私立中山醫學院醫學研究所碩士論文

Master Thesis, Institute of Medicine, Chung Shan Medical and Dental College

對金黃色葡萄球菌上抗鎘腺核甘三磷酸酶穿膜結構之研究

Membrane Topology of CadA Cadmium Resistant ATPase in *Staphylococcus aureus*

指導教授：蔡淦仁 (Tsai, Kan-Jen) 博士

研究生：　林詠峰 (Lin, Yung-Feng) 撰

中華民國九十年六月

博碩士論文電子檔案上網授權書

本授權書所授權之論文爲授權人在中山醫學院醫學研究所 89 學年度第二學期取得碩士學位之論文。

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授權人

簽　名: 林詠峰　　中 華 民 國 90 年 6 月 28 日

本論文為中山醫學院授與理學碩士學位之必備條件之一，經中山醫學院醫學研究碩士論文考試委員會審查合格及口試通過。

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中華民國九十年六月

學生林詠峰論文題目為對金黃色葡萄球菌上抗鎘腺核甘三磷酸酶穿膜結構之研究，其論文已經私立中山醫學院醫學研究所論文碩士委員審查合格及口試通過，並由其指導教授校閱後無誤。

指導教授：蔡淦仁 博士　　簽名：_____

中華民國九十年六月二十二日

誌　謝

本篇論文的完成要感謝很多人的幫忙。除了感謝母校中山提供的研究機會之外，首要感謝的是我論文指導教授蔡淦仁老師。在這兩年期間，他教導我如何從一個學生變成一位學者。幽默風趣的教學方法，理性尊重的待人風格，讓我著實受益良多。再者感謝林嬪嬪老師及劉昭君老師，在我邁向碩士學位的最後關頭助我一臂之力。另外也要感謝美國 Rosen 博士的實驗室提供了重要的實驗材料和許多的建議，以及日本 Nakae 博士的實驗室贈送了實驗用的質體。還有我們實驗室的淑娟、宏基和佳瑜，已畢業的學樑和秉儒，隔壁實驗室的澤民以及美國朋友范斌和 Marco 提供了實驗上的協助。也感謝醫研所和醫技系的老師們，整個大實驗室裏的所有夥伴們以及其他不勝列舉的朋友們，提供有形和無形的幫助。最後謹將這份小小的成就和喜悅獻給我的母親詹氏和夫人惠雯以及我親愛的家人們。

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摘　要

細菌對於有毒金屬之抗性起初是來自於自然環境裏的暴露，而最近則被發現有來自於藥品的使用。有一些細菌的抗性已知是由添加性的突變所產生的。之前的研究發現有一盤尼西林(penicillin)抗性質體 pI258 提供了金黃色葡萄球菌(*Staphylococcus aureus*)對鎘離子的抗性。生化學上對於該種抗性的探討則已發現這是由 pI258 上 *cadA* 基因所轉錄的 CadA 抗鎘腺核甘三磷酸酶(Cd^{2+}-ATPase)所提供的抗性。序列比對的結果，將此 CadA 蛋白歸類於 P 型腺核甘三磷酸酶(P-type ATPase)。另因 CadA 這類蛋白在結構上有另外的特徵，該蛋白和其它一些能轉運重金屬離子的腺核甘三磷酸酶則進一步被歸類在一新的 CPx 型腺核甘三磷酸酶(CPx-type ATPase)次族群裏。在這次的研究當中，我們已經探查出 CadA 蛋白的穿膜結構以及 CPx 型腺核甘三磷酸酶這一類蛋白所特有的一些功能性區域所在的位置。利用鹼性磷酸酶(*phoA*)和乙型半乳糖酶(*lacZ*)基因融合的方法，我們製造出各二十二種的 *cadA-phoA* 和 *cadA-lacZ* 的基因融合，涵蓋了該轉運蛋白的全長。然後根據這些融合酵素活性的高低，我們分析後得到的結果是 CadA 蛋白擁有八個穿膜片段(TMs)，其中第三和第四片段可能不完全穿膜；氮端(N)和碳端(C)都留在細胞質中。這樣的結果跟另外一個發現於幽門桿菌(*Helicobacter pylori*)的 CadA 同源蛋白的穿膜結構研究的結果很相近，除了對第三和第四穿膜片段的描述之外。另外則有一些結構上的差異被發現存在於這兩個同源蛋白之間，包括葡萄球菌上的 CadA 含有一高厭水性的區域緊接在假設的腺核甘三磷酸結合位置(ATP binding site)後面，以及在這兩個蛋白之間其帶電胺基酸的分佈也是不相稱的。總而言之, 以葡萄球菌上的 CadA 當一個例子, 我們提供了 CPx 型腺核甘三磷酸酶這類蛋白更完整的結構模型。

Abstract

Bacterial metal resistances evolved originally from the exposure of natural sources, and become more severe in recent years after the usages of therapeutic drugs. Some of these bacterial resistant mechanisms are raised from additive mutations of bacterial genes. Previously, the staphylococcal resistance to cadmium carried by pI258 penicillinase plasmid has been extensively studied. Biochemical characterization of the resistance has revealed that a CadA Cd^{2+}-ATPase mediates the resistance phenomena and this protein has been categorized into a P-type ATPase enzyme family. Due to their difference in certain structural features, CadA and other P-type ATPases for transporting heavy metal ions have been grouped into a new enzyme family, called CPx-type ATPases. In this proposed study, we have investigated the CadA topography and, as a model, to characterize those functional domains unique for the CPx-ATPases. Using the *phoA* (alkaline phosphatase) and *lacZ* (β-galactosidase) gene fusion methods, a series of 22 *cadA-phoA* and 22 *cadA-lacZ* fusions were created throughout the *cadA* gene. Based on the reporter enzyme activities measured in this study, we have determined that there are eight transmembrane segments (TMs) in CadA; however, the third and forth segments might not traverse the membrane completely. Both the N-terminus and the C-terminus of CadA protein are located within the cytoplasm. This topological model is similar to that of the homologous ATPase found in *Helicobacter pylori* except the description of the TM3 and TM4. Besides, some significant differences have been found between these two pumps,

including a highly hydrophobic region just behind the putative ATP binding site found in staphylococcal CadA but not in the helicobacter version of CadA, and an asymmetric arrangement of charged residues between these two ATPases. Anyway, we proposed a more detailed structure model of this kind of ATPases using staphylococcal CadA as an example.

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Yung-Feng Lin

June, 2001

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Chapter 1. Introduction

1.1 Bacterial resistance to toxic metals

Bacterial resistance to toxic substances is ubiquitously found in the contaminated environment (Foye 1977, Knowles et al. 1983). For their survivals in the presence of toxic metal ions, various mechanisms are developed to confer resistance to these environmental toxins, including those resistances to arsenate, arsenite, antimony, lead, bismuth, zinc, mercury, as well as cadmium (Novick and Roth 1968, Foster 1983). Some of these resistance mechanisms are adaptive from additive mutations of some existed bacterial genes, and some are from alternative substrate usages of the bacterial genes (Aguiar *et al.*, 1990; Inbar and Ron, 1993; Hustavova *et al.*, 1995; LaRossa *et al.*, 1995; Crupper *et al.*, 1999). And the resistance genes spread among closely related species and even to other species through transformation, transduction and transposition in the bacterial world. Particularly, plasmid-mediated resistances to metal ions are frequently found in prokaryotes (Rensing *et al.*, 1999).

1.1.1 Cadmium resistance

Cadmium is a hazard element universally found on earth, and this metal ion is one of the most likely contaminants found during the industry evolution. Bacteria and some other microorganisms have developed certain mechanisms to cop with the cadmium toxicity (Aguiar *et al.*, 1990; Inbar and Ron, 1993; Hustavova *et al.*, 1995; LaRossa *et al.*, 1995; Crupper *et al.*, 1999). In fact, many bacterial plasmids encoding

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June, 2001

resistance systems to cadmium have been reported to date. For example, Nies *et al.* (1987) isolated a 9.1-kb *EcoR*I fragment from a 238-kb plasmid, pMOL30, found in *Alcligenes eutrophus* CH34, which confers resistance to cadmium, zinc and cobalt salts in sensitive strains. Later, Nies *et al.* has also reported that the genes contribute to these three metals resistance arranged as a *czc* operon, which encodes five polypeptides called CzcA, B, C, D, and R. The CzcR and Czc D are regulatory elements for the expression of CzcA, B, and C, the structure genes of the resistance system, in which the CzcA, CzcB and CzcC are essential for a full resistance to all three metals cations (Nies and Silver, 1989; Nies *et al.*, 1989; Nies, 1992). Similarly, some cadmium resistance systems were found in *Staphylococci spp.* In *S. aureus*, two resistant determinants to cadmium were identified in staphylococcal plasmid pI258, called *cadA* and *cadB* (Novick and Roth 1968; Smith and Novick, 1972; Weiss *et al.*, 1978; Novick *et al.*, 1979). Recently, Chaouni *et al.* (1996) reported a third cadmium resistant system exists in pLUG10 plasmid from *S. lugdunensis*. In this latter resistant system, two genes were identified, which includes a *cadB*-like cadmium resistance gene and a regulatory gene, called *cadX*, which functions in a high-level of cadmium resistance.

