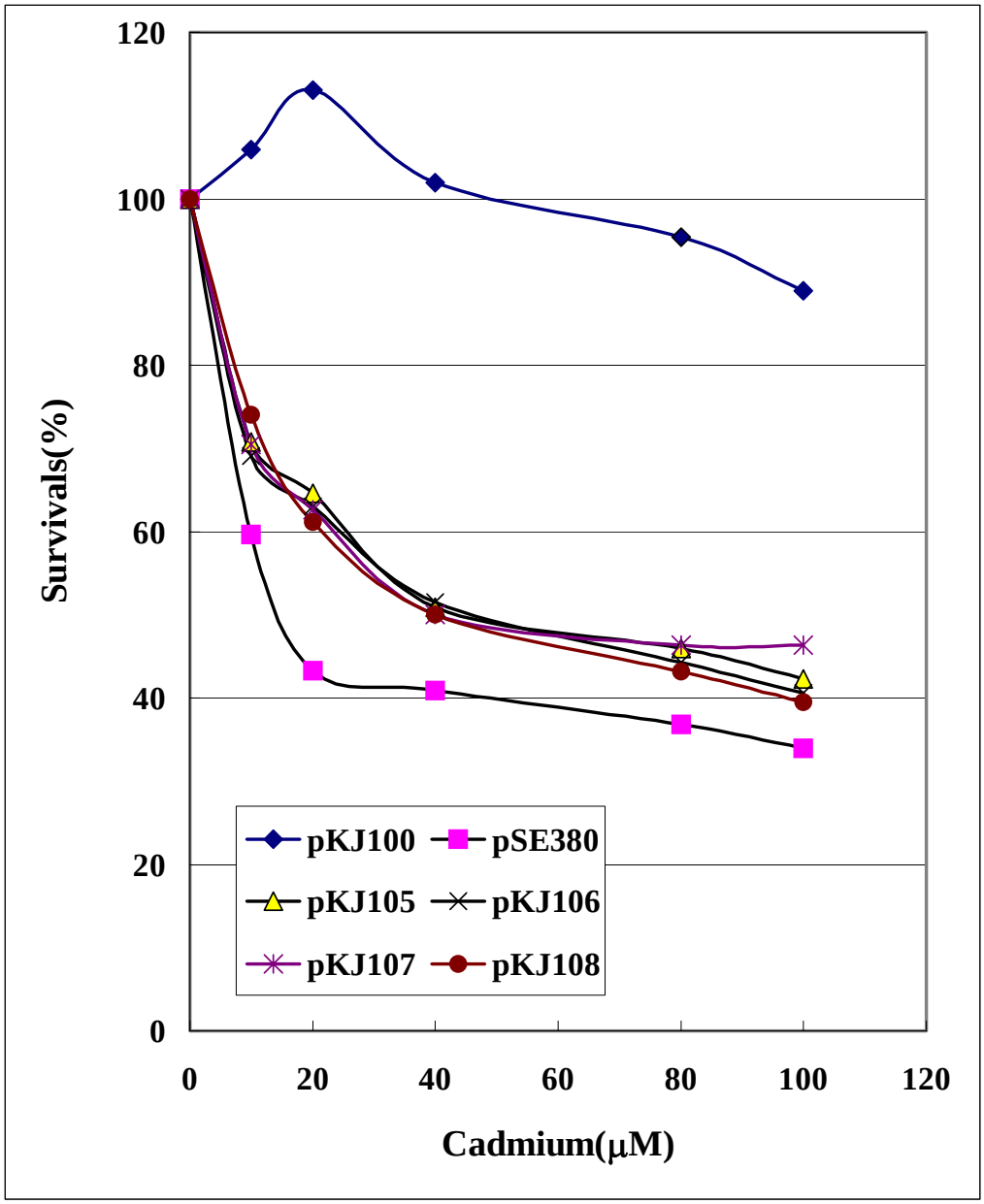


Fig. 3.4. Growth curves of CPC mutants and wild-type CadA in LB in the presence of different concentrations of cadmium. Overnight cultures of RW3110 were diluted 50x in fresh LB and incubated at 37^{o}C for 6 hours with shaking at 200r.p.m. The survival rates of cultures were normalized using the reading from the cultures without cadmium and shown as percentage (%).

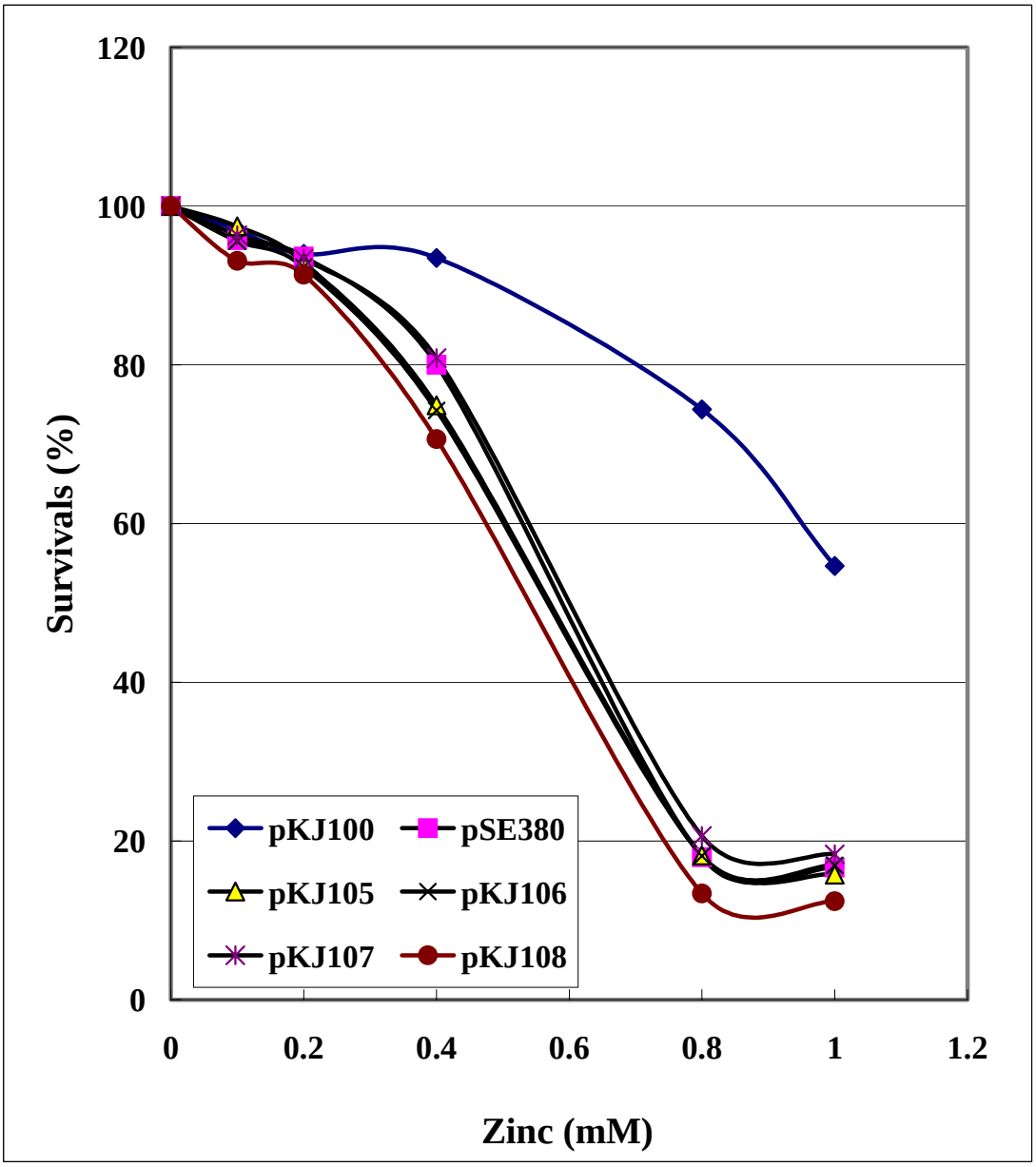


Fig. 3.5. Growth curves of CPC mutants and wild-type CadA in LB in the presence of different concentrations of zinc. Overnight cultures of RW3110 were diluted 50x in fresh LB and incubated at 37^{o}C for 6 hours with shaking at 200r.p.m. The survival rates of cultures were normalized using the reading from the cultures without znic and shown as percentage (%).