Studies have shown that the *cadA* and *cadB* resistant determinants are different in their resistance mechanisms even though they are both carried by plasmid pI258 in *S. aureus* (Novick and Roth 1968; Smith and Novick, 1972; Weiss *et al.*, 1978; Novick *et al.*, 1979). It was suggested that the *cadA* resistant determinant is mediated by a 727-amino-acid protein, which shows sequence similarity to the P-type ATPase (Nucifora *et al.*, 1989). On the other hand, the cadmium resistance mediated by the *cadB* resistant determinant was poorly defined. However, it has been

reported that cadmium resistance of the *cadB* resistant determinant does not due to the decrease of cadmium accumulation within the resistant cells (Smith and Novick, 1972; Perry and Silver, 1982). It was proposed that CadB does not promote cation efflux as that of CadA, the latter system was characterized as an efflux pump for the cadmium resistance, and the CadB may provide a protection through the binding of cadmium on the membrane (Perry and Silver, 1982). Another cadmium resistance gene found in *S. aureus*, *cadD*, has also been recently identified from the plasmid pRW001 (Crupper *et al.*, 1999). A high degree of sequence similarity was found between *cadD* and the *cadB*-like gene from *S. lugdunensis*; however, no evidence related to either *cadA* or *cadB* was found. The expression of *cadD* in *S. aureus* as well as *Bacillus subtilis* has shown a low-level resistance to cadmium when compared it with that of *cadA* and *cadB* (Crupper *et al.*, 1999). Suggesting that the resistance to cadmium mediated by *cadD* gene plays a minor role and only functions when other cadmium resistant determinants were absence.

1.1.2 CadA is a cadmium efflux in *Staphylococcus aureus*

The *cadA* gene from staphylococcal penicillinase plasmid pI258 was identified along with many other resistant determinants to heavy metal ions in *S. aureus* (Novick and Roth, 1968). Early study from Chopra (1975) has shown that protein synthesis in cell-free extracts from resistance or sensitive bacteria was equally susceptible to inhibition by Cd^{2+}; however, the spheroplasts prepared from resistant bacteria retained their resistance. Based on these findings, a cadmium resistance due to the change in the orientation of the membrane protein or phospholipids was

proposed. The Cd^{2+} enters into cells via an energy-dependent Mn^{2+} transport system as an alternative substrate for the transport system in *S. aureus* and maybe same to other Gram-positive bacteria (Tynecka *et al.*, 1981). Similarly, Weiss *et al.* (1978) demonstrated that Cd^{2+} inhibits the uptake of Mn^{2+} and accelerates the loss of intracellular of Mn^{2+} in the susceptible cells, but no effect was found on Mn^{2+} transport in resistant *S. aureus*. Weiss *et al.* (1978) has also suggested that the cadA resistance mechanism is dependent upon a blockage of energy-dependent Cd^{2+} transport. On the other hand, Tynecka *et al.* (1981) found that the resistance was mediated by an active efflux pump to reduce the intracellular accumulation of the toxic metal ions and the net efflux of Cd^{2+} was blocked in the presence of 2,4-dinitrophenol, *N, N,* -dicylohexylcarbodiimide (DCCD) or incubation at 4˚C. Indicating that the resistance is in an energy-dependent fashion (Tynecka *et al.*, 1981). Furthermore, they also found that valinomycin had no effect on the cadmium resistance and a $Cd^{2+}/2H^{+}$ antiporter for cadmium resistance was then proposed (Tynecka *et al.*, 1981).

1.1.3 The *cadA* operon in *S. aureus* pI258

The most striking finding in *cadA* cadmium resistant determinant was come from the identification of the genes involved in the resistance (Nucifora *et al.* 1989). Two open reading frames (ORFs) were found within the cadmium resistant determinant, called *cadC* and *cadA* genes, and later the cadmium determinant was named *cad* operon. In their report, they found that a 3.5-kb *Bgl*II-*Xba*I DNA fragment from plasmid pI258 contains the *cadA* operon to confer a full cadmium resistance, however,

the large ORF, the *cadA* gene, seemed more essential for the resistance phenomena (Nucifora *et al.* 1989). When *cadA* and *cadC* were both expressed in *S. aureus*, the full complementation of cadmium resistance was observed when compared it with that of *cadA* or *cadC* gene alone (Yoon and Silver, 1991).

When aligned the deduced amino acid sequence of *cadA* gene with the sequences from other known proteins, it was found that the predicted CadA protein shows a high homology to a class of enzymes called E_1E_2-ATPase or P-type ATPase (Nucifora *et al.*, 1989; Silver *et al.*, 1989). On the other hand, the smaller ORF with a 3' end of the gene overlaps 8 base pairs to *cadA* encodes for a short polypeptide of 122 amino acids in length was named *cadC* (Nucifora *et al.*, 1989). Furthermore, it was found that purified CadC protein could bind to the proposed *cadA* operator/promoter DNA sequences, and Cd^{2+}, Bi^{3+} and Pb^{2+} caused the releases of CadCs from DNA in gel retardation assays and DNaseI footprinting experiments (Endo and Silver, 1995). These observation strongly suggested that *cadC* is a regulatory element in *cad* operon, as it was previously found that the predicted *cadC* gene product shown homology only to the regulatory element of Ars operon, the ArsR gene (Wu and Rosen, 1991). 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). Therefore, the regulatory role of *cadC* to modulate the *cadA* expression in the *cad* operon in *S. aureus* should be without any suspicion.

1.2 CadA is a metal transporting P-type ATPase

1.2.1 CadA is a member of P-type ATPases

As mentioned in the above section, the amino acid sequences of predicted *cadA* gene product is similar to other P-type ATPase transporters (Nucifora *et al.*, 1989; Silver *et al.*, 1989; Silver and Walderhaug, 1992). The *cadA* gene was cloned and expressed it in *Bacillus substilis* (Nucifora *et al.*, 1989). *In vitro* transport study has confirmed that CadA mediates an ATP-dependent cadmium efflux to confer resistance to cadmium (Tsai *et al.*, 1992). Using everted membrane vesicles prepared from *B. subtilis* harboring *cadA* gene and in the presence of different energy sources in the cadmium transport assay. They showed that the cadmium transport was driven only in the presence of ATP, but not NADH or phenazine methosulfate plus sodium ascorbate, which together generate a proton motive force. They also showed that bafilomycin A_1 inhibited to cadmium transport at a concentration of micro molar range, which is comparable to other P-type ATPases. Furthermore, a cadmium-dependent CadA protein phosphorylated intermediate was demonstrated within the CadA enzyme cycle (Tsai and Linet, 1993). Together all these evidence, the role of CadA as a cation-translocating ATPase similar to those of P-type ATPases was established.

The P-type ATPases are polytopic membrane proteins and their major functions are transporting cations against their concentration gradients with the expenses of chemical energy, ATP. Characteristically, these ATPases form a covalently phosphorylated intermediate in their reaction cycles (Pedersen and Carafoli, 1987a and 1987b). Most P-type ATPases are single, large, and catalytic monomers ranging from 70 to 200 kDa in

size, with an exception of Na^+/K^+-ATPase found in eukaryotes, in which heterodimer mediated the transport activity, and this type of ATPases are inhibited by micro molar concentrations of vanadate. Additionally, all P-type ATPases share a unique characteristic that is the formation of phosphorylated aspartate, which is formed transiently when the energy from ATP hydrolyzation is used to translocate the substrate, as the hallmark of this enzyme family. More recently, it was found that the invariant aspartic acid (D) residue in the conserved sequence DKTGT is first phosphorylated while ATP binds to the ATP-binding domain (Solioz and Vulpe, 1996, Fig.1). The conserved sequences found in these ATPases include the kinase domain (DKTGT) as described, an ATP binding domain (GDGXNDXP) and a phosphatase domain (TGES/A) (Solioz and Vulpe, 1996; Fig. 1).