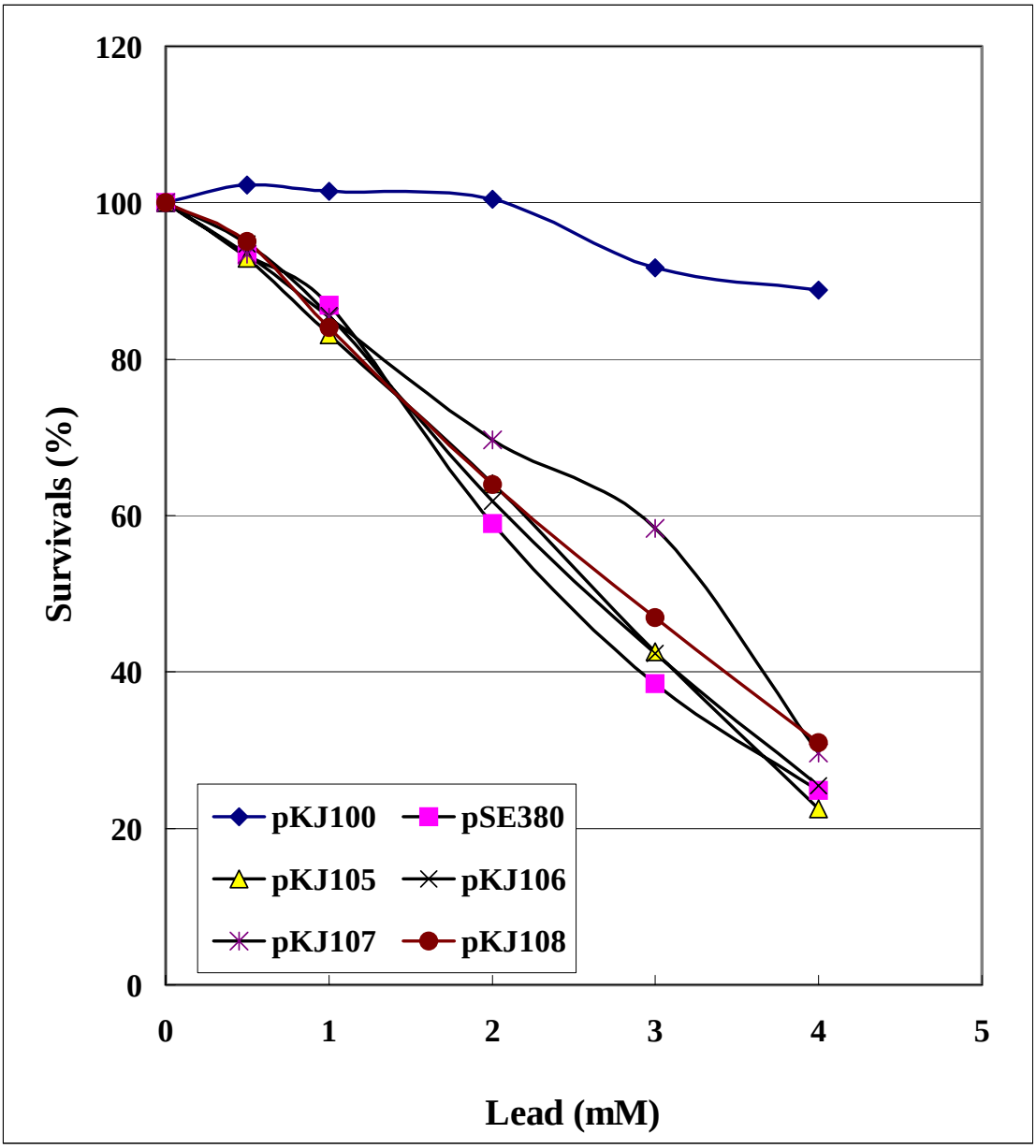


Fig. 3.6. Growth curves of CPC mutants and wild-type CadA in LB in the presence of different concentrations of lead. Overnight cultures of RW3110 were diluted 50x in fresh LB and incubated at 37^{o}C for 6 hours with shaking at 200r.p.m. The survival rates of cultures were normalized using the reading from the cultures without lead and shown as percentage (%).

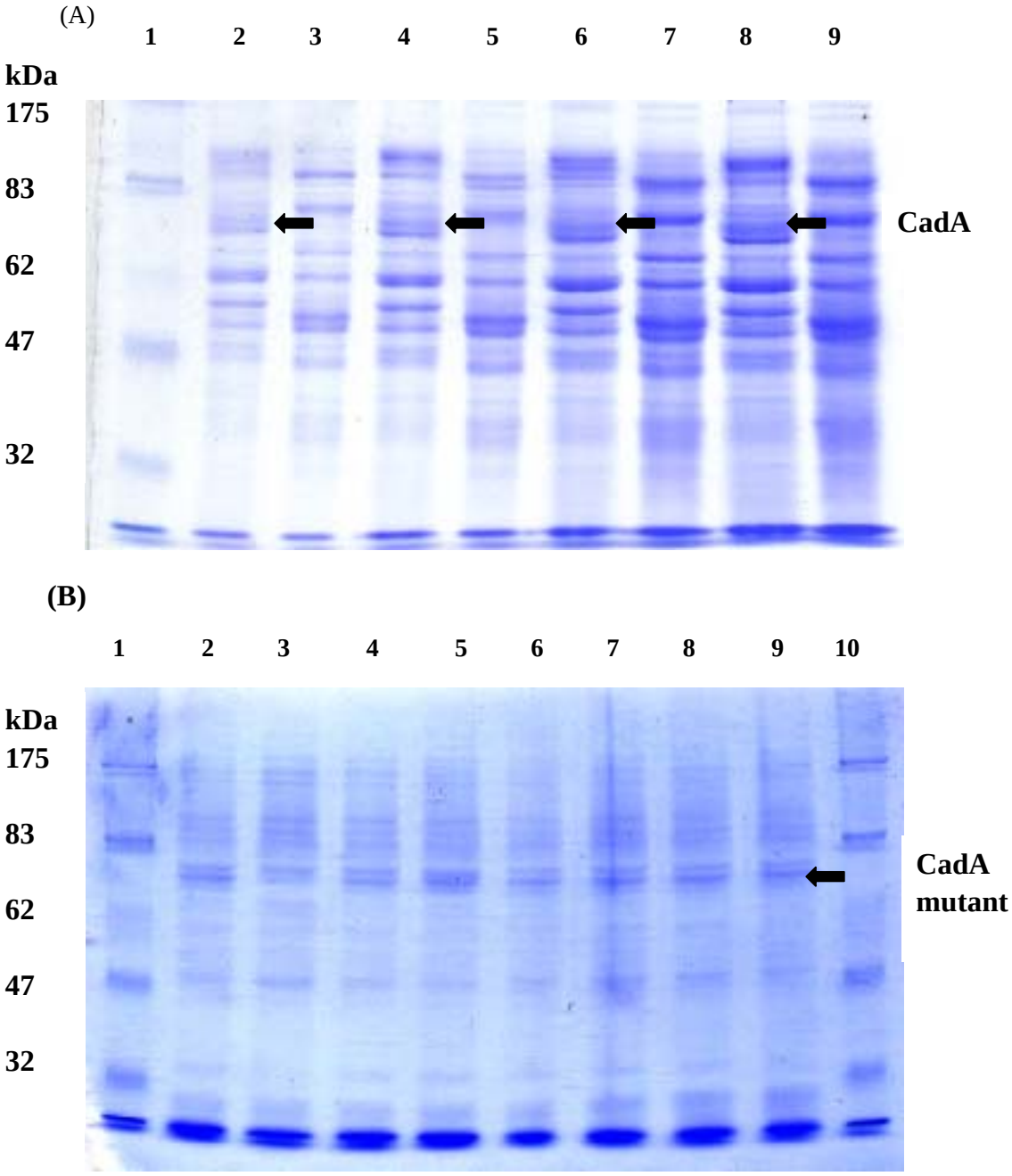


Fig. 3.7. Membrane fractions of RW3110 harboring CadA mutants on 10 % SDS-PAGE. (A) Different concentrations (10, 25, 50, 100 μ g) of membrane proteins prepared from cells harboring either pKJ100 or pSE380 are shown.(lane1. Molecular weight marker; lane 2, 4, 6, 8 , pKJ100; lane 3, 5, 7, 9, pSE380). (B) CxxC and CPC mutants are also shown to ensure protein production (lane 1 and 10, Molecular weight marker; lane 2-9, pKJ101-pKJ108).

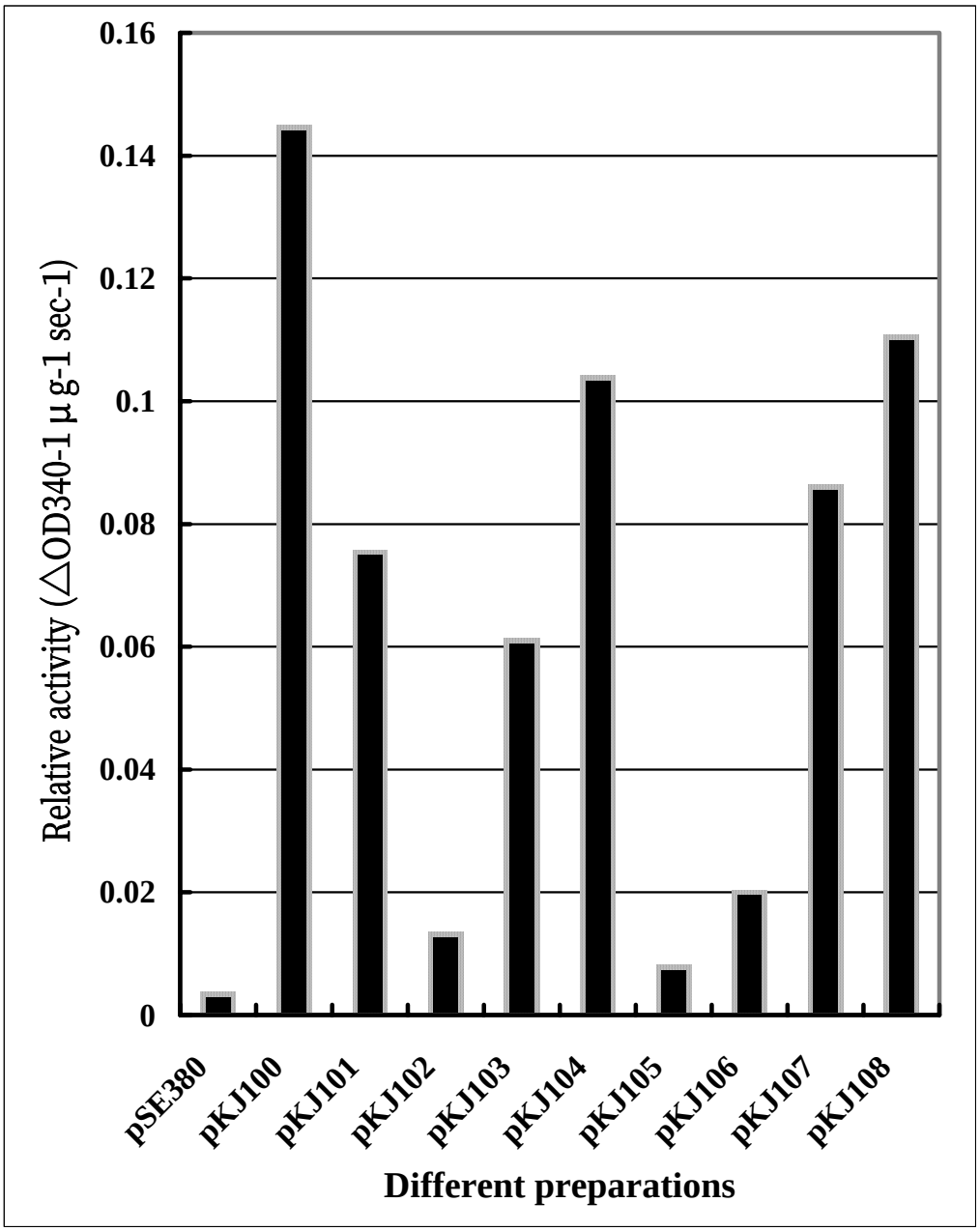


Fig 3.8. Comparison of ATPase kinetics using coupling ATPase assays. The CxxC, CPC mutants as well as CadA are subjected to measure their ATP hydrolysis activities. The optical absorbance of NADH at 340 nm is declined upon ATP regerneration. The changes of OD_{340} ($\triangle OD_{340}$) were reversed and normalized as $\triangle OD_{340}^{-1}\ \mu\ g^{-1}\ sec^{-1}$.

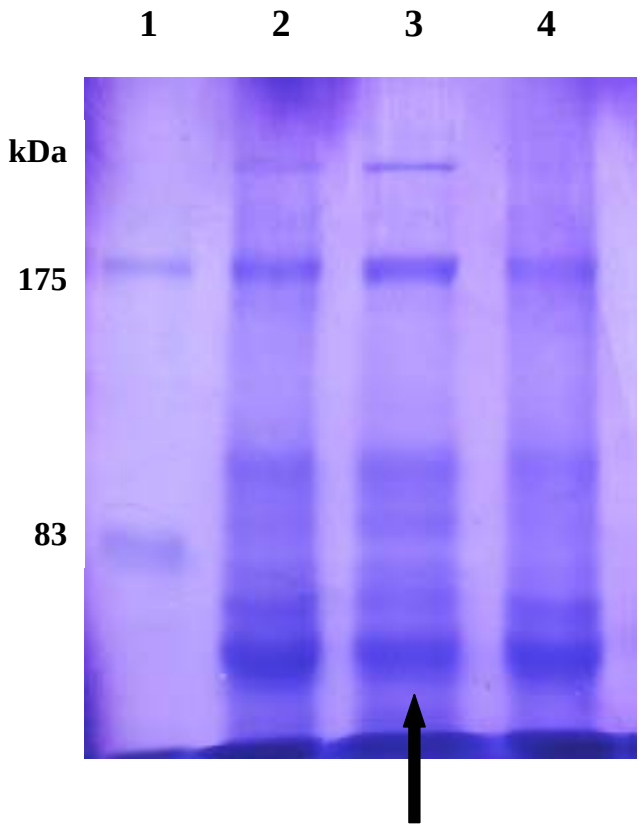


Fig. 3.9. Solubilization of RW3110 harboring CadA with or without Triton X-100 (1 %) on 7 % SDS-PAGE gel. (Lane 1. Molecular weight marker, 2. membrane of pKJ100, 3. supernatant fraction of membrane with Triton x-100, 4. pellet fraction of membrane)