The best-known P-type ATPases include eukaryotic plasma membrane Na^+/ K^+-ATPase, sarcoplasmic reticulum Ca^{2+}-ATPase (Shull *et al.*, 1985; MacLennan *et al.*, 1985), yeast plasma membrane Na^+/K^+-, K^+- and Ca^{2+}-like ATPases (Serrano *et al.*, 1986), and bacterial K^+-ATPase from *E. coli.* More recently, some newly found eukaryotic and prokaryotic ATPases were also categorized into this enzyme family, which include the iron-induced mammalian iron ATPase (Baranano *et al.*, 2000), the facultatively anaerobic alkaliphile Na^+-ATPase from *Exiguobacterium aurantiacum* (Ueno *et al.*, 2000), and a soluble P-type ATPase from *Methanococcus jannaschii* (Ogawa *et al.*, 2000). In addition to these essential metal-translocating pumps, P-type ATPases were also found in the transporting of heavy metal ions, such as CadA transports Cd^{2+} in *S. aureus*, CopA and CopB transport Cu^+ in *E. coli*, and ZntA

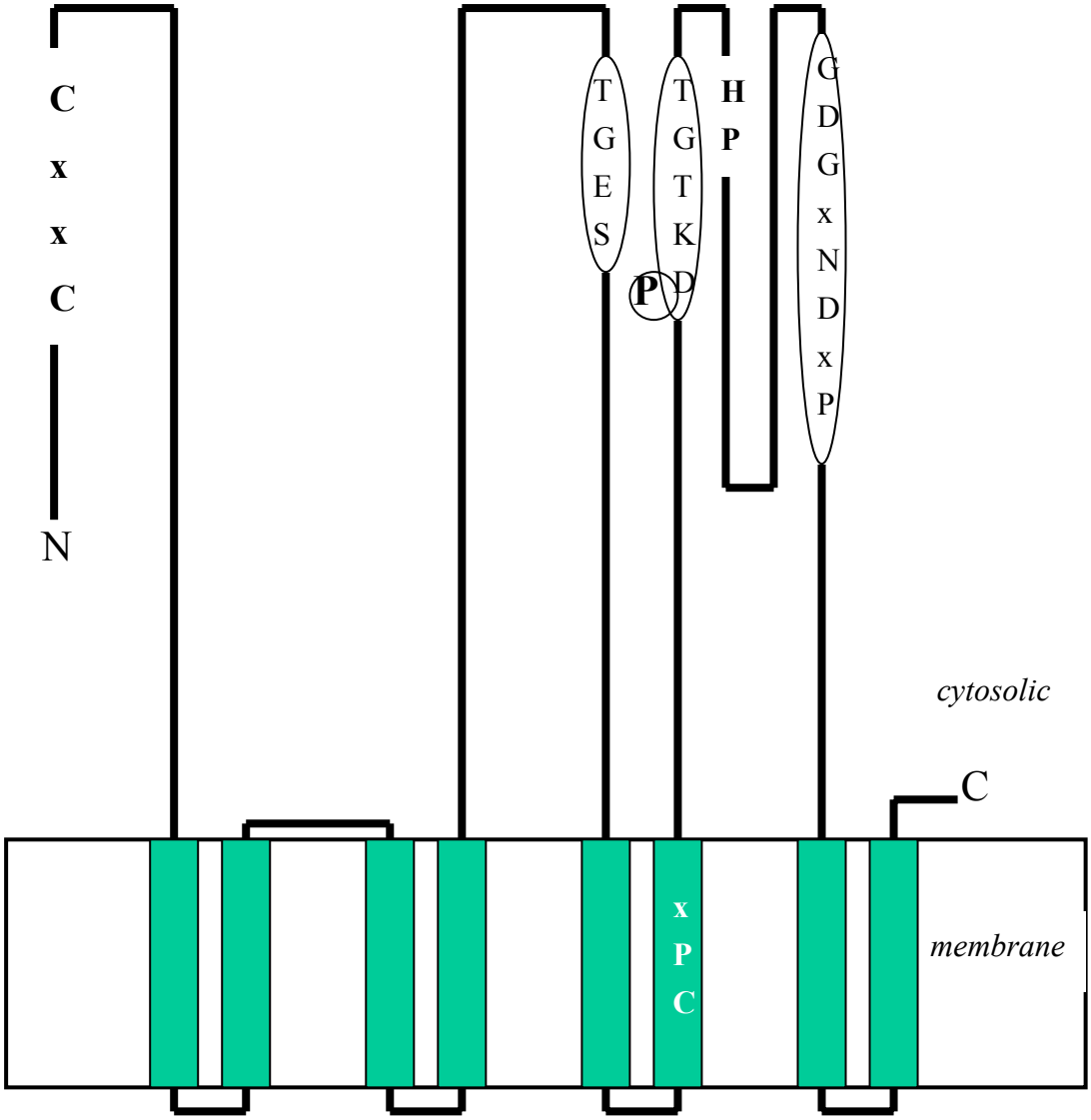


Fig. 1 The schematic model of CPx-type ATPases. The features belonged to P-type ATPases are shown in ellipses. The phosphate group interacting with aspartate residue of CadA is shown in circle. Other conserved motifs of CPx-type ATPases are also indicated but only in letters. The putative transmembrane segments are drawn in rectangle.

transports Zn^{2+}, Cd^{2+} and probably Pb^{2+} in *E. coli* (Silver *et al.*, 1989; Odermatt *et al.*, 1992; Rensing *et al.*1997).

1.2.2 The heavy metal transporting CPx-type ATPase

Heavy-metal translocating ATPases including CadA, CopA and CopB contain elements common to all P-type ATPases as well as several unique features only found in the membrane transporters for heavy metals, which are: (1) putative heavy metal-binding sites (CXXC motif) in the polar amino-terminal region; (2) a conserved intramembranous CPC or CPH motif (so called CPx motif); (3) a conserved histidine-proline dipeptide (HP locus) 34 to 43 amino acids carboxyl-terminal to the CPx motif; and (4) a unique number of the membrane-spanning domains (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996; Fig. 1). Suggesting that these heavy metal ATPases might form a distinct subgroup of the P-type ATPases. And this subgroup of enzyme family was reclassified as CPx-type ATPases or P_1-type ATPases for their sharing the distinctive features beside those characteristics of P-type ATPases (Lutsenko and Kaplan, 1995; Solioz and Vulpe, 1996).

In addition to CopA, B and CadA, some other heavy-metal translocating ATPases have also been characterized and grouped into CPx-type ATPase enzyme family. For example, the copper transporting MNK protein, in which the mutation of the protein resulted in a human Menkes disease, similar diseases were also found in hamster and mouse (Mercer *et al.*, 1993; Vulpe *et al.*, 1993; Chelly *et al.*, 1993). Another human disease gene, the Wilson's disease gene, WND, was also a member of this enzyme family (Tanzi *et al.*, 1993; Yamaguchi *et al.*, 1993;

Bull *et al.*, 1993). Furthermore, a high copper tolerant P-type ATPase, CaCRP1, in *Candida albicans* (Weissman *et al.*, 2000) was also identified recently and was placed into the CPx-type ATPase family. However, only few of these ATPases has been demonstrated to transport metals, except for CadA transports cadmium, zinc, and lead ions (Tsai *et al.*, 1992; Rensing *et al.*, 1998) and CopB transports copper and silver ions in *Enterococcus hirae* (Solioz and Odermatt, 1995), ZntA, a homologue of CadA in *E. coli* chromosome, transports cadmium, zinc and lead ions (Beard *et al.*, 1997; Rensing *et al.*, 1997 and 1998; Binet and Poole, 2000), and CopA, involved in the copper homeostasis in *E. coli*, transports copper ion (Rensing *et al.*, 2000). There were also some CPx-type ATPases found in other bacteria, but without transport evidence. Using a mixture of oligonucleotides coding for the DKTGT (I/L) T consensus sequence, specific for the phosphorylation site of this family of ATPases, to screen the genomic *Helicobacter pylori* library led to discover a P-type ATPase gene (Melchers *et al.*, 1996). This protein of 686 amino acids carries the consensus sites for phosphorylation and ATP binding and exhibits a 25-30% identity to bacterial Cd^{2+}- and Cu^{+}-ATPases, together with the postulated N-terminal CXXC ion binding motif and the intramembranous CPC sequences found in CPx-type ATPases. Others were the *cop* operons of *Helicobacter pylori* and *Helicobacter felis*, which genes were also cloned by gene library screening (Bayle *et al.*, 1998) and both operons contain open reading frame for a CPx-type ATPase (CopA) with homology to Cd^{2+}- and Cu^{+}-ATPases including the conserved CXXC and CPC motifs, in which the N-terminatl metal binding property has been reported (Bayle *et al.*, 1998). And more to come to this enzyme family should then be expected

in the future.

1.3 The topological study of CPx-type ATPases

Although CPx-type ATPases like CadA has been studied both genetically and biochemically (Nucifora *et al.*, 1989; Tsai *et al.*, 1992; Rensing *et al.*, 1998), more detail investigations in cadmium binding, phosphatase activity and aspartyl kinase activity have yet been defined. For a better understanding of the detail mechanism underlying the enzyme activity of CPx-type ATPase, the knowledge of the structural aspects of these membrane proteins will be essential. Considering of the fact that there will be years before high-resolution crystal structures are determined, the relatively crude two-dimensional models, are still the best strategy to explore the structure-function relationship of a membrane protein like CPx-ATPase. In addition, the two-dimensional "topography", so-called topology map, places major constraints on the possible folding patterns of a membrane protein (Jennings, 1989). The basic idea of integral membrane protein structure in this two-dimensional topography is to predict the transmembrane segments of the polypeptide chain, i.e., the disposition of the chain relative to the lipid bilayer in which it is embedded (Lee and Manoil, 1996).

1.3.1 Inferences from sequence- hydropathy analysis

To set up a topological working model, computer programs were used to analyze the possible transmembrane locations of membrane ATPases. As described by Kyte and Doolittle (1982), a hydropathy profile

was drawn using the hydrophobicity analysis of each individual amino acid within the protein measured by the Goldman-Engelman-Steitz method (GES-scale) (Engelman *et al.* 1986) followed by a positive-inside rule (von Heijne 1992).

The GES-scale is calculated from the free energies that require for transferring an amino acid residue in an α helix from the membrane interior to water. It is well known that the hydrophobic interior of a lipid bilayer is an energetically very unfavorable environment for peptide bonds, unless the polar groups are hydrogen bonded (Engelman *et al.* 1986). For this reason, and because of the tendency of hydrophobic amino acid side chains to partition into lipid bilayer, a membrane-spanning α helix is an energetically favorable structure for a stretch of hydrophobic amino acid residues (Jennings, 1989).

The hydrophobicity analysis method that originally used is the SOAP program (Kyte and Doolittle, 1982; Klein *et al.*, 1985). It is considered that the hydrocarbon core of membranes is typically 30 Å width, which can be traversed by a α-helix consisting of about 20 residues, and forms a membrane-spanning domain (Kyte and Doolittle 1982). The hydrophobic analysis was usually carried out using the GES-scale (Engelman *et al.* 1986) and a window composed of a central 7 or 11 residue was selected. The free energies of the 7 or 11 residues in a window were calculated as a sum or average of all the amino acid residues included. When the position of the window changes from the first residue to the end of the sequence, a plot of the free-energy change versus the residue positions could identify the peaks of high hydrophobic domains. When the peaks of greater than a given criterion level in hydropathy plots as well as a stretch of amino acid number higher than 20

residues are indicative of potential transmembrane helices.

The positive-inside rule is based on the observation that positive charged amino acids are manifold more abundant in cytoplasmic location, as compared to periplasmic location. This is consistent with positively charged loops serving as cytoplasmic anchors for membrane-spanning regions (von Heijne 1989, 1992).

There were also some other hydropathy systems used to predict transmembrane domains of membrane proteins, such as the TopPred (Von Heijne, 1992; Sipos and Von Heijne, 1993) and TMpred (Hofmann and Stoffel, 1993). All these methods are following the similar principles as described above but more complex. There are examples of hydropathy plot shown in Fig. 2.

1.3.2 Experimental approaches to membrane topology

A variety of biochemical techniques have been employed to elucidate topological structure, including chemical labeling, *in situ* proteolysis, immunological methods, and molecular genetic approaches (Jennings, 1989). Recently, a method of using the *in vitro* transcription/translation technique was also developed to detect the topological structure of certain membrane proteins (Holland and Drickamer, 1986).

The chemical labeling is a well-established and simple method in its principle. It requires a labeling agent that only accesses to one side of the membrane. Labeling agents can be covalently reacting radioactive, fluorescent, or spin-labeled small molecules, or they can be antibodies or proteolytic enzymes (Jennings, 1989). The most frequently used chemical

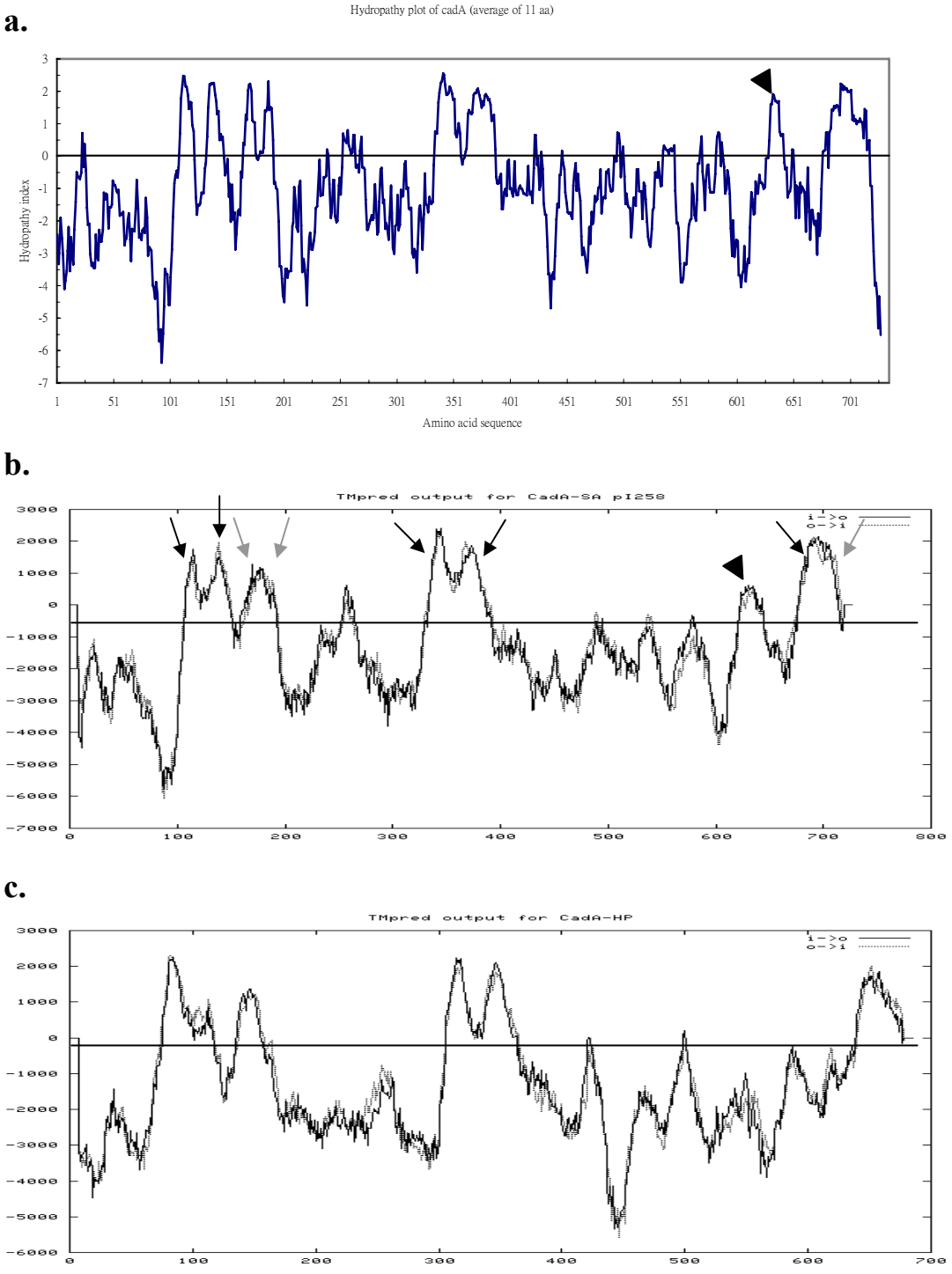


Fig. 2 Hydropathy plots. (a.) Staphylococcal CadA by Kyte and Dollittle method (SOAP). **(b.)** Staphylococcal CadA by TMpred program. **(c.)** H. pylori CadA by TMpred program. The arrows that indicate the peaks of the hydropathy plot are high hydrophobic regions. They usually serve as transmembrane segments in membrane proteins. The black triangles shown in (a.) and (b.) indicate the extra hydrophobic region in staphylococcal CadA but not in H. pylori CadA.

is the site-directed chemical labeling of cysteine residues using a sulfhydryl reagent (Loo and Clarke, 1995). From generating a collection of mutants, each with a single unique cysteine residue, these mutated proteins can be probed with sulfhydryl reagents in oriented membranes to determine the surface accessibility at different residues (Long *et al.*, 1998).