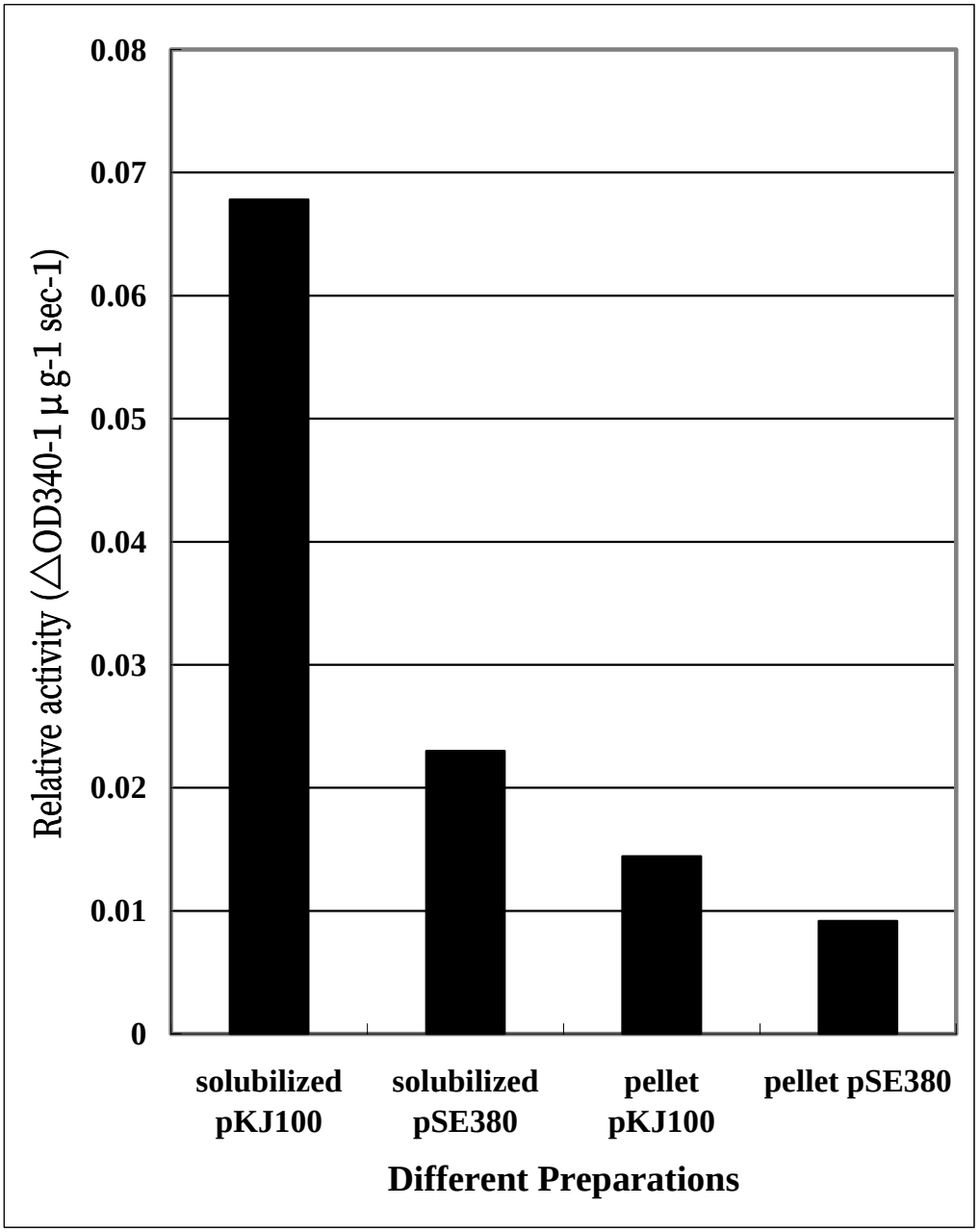


Fig 3.10. Comparison of ATPase kinetics using coupling ATPase assays and Triton X-100 solubilized protein preparations. The pKJ100 and pSE380 were solubilized by 1% Triton X-100. Following by centrifuged at 10,000 x g, both the supernatant and pellet were subjected to measure their ATP hydrolysis activities. The optical absorbance of NADH at 340 nm is declined upon ATP regerneration. The changes of OD_{340} ($\triangle OD_{340}$) were reversed and normalized as $\triangle OD_{340}^{-1}\ \mu\ g^{-1}\ sec^{-1}$

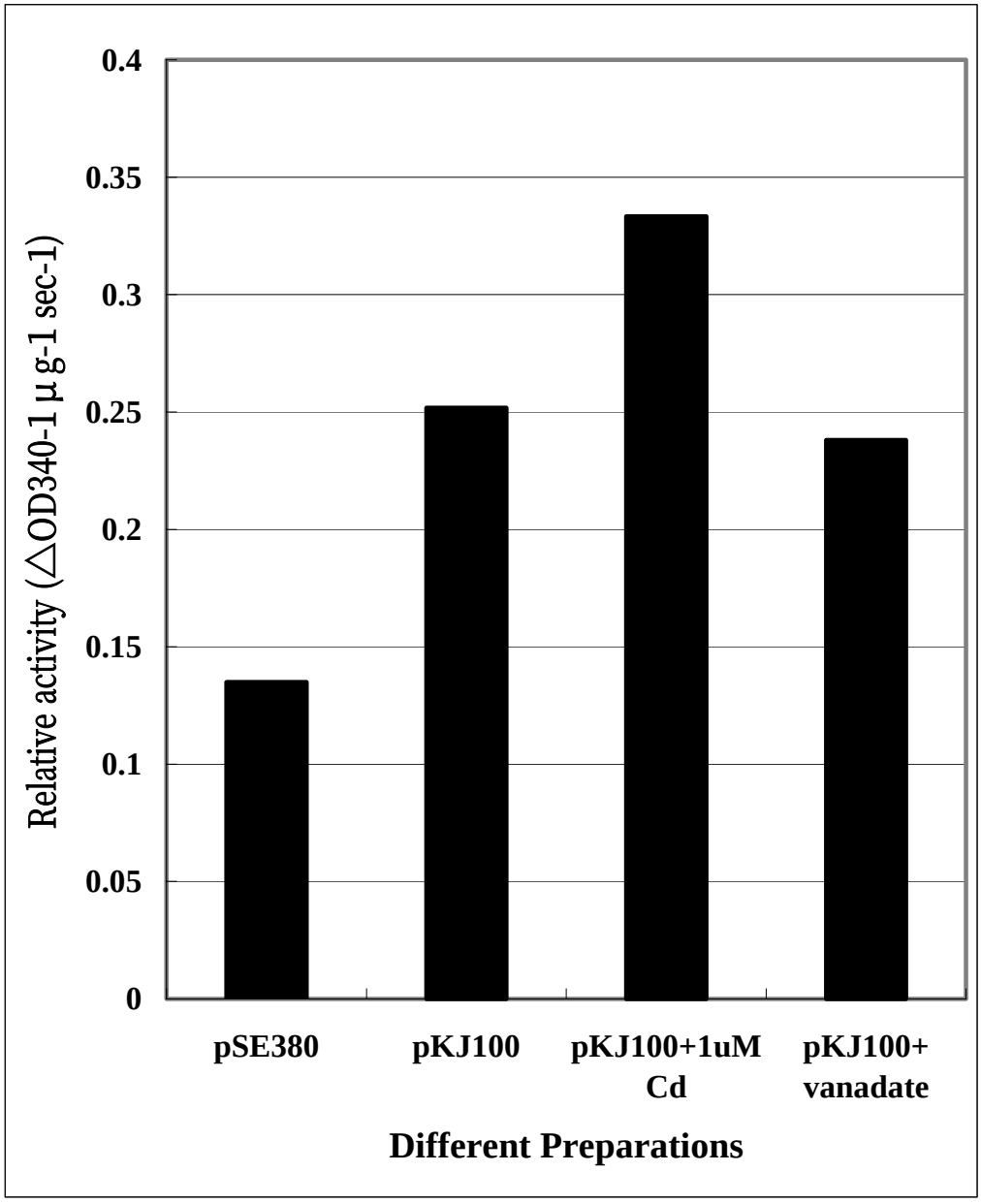
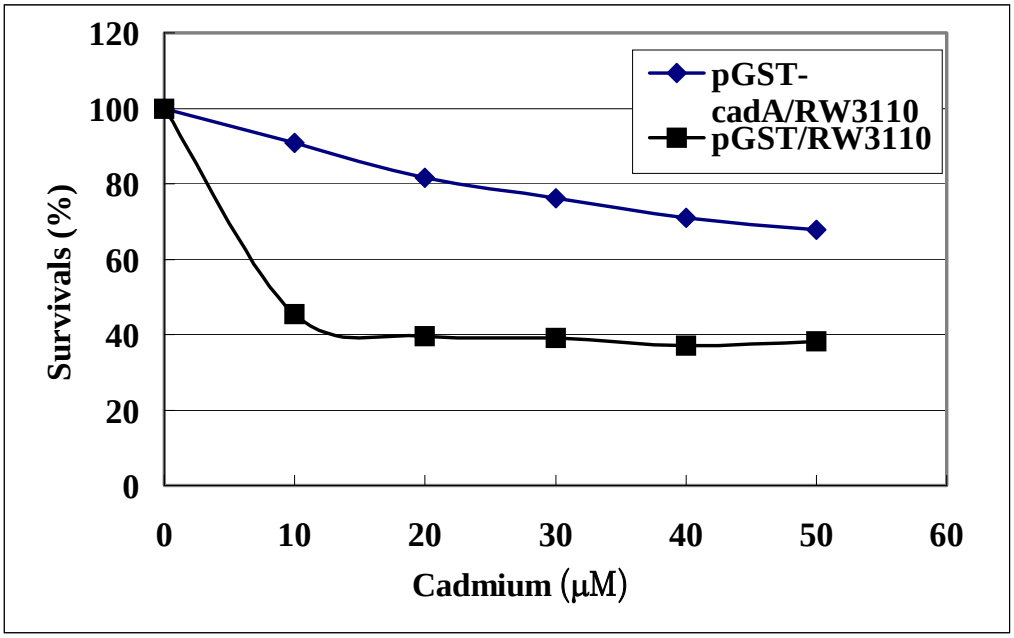