In situ proteolysis method is using the proteolytic enzymes, which have been used extensively to identify sites that are exposed to the surfaces of membrane proteins. The most common use of this method is to generate and isolate relatively large hydrophobic fragments (Steck *et al.*, 1976; Jennings *et al.*, 1986). End group determination using the proteolytic method makes it possible to localize the cleavage site in the primary structure. Proteolytic enzymes are usually used on the outer surface of sealed membranes, or for bilateral cleavage of an unsealed membrane. However, sealing proteolytic enzymes may also work inside RBC ghosts to establish intracellular cleavage sites (Lepke and passow, 1976; Jennings *et al.*, 1986).

Immunological tools have been used increasingly in the study of membrane protein topography recently. In order to be useful for such purposes, the antibody is prepared directly against a specific portion of the primary structure of the protein or an extra epitope inserted to the protein using molecular techniques. The antibodies can be used in topographical work by determining the accessibility of a particular antibody to its target portion of a membrane protein at which side of a sealed membrane. The work of Eckhardt *et al.* (1999) on mammalian CMP-sialic acid transporter has exemplified this approach in membrane topology studies.

In 1986, Manoil and Beckwith reported a new method for assessing membrane protein topography based on the sidedness of fusion proteins formed from portions of the membrane protein of interest attached to reporter enzyme alkaline phosphatase, which is active only when exported to the periplasm (Hoffman and Wright, 1985). The fusion protein is constructed with the membrane polypeptide upstream from alkaline phosphatase, and the eventual location of the phosphatase will reflect the sidedness of the C-terminus of the membrane protein. Years later, Froshauer *et al.* (1988) developed a complementary method that employed fusions with β-galactosidase, which is active only in the cytoplasmic location. On the other hand, fusions to periplasmic C-termini of a membrane polypeptide give low β-galactosidase activity. The combined uses of periplasmic- and cytoplasmic- fusions provide positive activity signals for the domain determinations on both sides of the membrane (Manoil *et al.*, 1990; Manoil, 1991, Fig. 3). Hirata et al. (1998) did another example for the combined uses of reporter enzymes in staphylococcal tetracycline efflux protein, Tet (K), using periplasmic β-lactamase and cytoplasmic chloramphenicol acetyl transferase.

A novel method described for the membrane protein topography was derived from using *in vitro* transcription/translation of DNA constructs encoding fusion proteins containing potential transmembrane segments in the presence of microsomes (Holland and Drickamer, 1986). When the fused target peptide is transmembraneous, the C-terminus-fused signal protein will be transferred into the microsome and glycosylated after *in vitro* transcription/translation. On the other hand, when the fused peptide is not behind a transmembrane segment, the signal protein will be stayed out of the microsome, and

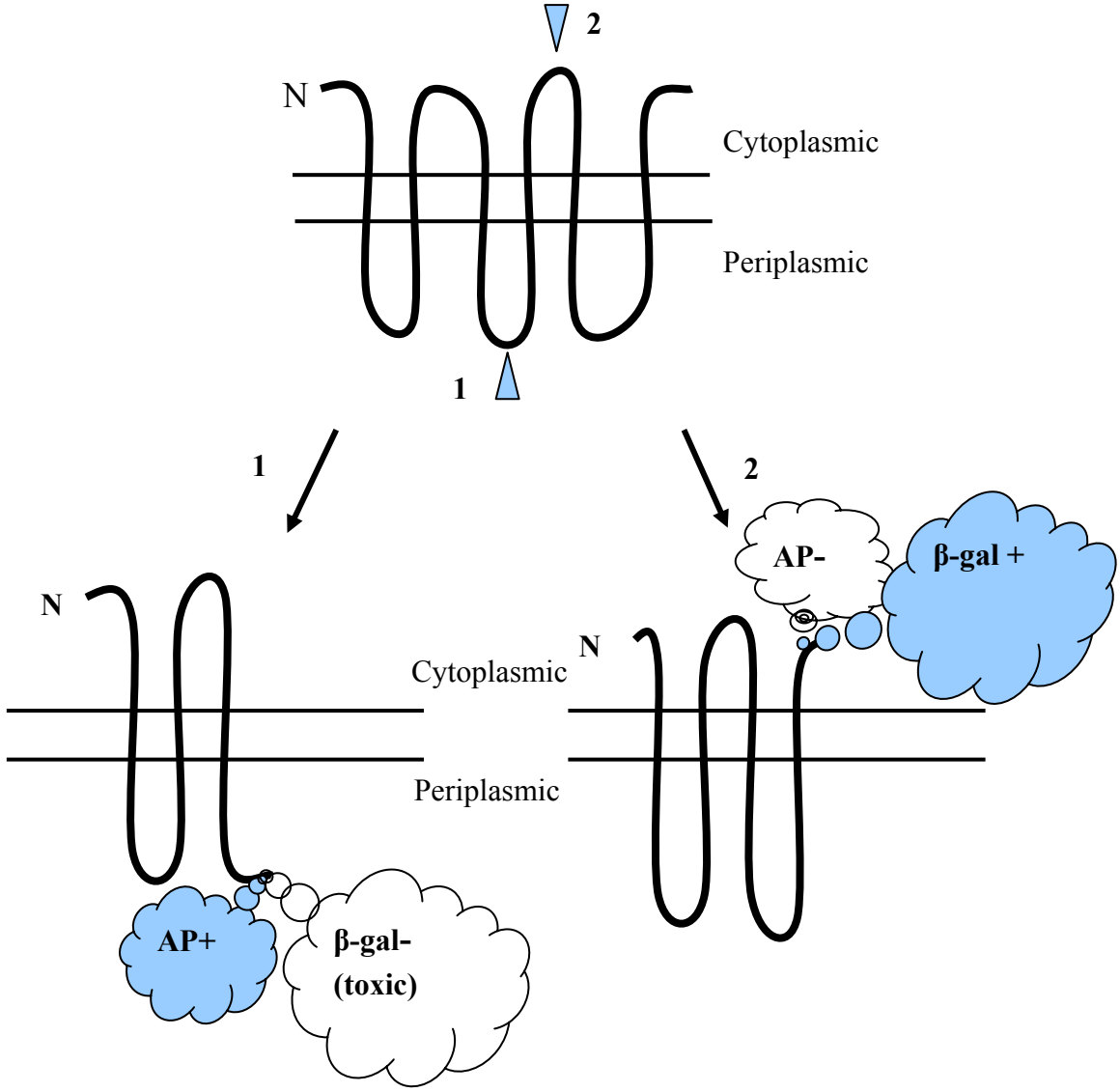


Fig. 3. Use of fusions to analyze membrane protein topology. Fusion of alkaline phosphatase to a cytoplasmic membrane protein at a periplasmic site (site 1) yields a hybrid alkaline phosphatase moiety that it is enzymatically active. Fusion of alkaline phosphatase at a cytoplasmic site (site 2) yields an inactive enzyme. β-galactosidase hybrids show reciprocal behavior, with fusions at periplasmic sites yielding proteins with low specific activities when expressed at low levels, which are usually toxic when expressed at high levels. Cytoplasmic sites of β-galactosidase attachment yield high-activity proteins that are relatively nontoxic.

without glycosylation. Using the protein glycosylation or not, the transmembrane segments could then be defined (Holland and Drickamer, 1986). The topographies of a mammalian P-type ATPase, H^+,K^+-ATPase (Bamberg and Sachs, 1994), and two CPx-type ATPases from *Helicobacter pylori* (Melchers *et al.*, 1996; Bayle *et al.*, 1998) were established using the approach.