Fig 3.11. Comparison of ATPase kinetics using coupling ATPase assays and *in vitro* transcription/translation products. The *in vitro* transcription/translation products of pKJ100 and pSE380 were subjected to measure their activities. The optical absorbance of NADH at 340 nm is declined upon ATP regerneration. The changes of OD_{340} ($\triangle OD_{340}$) were reversed and normalized as $\triangle OD_{340}^{-1}\ \mu\ g^{-1}\ sec^{-1}$

(A)



(B)

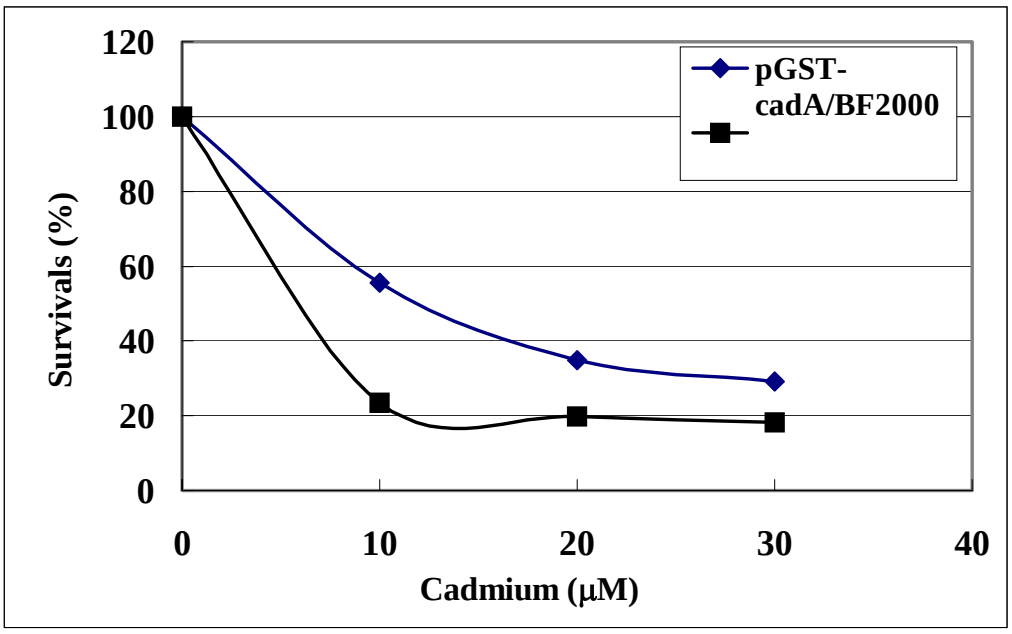


Fig. 3.12. The cadmium resistance of RW3110 (A) or BF2000 cells harboring either pGST-cadA or pGST plasmids. The cadmium resistances were measured as described above.