1.3.3 Previous membrane topology studies in CPx-type ATPase related proteins

Two Helicobacter CPx-type ATPases have been determined their topographies using the *in vitro* transcription/translation method described above (Melchers *et al.*, 1996). Among them, one was suggested as a Helicobacter homologous CadA, since this protein includes a 31% in overall sequence identity among its 686 amino acids to the staphylococcal CadA (Melchers *et al.*, 1996). The amino acid sequence alignment of CadA from S. aureus pI258 and that from H. pylori was shown in Fig. 4. According to Helicobacter topological study, there are 8 transmembrane segments (TM1 to TM8) found in this ATPase (Melchers *et al.*, 1996). Both the N- and C-termini are located in the cytoplasm of the cells. The putative phosphatase domain is between forth and fifth transmembrane segments (TMs) and the phosphorylation site and ATP binding site are both between sixth and seventh TMs in this P-type ATPase (Fig. 1). Other than those, the signatures of CPx-ATPases are also present in this enzyme just as predicted (Solioz and Vulpe, 1996). Later, using alkaline phosphatase fusion has confirmed the model (Melchers *et al.*, 1999; Fig. 4). The other *Helicobacter* CPx-type ATPase was suggested as a CopA

Fig. 4. Comparison of *S. aureus* CadA and the homologous *H. polori* ATPase. The amino acid sequences of CadA homologous pumps are 727 (*S. aureus* pI258) and 686 (*H. pylori*) amino acids in length. The overall sequence identity level between the two pumps is about 31%. The occurrence of transmembrane segments as experimentally determined in the *H. pylori* pump by *in vitro* method (Melchers, 1996) are highlighted; by *in vivo* alkaline phosphatase fusions (Melchers, 1999) are indecated by triangles. That corresponding to the conserved motifs characteristic for CPx-type ATPases are boxed.

homology, based on the reasons of the high sequence similarity to *Enterococcus hirae* CopA and the special Cu^{2+} binding affinity to the N-terminal peptide of this ATPase (Bayle *et al.*, 1998). As determined experimentally by *in vitro* transcription/translation method, *H. pylori* CopA also contains four pairs of TMs, and shares the same patterns of motifs with the predicted Helicobacter CadA.

Alignment of all the P-type ATPases suggests that the Helicobacter pumps and other homologous heavy metal ATPases have an additional pair of membrane sequences preceding the first pair of membrane segments that are present in the non-heavy metal ATPases. The Helicobacter pumps and other similar P-type ATPases, on the other hand, do not have sequences following the TM8 (Melchers *et al.*, 1996). The heavy metal transporting CPx-type ATPases set themselves apart from the rest of the P-type ATPases not only at the sequence aspect, but also at the membrane topology concerns (Solioz and Vulpe, 1996).

1.4 Motivations for CadA topology

Although the topological study of CPx-type ATPases has been done in Helicobacter models, some uncertainties were remained. Firstly, the experimental data presented to this topology model for this transition metal ATPase was from *in vitro* study and only 9 *in vivo phoA* fusion data in a subsequent study (Melchers *et al.*, 1996; Melchers *et al.*, 1999; Fig. 4). However, there are still 3 large cytoplasmic domains of this protein has not been explored, which are the N-terminal first 70 residues, and the regions ranging from amino acid positions 177 to 309 and 371 to 493. Additionally, the third and forth TMs (TM3 and TM4) suggested in these

studies were originally predicted to be as low hydrophobic domains, according to hydropathy programs (Fig. 2c). It seems that they were not able to traverse the lipid bilayer. In the *in vitro* study, the suggested TM4 of the CadA homologous ATPase was not present as completed transmembrane domain (Melchers *et al.*, 1996). The *in vivo* alkaline phosphatase fusions were too few to cover the full length of the protein including this region (TM3 to TM4) (Melchers *et al.*, 1999). Therefore, this information would not be possible to serve as the perfect topological model for the CPx-type ATPases.

In addition to the reasons we have argued above, some differences exist between these two ATPases. From the sequence comparison data shown in Fig. 4, staphylococcal CadA has a longer N-terminal portion of amino acid sequences and more charged C-terminal portion of amino acid residues than those of the Helicobacter one. Moreover, some CPx-type ATPases including staphylococcal CadA have another putative transmembrane domain, just behind the GDGXNDXP conserved motif, which were not predicted as a transmembrane domain in the ATPases of *Helicobacter spp* (Fig. 2). The similar domain was also shown in other CPx-type ATPases like CadA ATPases from *S. aureus* Tn554, *Bacillus firmus*, *Listeria monocytogenes* as well as PacS and CtaA from *Synechococcus spp* (Chikramane and Dubin, unpublished; Ivey *et al.*, 1992; Lebrun *et al.*, 1994; Kanamaru *et al.*, 1993; Phung *et al.*, 1994). Therefore, another membrane topology data would be necessary to clarify these suspicions.

1.4.1 Gene fusion strategy for CadA topology

Since the topography acquired from Helicobacter studies were not completed to truly represent the CPx-type ATPases, therefore, it will be necessary to re-examine the protein structure of the family. In this thesis study, we decide to use CadA as a model to further disclose the structural aspect of the CPx-type ATPase. There are many experimental techniques would be able to study the topological structure of membrane proteins, including side chain covalent modification, proteolysis, and epitope recognition (Jennings, 1989). However, these techniques are often unable to provide a full topological description of a membrane protein because of their reliance on the natural occurrence of residues that are susceptible to proteolytic cleavage, reactive side chains or accessible to antibody binding (Lee and Manoil, 1996). Another method, the *in vitro* transcription/translation technique, seems amazing (Holland and Drickamer, 1986), but difficult to reflect the intact protein folding in cell membrane because of using only those putative transmembrane fragments for detection. These limitations could be circumvented by the supplement with data from some other molecular biological methods, especially those of gene fusion strategies. The best-established gene fusion strategy for the study of this kind is the *phoA*- and *lacZ*-fusion combination method (Manoil *et al.*, 1990; Manoil, 1991; Fig. 3). This method has been used in many membrane topological studies, for example, the *E. coli* ArsB done by Wu *et al.* (1992), *E. coli* K-12 Mtr permease done by Sarsero and Pittard (1995), *B. subtilis* BofA done by Varcamonti *et al.* (1997), *Lactococcal* LcnC done by Franke *et al.* (1999), *Helicobacter* NixA done by Fulkerson and Mobley (2000). In this study, we take the similar strategy of using the *phoA*- and *lacZ*-fusion to determine the membrane topography of CadA for the first time.

1.4.2 Properties of reporter enzymes

Alkaline phosphatase is a nonspecific phosphomonoesterase, which has a total of 471 amino acids including a signal sequence of 21 amino acids (Chang, 1986). It is ordinarily found in the periplasm of *E. coli* under the condition of phosphate starvation (Wanner 1996). If retained in the cytoplasm, alkaline phosphatase is enzymatically inactive (Michaelis 1983, Hoffman 1985), due to the inability of forming disulfide bonds (Derman and Beckwith, 1991). This property of alkaline phosphatase forms the basis for a convenient *in vivo* strategy for monitoring protein translocation across the cytoplasmic membrane (Manoil *et al.* 1990).

On the other hand, β-galactosidase is a tetramer, and each identical monomer is 116,353 Dalton in molecular weigth and 1,023 amino acid residues in length (Fowler and Zabin, 1978). It is active normally in the cytoplasm (Manoil *et al.*, 1988; Slauch and Silhavy, 1991), but inactive or even toxic when secreted to the periplasm of *E. coli* (Snyder and Silhavy, 1995).

Using these two differently orientated reporter genes to fuse with membrane protein at different locations, completed membrane protein topography will then be possible (Froshauer *et al.*, 1988).

1.5 Rationale and experimental approach

The main purpose of this thesis study is to characterize the topography of CadA protein. To determine how CadA spans on the plasma membrane of bacteria cells; how many TMs are involved in this

protein; where the amino and carboxyl termini are located; and which side the functional groups or the conserved motifs are placed. Furthermore, it is expected to provide a better topological model for CPx-type ATPases from this study.

Special arm:

1. To determine the computer predicted CadA hydrophathy.

2. Using alkaline phosphatase and β-galactosidase as the reporter genes to determine the presence of transmembrane segments in CadA.

3. Prepare a detail CadA topological model using the enzymatic data provide from this study.

4. Disclose the similarities and differences between the staphylococcal CadA and its homologous *Helicobacterial* CadA in this study.

Chapter 2. Materials and Methods

2.1 Bacterial strains, plasmids, and culture conditions

E. coli strains and plasmids used in this study are described in Table I. *E. coli* strain JM109 was used as host for gene cloning purpose. The *E. coli* strain LMG194 was used for *phoA* fusion protein expression, and strain MC1000 was used for *lacZ* fusion protein expression. In all experiments indicated in this study, bacterial cells were grown in LB medium (1% tryptone, 1% NaCl, and 0.5% yeast extract in water) at 37ºC with 200 rpm shaking and supplement with 100μg/ml of ampicillin. A 400μg of either 5-bromo-4-chloro-3-indolyl phosphate (BCIP) or 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-GAL) were spread onto LB agar plates (1.5% agar in LB medium) for screening alkaline phosphatase or β-galactosidase containing bacterial clones.

2.2 Molecular biology techniques

Gene cloning was performed using the standard procedures and described as follow. Polymerase Chain Reactions (PCR) were preformed using Takara Ex Taq (Takara) enzyme in a PTC-200 thermocycler (MJ Research). Plasmids and fragment DNAs, including PCR products and digested DNA, were purified using NucleoSpin Plus Plasmid Miniprep Kit and NucleoSpin Extraction Kit (Clontech), respectively. Restriction enzymes were purchased from New England Biolabs and DNA ligation reactions were performed using a T4 DNA ligase (Promega). Transformation was done using electroporation method of Gene Pulser II

Table I Strains and plasmids

Strain/plasmid	Genotype/description	Refercnce
E. coli strains		
JM109	F' *traD36 lacIq* Δ*(lacZ)M15 proA*$^+$*B*$^+$ / e14$^-$(McrA$^-$)Δ*(lac- proAB) thi gyrA96* (Nal^r) *endA1 hsdR17*(r_K^- m_K^+) *recA1 relA1 supE44*	Promega
LMG194	F$^-$ Δ*(lacIPOZY)X74 galE galK thi rpsL* Δ*phoA ra714*	Invitrogen
MC1000	F$^-$ *araD139* Δ*(araABC-leu)*7679 *galU galK* Δ*(lac)*X74 *rpsL thi*	Ellis *et al.* 1995
RW3110	F$^-$ *mcrA mcrB IN(rrnD-rrnE)1 lambda- ZntA::km*	Rensing *et al.* 1997
Plasmids		
pKJ3	2.6-kilobase pair *XbaI* fragmeng containing the 3' end of *cadC* and the complete *cadA* gene from *S. aureus* in pET11a	K. J. Tsai
pFU3K	entire *cadA* gene, 2184 base pair *NcoI-EcoRI* fragment without internal *NcoI* and *EcoRI*, ligated into pBADMycHisA	S. L. Fu
pSE380	an expression vector offering *trc* promoter, *lacO* operator, *lacI*^{q} repressor and Amp^R selection marker	Invitrogen
pMM1	a transposon delivery plasmid carrying a mini-transposon TnTAP and Tn5 transposase	Guan *et al.* 1999
pMLB1069	pBR322 Δ(tet^r) *lac'ZY'*	Ellis *et al.* 1995
pKJ100	*NcoI- Xba*I fragment of entire *cadA* gene in vector plasmid pSE380	This study
pXxP series	Fusions of *phoA* to various PCR fragments of *cadA* gene	This study
pXxL series	Fusions of *lacZ* to various fragments of *cadA*	This study

(Bio-Rad).

2.3 Construction of inducible *cadA* expression system

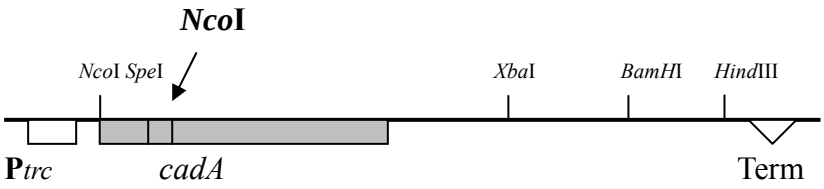
In order to obtain a well-controlled *cadA* expression system, the plasmid pSE380 (Invitrogen) was chosen as the vector for cloning purpose. The pSE380 was digested with *Xba*I and *Hind*III, and then filled with a 368-bp *Xba*I-*Hind*III fragment from pET11a to yield pSE380a plasmid, in which the *Spe*I site was removed. A 2.2-kb *Nco*I-*EcoR*I *cadA* gene from pFU3K was cut out and ligated into *Nco*I-*EcoR*I digested pSE380a to make pSE-FU3K plasmid. Then replaced the 1.7kb *Spe*I-*Xba*I fragment of pSE-FU3K with a *Spe*I-*Xba*I fragment from pKJ3 to generate plasmid pKJ100 (Fig. 5a). In this construction, an approximately entire *cadA* gene is controlled by the *trc* promoter, which is a hybrid promoter from *trp* and *lac* operon.

2.4 Cadmium resistance assays

Cd^{2+} resistance assays were performed using the method previously described (Nucifora *et al.* 1989). RW3110 cells harboring pKJ100 or pSE380 were cultured in LB medium in the presence of 100 μg/ml of ampicillin at 37ºC overnight. Aliquots of cultures were diluted 1: 50 with fresh LB containing 0, 10, 20, 40, and 80 μM $CdCl_2$, respectively and grew for another 6 hours. The cell survivals were measured spectrometrically at a wavelength of 600 nm.

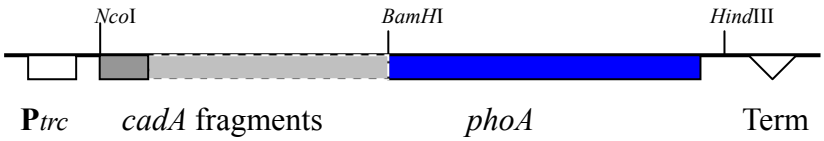
a.

pKJ100



b.

pXxP*



c.

pXxL*

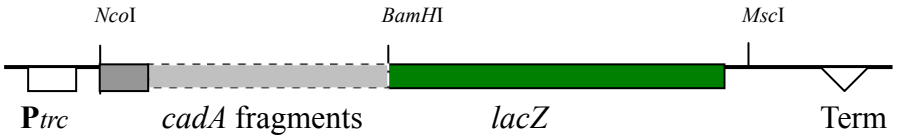


Fig. 5. Constructed plasmids. (a.) The plasmid pKJ100 contains the full-length *cadA* gene. **(b.)** The plasmids contain various *cadA-phoA* fusions. **(c.)** The various plasmids contain the *cadA-lacZ* fusions. The vector plasmid used for these constructions was pSE380. The regulation pattern of this vector is the *trc* promoter, *lac* operator, and it's own terminator. Restriction sites used in construction were also shown. An internal *Nco*I site in the *cadA* gene was marked. ***** The capital "**X**" represents the residue name on CadA protein just before the fusion site. The lowercase "**x**" represents this residue sequence number.

2.5 Construction of *cadA-phoA* and *cadA-lacZ* fusions

Either *phoA* or *lacZ* fusion with the truncated *cadA* were prepared by ligating the PCR fragments of cadA to the 5'-end of these reporter genes, as described below

2.5.1 CadA-phoA fusion

The *cadA-phoA* gene fusions were constructed *in vitro* and cloned in *E. coli* strain JM109. The signal sequenceless *phoA* gene in the 1.9-kb *BamH*I-*Hind*III fragment from pMM1 (Ehrmann *et al.*, 1997; Guan *et al.* 1999) was cut out and ligated to pSE380 digesting with the same enzymes, producing the pSE-phoA clones. PCR fragments, summing up 22 different lengths of *cadA* gene, were produced using pKJ3 as template. And used the same positive-strained primer and different negative-strained primers (Tab. IIa). The first 6 PCR products that are without internal *Nco*I site were digested with *Nco*I and *BamH*I and cloned into digested pSE-phoA to generate clones pL109P, pL130P, pL155P, pE181P, pR204P and pV326P. The rest of the 16 fragments were digested with *Spe*I and *BamH*I to prevent the *Nco*I sites in these PCR fragments and placed onto the pV326P plasmid digested with the same enzymes. Totally, 22 *cadA-phoA* fusions were achieved, and named them as pXxP according to the residue name (X) and number (x) before the fusion junction sites of *cadA-phoA* fusions (Fig. 5b).