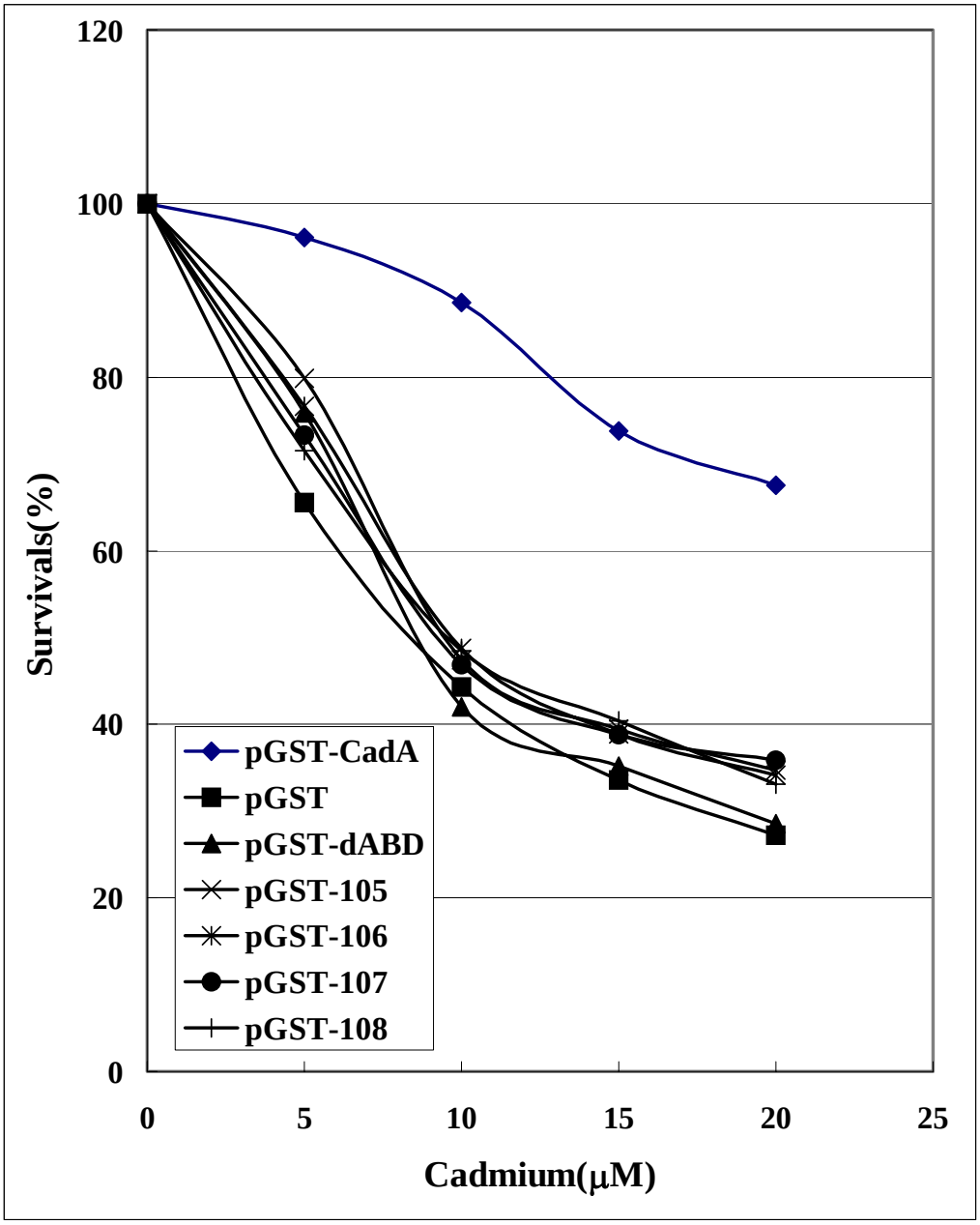


Fig. 3.13. Growth curves of wild-type CadA, CPC and ABD deletion mutants in modified LB medium (supplement with 50 mM KCl) in the presence of different concentrations of cadmium. Overnight cultures of BF2000 were diluted 50x and incubated at 37^{o}C for 6 hours with 200 rpm of shaking. The survival rates of cultures were normalized by readings from cultures without cadmium and shown as percentage (%).

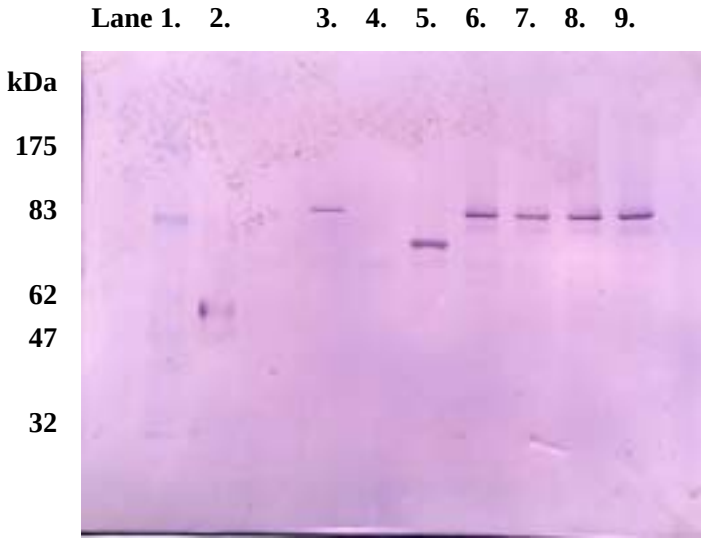


Fig. 3.14. Western blot analysis of BF2000 strain harboring either pGST-cadA, pGST-dABD or four other CPC mutants (pGST105-108). Lane 1, Molecular weight marker; Lane 2, GST protein control; Lane 3, pGST-cadA; Lane 4, pGST vecter control; Lane 5, pGST-dABD; Lane 6, pGST-105; Lane 7, pGST-106; Lane 8, pGST-107; and Lane 9, pGST-108.

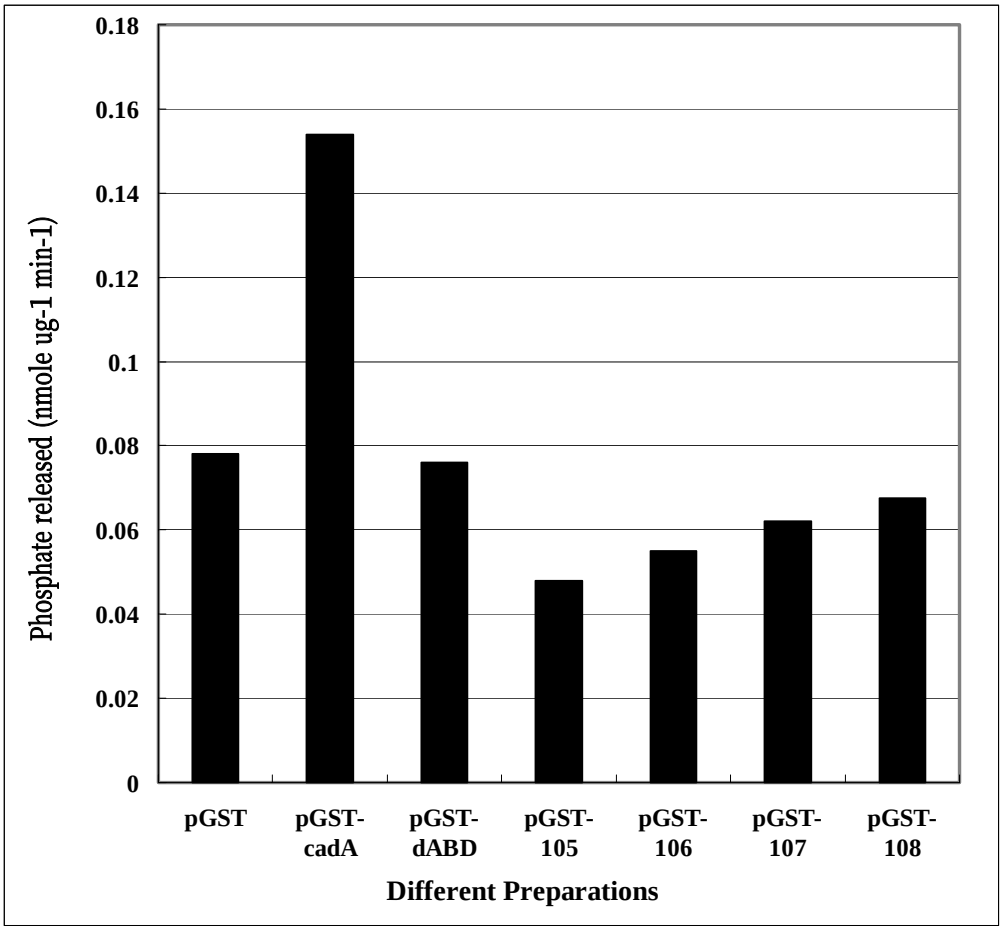


Fig 3.15. Comparison of ATPase kinetics using phosphate precipitation method with CadA mutants as described. The membrane vesicles were prepared from BF2000 cells harboring either pGST, pGST-cadA or *CadA* mutants are subjected to measure their ATP hydrolysis activities. The amounts of inorganic phosphates released from ATP were recorded at different time intervals. And the fusion protein production was normalized with their western blots.

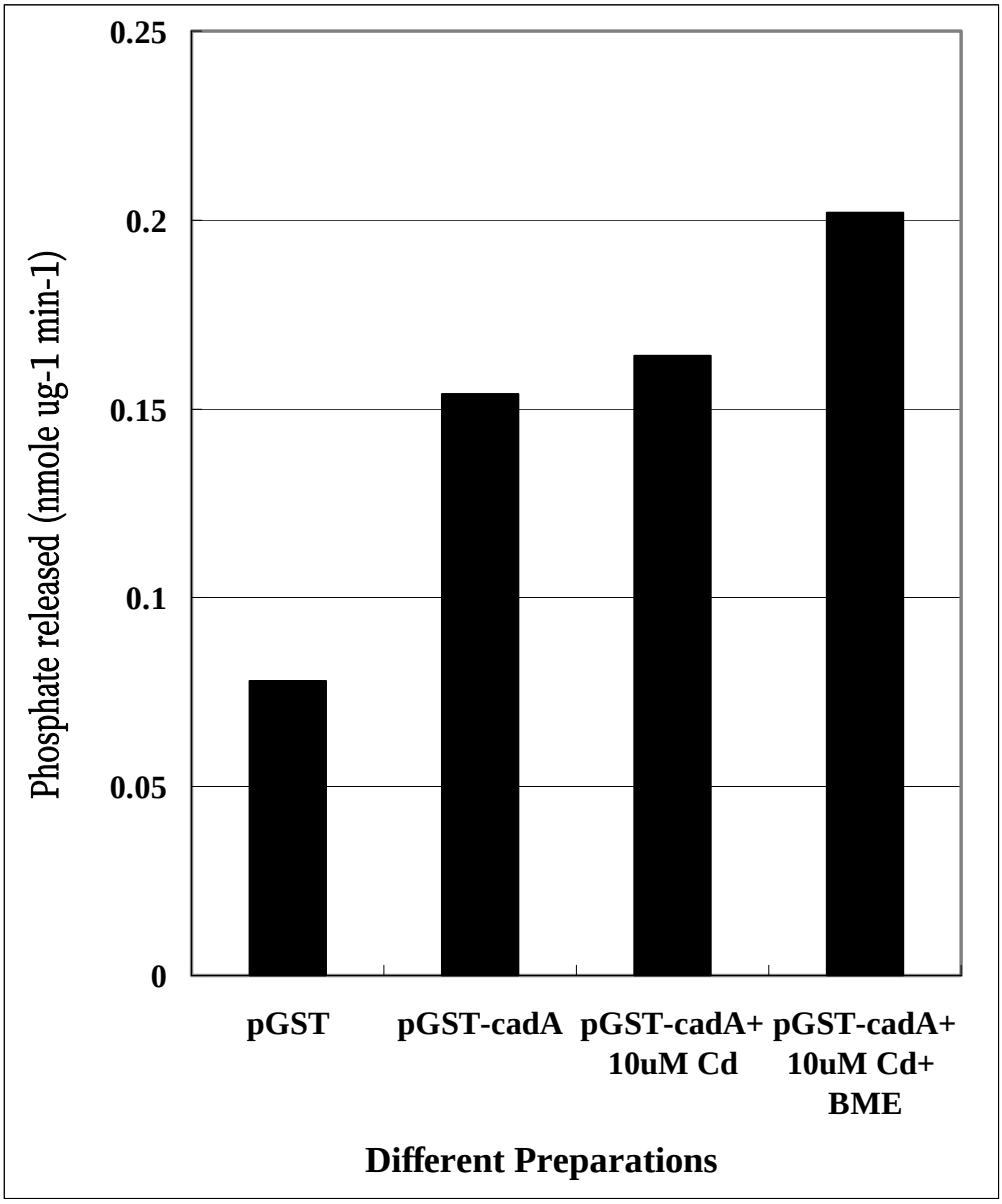


Fig 3.16. Comparison of ATPase kinetics using phosphate precipitation method with or without cadmium and BME. The membrane vesicles were prepared from BF2000 cells harboring either pGST, pGST-cadA are subjected to measure their ATP hydrolysis activities. The preparations with or without Cadmium ion and β-Mercaptoethanol (BME)were also included. The amounts of inorganic phosphates released from ATP were recorded at different time intervals. And the fusion protein production was normalized with their western blots.

Chapter 4. Discussion

The unique structure of CPx-ATPase has been speculated for a while (Solioz and Vulpe, 1996), however, little enzymatic evidence were achieved to disclose their crucial relationship with the protein function. Recently, works done by this laboratory has revealed the CadA topography (Fig. 1) (Tsai *et al.,* 2002), and the locations of those CPx-ATPase signature structures were identified. Among them, the N-terminal CxxC motif positions ahead of the first transmembrane segment, the CPC motif buries within the sixth transmembrane segment, and the large cytoplasmic ATPase domain were also found (Fig. 1; Tsai *et al.,* 2002).

To date, the role of the unique CxxC motif in CPx-ATPase is still controversial. Based on its conserved sequences similar to those of mercury binding proteins, Silver *et al.* (1989) have proposed the cysteine pairs of CadA are the metal-binding sites for cadmium resistant mechanism. Previous studies in this lab has demonstrated that CxxC motif is important for the CadA function, presumably through the cadmium binding (unpublished data). Either serine or glycine substitution of the cysteine residue within this CxxC motif resulted in the functional loss (unpublished data). However, recent studies have shown that the cysteine mutations within this conserved motif did not affect the metal resistance and transport (Fan *et al.,* 2001). In this thesis study, we found that the mutations in CxxC motif are not necessary to abolish the

complete cadmium resistance of CadA, especially for those cells harboring pKJ101 (C23S) and pKJ103 (C26S) plasmids (Fig. 3.1). Similarly, some of these cysteine mutants in CxxC motif displayed partial resistance to znic or lead (Fig. 3.2, 3.3 respectively). On the other hand, completely demolished of heavy metal resistances were found in those cysteine mutants of CPC motif (Fig. 3.4- 3.6). Suggesting that the crucial role for cysteine residues within the CPC motif is obvious.

Whether or not the defect in cadmium resistance found in those cysteine residues in CxxC and CPC motif affect the enzymatic function of the CadA ATPase activity will be very interesting to disclose. Especially the ATP hydrolytic activity of CadA, which generates energy for conformational change during the catalytic cycle, a signature phenomenon for all P-type ATPases, and produces free phosphate for the intermediate enzyme formation, are most worthy to study. In order to determine the influence of CxxC or CPC motif to ATPase activity in CadA, membrane vesicles prepared from *E. coli* strain RW3110 harboring either wild type or mutant *cadA* gene were investigated their ATP hydrolysis activities by enzyme coupling method. As shown in the Fig. 3.8, all cysteine mutants of either CxxC or CPC motif were measured their ATPase activities. Interestingly, those cells harboring mutant genes displayed partial resistance to cadmium, including the cysteine mutants in CxxC motif (pKJ101 and pKJ103), showed no better ATPase activities than those of the CPC mutants (pKJ107 and pKJ108) and pKJ104. Suggesting that the partial inhibition of CxxC mutants in metal resistance was not directly related to their ATPase activities. Therefore, the possible

function of CxxC motif in CadA might be involved in other mechanisms necessary for survival in toxic metals, rather than actively involved in energy-dependent cadmium translocation. Another explanation for this phenomenon, for example, the pKJ101 (C23S) and pKJ103 (C26S) displayed partial resistance to heavy metal, was due to their conserved amino acid substitutions (cysteine to serine) might reserve some CxxC activity. Since the residue serine is similar to cysteine in structure, thus the mutation might not completely abolish the function of CxxC motif. However, the assumption could not be applied to pKJ105 (C371S) and pKJ107 (C373S) because of their resistance to heavy metal were exactly the same as those nonconserved mutations, pKJ106 (C371G) and pKJ108 (C373G). Therefore, it remains an unanswered question for the role of CxxC motif in the mechanism of cadmium translocation.

A major concern about the use of *E. coli* cell membrane in our ATPase experiments was the presence of high activity background from ATPases other than CadA ATPase itself. In fact, it was difficult to reproduce satisfactory results because of the strong interference possibly from F_0F_1-ATPase, which possess remarkable activity than CadA (Hugentobler *et al.,* 1983). In order to reduce the background activity acquired from F_0F_1-ATPase, we have tried to remove the F_1-portion of the ATPase, the ATPase subunit of the respiratory ATPase on *E. coli* membrane, using a hypotonic buffer (1 mM EDTA), but without success. On the other hand, so far, we do not have the protocol for CadA purification, and a purified CadA for ATPase activity measurement would not be possible at this moment. Alternatively, we took advantage of using

mild detergent to solubilize CadA from cell membrane to get rid of those ATPases other than CadA. As shown in the Fig. 3.9, using Triton X-100, a respective amount of solubilized CadA was obtained. The Triton X-100 solubilized CadA proteins, along with the remained membrane fraction from RW3110 were used for our ATPase activity determination (Fig. 3.10). Although the solubilized-CadA seems more active than other preparations, however, the relative activity was only half of the crude membrane extract prepared from cells harboring the *cadA* gene as shown in Fig. 3.10. The difference is possibly due to the detergent inactivation of the ATPase assay itself. Whether or not the Triton X-100 solubilized CadA remains function needed to be further determined. However, it is also possible that only a portion of CadA was solubilized as it was found some unsolubilized CadA was present in the pellet fraction (membrane fraction) after ultracentrifugation (Fig. 3.9, lane 4). Together, the ATPase activity observed in Fig. 3.10 might be from either inactivated solubilized or a small portion of the functional CadA protein, and therefore, the ATPase activity is lower than the whole cell preparation. A further problem for this strategy is that Triton X-100 might interfere the delicate components in coupling assay generates a considerable problem in our ATPase assay (Garavito and Ferguson-Miller, 2001).

To prevent the possible interference from detergent addition, the *in vitro* translation strategy for CadA protein synthesis was then adapted to use for our ATPase activity determination. The activity of CadA was about 2 folds higher than that of vector control (Fig. 3.11), however, a visible CadA protein band in SDS-PAGE gel was not found (data not

shown). Furthermore, the ATPase activity of the *in vitro* synthesized CadA was slightly increased in the presence of trace amount of cadmium in the assay (Fig. 3.11). However, a clear-cut cadmium-dependent ATPase activity was not displayed, as we previously expected, when CadA ATPase activity was measured in the presence of different concentrations of cadmium (data not shown). There are several possibilities for not being able to demonstrate the cadmium-dependence in CadA ATPase; firstly, the ATPase domain of CadA protein may consistently active, as a separated domain from rest of the CadA protein; and secondly, there might be some crucial regulatory factors for CadA ATPase that are missing from the *in vitro* system. Similar observation was also found in ABC transporters (ATP-binding cassette protein), in which the transport activity was evidently reduced in the absence of SBP (solute-binding protein)(Driessen *et al.*, 2000). Thirdly, the *in vitro* translation system may not provide an appropriate environment to generate a well-folded CadA protein, and the ATPase activity measured (Fig. 3.11) was only from a small portion of folded CadA. And therefore, only some activities were measured in our experiments. Alternatively, our data might also suggest that cadmium might be in different chemical forms as the substrate for CadA activity.

In order to confirm the activity measured using the *in vitro* translation system, several transport inhibitors were also applied in these experiments. Among them, vanadate, which is the typical inhibitor for all P-type ATPases, oligomycin and nigericin, both are the potent inhibitors for membrane transporters, were added to the ATPase assays. However,

none of these transport inhibitors showed significant inhibitions to *in vitro* prepared CadA, even in the presence of vanadate, the CadA ATPase activity measured was not affected (Fig. 3.11). Based on these results described above, we concluded that an incorrectly folded CadA was produced using the *in vitro* translation system, and thus a proper ATPase activity was not achieved.

In attempt to overcome the problems of no purified CadA protein for ATPase assay, we have recently acquired a triple mutation *E. coli* strain BF2000 from Dr. Rosen's lab. The *E. coli* BF2000 (*kdp*$^-$, *unc*$^-$, *zntA*$^-$) strain was created by insertional disruptions of two major ATPase genes in *E. coli*, the Kdp-ATPase gene for potassium transport and the *unc* gene for F-type ATPase. Other than the mutations in those two ATPase genes, the *zntA* gene, the gene for cadmium, zinc and lead resistance in *E. coli*, of BF2000 was also inactivated. Thus, a very low ATPase activity was expected in this triple mutated *E. coli*. Alone with this newly created *E. coli* strain, an expression system for CadA using GST fusion was also obtained from Dr. Rosen's lab. The GST-CadA fusion plasmid, the pGST-*cadA*, displayed cadmium resistance when transformed into BF2000 (Fig. 3.12.B). However, we also noticed, probably due to its triple genetic defects, the BF2000 strain seems to be more sensitive to cadmium than our previous strain, RW3110 (Fig. 3.12.A). The Kdp-ATPase deficient cells are more vulnerable to osmolarity changed like its parent strain TKR2000, and therefore, we have modify the growth conditions and supplements of potassium were added to the cultures as described previously (Kollmann and Altendorf, 1993). Therefore, when a

modified medium of pH7.0 was used, a better cadmium resistance was observed (Fig. 3.13).

As mentioned previously, the CPC motif of CPx-ATPase would be more essential for CadA activity through its proposed function of cation-translocation within the CadA enzyme cycle. The hypothesis of the CPC motif as the cation-translocating domain is important for CadA activity through its participating the formation of ion channel for cadmium. Therefore, we decided to determine if its crucial role in CadA function related to the ATPase activity or not. Taking the advantage of using the BF2000 triple mutation strain and the GST fusion, four cysteine mutants in CPC motif were prepared and their resistances to cadmium were determined (Fig. 3.13). As shown in the Fig. 3.13, all CPC mutants were shown sensitivities to cadmium, as we previously observed (Fig. 3.4). On the other hand, membrane preparations from all these mutants were also measured their ATPase activities (Fig. 3.15). In order to differentiate whether or not the ATPase activities measured were from the CadA, a deletion mutation at the proposed ATPase domain was also prepared recently and included into these experiments. Also, an alternative ATPase assays by measuring the inorganic phosphate released from ATP was employed (Cogan *et al.*, 1999) in order to achieve a more sensitive result. In these experiments, the ATP hydrolytic activity of GST-CadA was almost 2 folds than that of control (Fig. 3.15), however, the activity of GST-CadA was less than ZntA (Sharma *et al.*, 2000). The major reason for the reduced activity than its close relative might be the GST protein tag, which may interfere the correct protein folding in cell

membrane. Unless the GST-tag was removed, the exact activity of CadA would then be able to precisely determine. As the five mutants, including ATP-binding domain deletion and CPC mutants, showed no ATPase activity at all (Fig. 3.15). However, the cadmium-dependent ATPase activity was not exhibited in these assays (data not shown). Interestingly, when β-mercaptoethanol (BME) was added with cadmium, the ATP hydrolytic activity of GST-CadA was increased slightly (Fig. 3.16). In fact, the reductant BME has been proposed to form complex with metal as thiolated-metal conjugate in ZntA (Gatti *et al.,* 2000). If the thiolated complex is the only substrate for CPx-ATPases, then it may open a window for disclosing the *in vivo* mechanism of metal transloaction of CPx-ATPases. Moreover, not only BME, but also cysteine and glutathione might form the thiolated complex (Gatti *et al.,* 2000), however their roles in CPx-ATPase activity remain to be determined.

In this thesis study, we have demonstrated that the resistant function lost when cysteine mutation in conserved CxxC and CPC motifs of CadA-ATPase. Also, solubilization of CadA by Triton X-100 was achieved and, however, the activity of solubilized CadA was shown less comparable to membrane vesicles. Meanwhile, the alternatives of *in vitro* synthesis or GST-fusion CadA were investigated to determine their ATP hydrolytic activity. However, the major problem as inconsistency has to be overcome in order to fully understand the enzymatic mechanism of CadA.

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