Table II Primers. (a.) Primers used in PCR that for *cadA-phoA* and *cadA-lacZ* constructions. The numbers in the primer names correspond to the DNA sequence of *cadA*. The added restriction sites are underlined. Altered nucleotides from *cadA* gene are written in lowercase letters. **(b.)** Primers used for DNA sequencing.

a.

NcoI(+)	5' GTGAAGGTCcATGgCTGAACAAAAG 3'
BamHI327(-)	5' GTGTGGgAtcCAGCAATGTACTATG 3'
BamHI390(-)	5'CATGGAAGgatCCAGGTTATCTTCTCC 3'
BamHI465(-)	5' AATCAAAGgGatcCAAATTTTGAAA 3'
BamHI543(-)	5' ACAACAATAGgatCCTCTGCCC 3'
BamHI612(-)	5' GAACGTATGGgaTccCTTGATCTGTCC 3'
BamHI690(-)	5' CCCACAGCGATAggaTCCACATGG 3'
BamHI765(-)	5' GCCGACAAGggATcCACAATGATTCC 3'
BamHI849(-)	5' TCGTTAAGCGgAtCcGCAAATACTTC 3'
BamHI978(-)	5' CGCAAATggATCcACGAATGCTTGGG 3'
BamHI1068(-)	5' AAACCCATGgATCCCAACTG 3'
BamHI1164(-)	5' TTTTTTCGCTGgATccCCAATTGCCG 3'
BamHI1242(-)	5' TTCCTGTTggATCcAATGCGACTGTC 3'
BamHI1335(-)	5' CTAAAGCTGgAtccATAGAGAATAGCTC 3'
BamHI1431(-)	5' CGAAGTGAATgaaTCCACTTGTAC 3'
BamHI1527(-)	5' GGCTAAAATCGGgAtCcTTTAATTCC 3'
BamHI1626(-)	5' CATCTGCAACGGgAtccACGCCGAGAATTG 3'
BamHI1731(-)	5' GCATTTGCAGgAtCcTGATTATCACC 3'
BamHI1833(-)	5' GCTACATTAggATcCTCCGATTGC 3'
BamHI1938(-)	5' ATCAGCTGgaTCcATTGCAGTATCCG 3'
BamHI2043(-)	5' TTTAATTCCGggAtCcAAAGTGATG 3'
BamHI2109(-)	5' ATATCGGAAgGAtcCGCTATCCAAAG 3'
BamHI2178(-)	5' TCTACCTATggATCCTTCACTC 3'

b.

SE204F	5' CAATTAATCATCCGGCTCG 3'
AP113R	5' GCAGTAATATCGCCCTGAGCAGC 3'
LAC1362R	5' GGGGATGTGCTGCAAGGCG 3'

2.5.2 CadA-lacZ fusion

The *cadA-lacZ* gene fusions were obtained following the similar procedure as that of *cadA-phoA* fusions. A 3.3-kb *BamH*I-*Msc*I *lacZ* fragment from plasmid pMLB1069 (Ellis *et al.* 1995) was prepared to replace the *BamH*I-*Msc*I region of the pSE380 plasmid to generate the pSE-lacZ plasmid. Similarly, the same 22 PCR fragments as mentioned above were cloned separately into this vector using either *Nco*I-*BamH*I or *Spe*I-*BamH*I digestion as described and named these clones as pXxL (Fig 5c). The plasmids are called pXxL followed the residue name and number before the fusion junction sites.

2.6 DNA Sequencing

All selected clones were subjected to DNA sequence determination using an ABI PRISM^{TM} Dye Terminator Cycle Sequencing System in core facility laboratory in this school. The sequencing primers used in these sequencing reactions were listed in the Table IIb.

2.7 Expression of the hybrid proteins

Plasmids that contained *cadA*-reporter gene fusions were transformed into either LMG194 for *phoA* fusions, or MC1000 for *lacZ* fusions, using electroporation for hybrid protein expressions. Transformed cells were cultured in 5 ml of LB medium in the presence of 100 μg of ampicillin at 37ºC overnight with 200 rpm shaking. Next morning, the cultures were transferred into a 200 ml fresh medium to

continuously grow until an optical density of $A_{600\ nm}$ between 0.5 and 0.6. A 0.1 mM Isopropyl β-D-Thiogalactopyranoside (IPTG) was then added to the culture to induce the protein expression at 30ºC for two hours. Finally, the cells were harvested and proteins were prepared for reporter enzyme activity assays and Western blotting analysis. In each culture, a portion of culture was withdrawn for mini-prep DNA preparation to confirm the presence of each hybrid gene using restriction fragment gel electrophoresis.

2.8 Enzyme activity assays

Alkaline phosphatase and β-galactosidase activity assays were performed using the methods of Manoil's (1991) and Miller's (1972), respectively. Some modification were made as described below. The data was collected and calculated from three to five independent experiments.

2.8.1 Alkaline phosphatase

The CadA-alkaline phosphatase fusion proteins were expressed in LMG194 cells as described above. Cells were harvested by centrifugation and washed once with Tris-HCl (pH8.0) buffer. Pellets were resuspended in 1 ml of the same buffer in the presence/absence of one drop of 0.01% SDS and chloroform. A 0.1 ml of the samples was mixed with 0.9 ml of AP buffer containing 100 mM NaCl, 5 mM $MgCl_2$, in 100 mM Tris-HCl (pH 9.5) and read in a spectrophotometer. The values of $A_{600\ nm}$ were recorded as the reference reading for the assay. Another 0.1 ml of the samples were mixed with 0.85 ml of AP buffer and 0.05 ml of 0.4%

ρ-nitrophenyl phosphate (PNPP) in 1 M Tris-HCl (pH 8.0) and make to a final volume of 1.0 ml. Mix the mixture well and measure the $A420_{nm}$ value every min for total 10 min. Alkaline phosphatase activities were calculated as $(\Delta A_{420\,nm} \cdot 1000) / (min \cdot ml \cdot A_{600\,nm})$ as described previously (Ouchane and Kaplan, 1999).

2.8.2 β-galactosidase

As described above, the CadA-β-galactosidase hybrid proteins were expressed in MC1000 cells. Similarly, the hybrid proteins prepared for the β-galactosidase activity assays were about the same as those for alkaline phosphatase activity assays, except for changing certain buffers in these assays. After harvesting the growing cells and washing the centrifuged cell pellets with 0.1 M sodium phosphate (pH 7.5), the cells were resuspended in the same buffer for the assays. A drop of 0.01% SDS and chloroform was added to the cell suspension. A 0.1 ml of the samples was mixed with 0.9 ml of Z buffer containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol at pH 8.0. The $A600_{nm}$ values were measured as a reference reading. The enzyme kinetic of β-galactosidase was measured of $A_{420\,nm}$ after mixing 0.1 ml of the samples with 0.85 ml of Z buffer in the presence of 0.05 ml of 0.4% *o*-nitrophenyl galactoside (ONPG) dissolved in 0.1 M sodium phosphate. The final calculation was done using the same formula as described above.

2.9 Cell fractionation

Cell harboring either *cadA-phoA* or *cadA-lacZ* gene fusions were grow, harvested as described. The cells were washed with 10 ml of 10 mM MOPS twice, and resuspended in a sucrose buffer (50 mM MOPS, 250 mM sucrose, 200 mM KCl, and 10 mM $MgCl_2$). Cells were then passing through a French press at 1,000 b.p.s, and the cell mixtures were centrifuged (4000 g, 15 min, 4˚C) to remove the cell debris. The cell lysates were ultracentrifuged (100,000 g, 90 min, 4˚C) to fractionate the cell contents. The supernatant solutions containing the cytosolic proteins were removed and stored. The membrane pellets were resuspended in 1.0 ml of 10 mM MOPS. The protein concentrations in cell lysates, supernatants, and the final membrane suspensions were measured using D_C protein assay kit (Bio-Rad).

2.10 Protein electrophoresis and Western blotting

To ensure the hybrid protein productions in those enzyme assays, the fractionated components from expressed cells were analyzed in a SDS-7% polyacrylamide gel electrophoresis as well as the Western blotting analysis. SDS-PAGEs of these hybrid proteins were conducted according the standard procedure. After the electrophoresis, proteins were blotted onto a PVDF membrane (Bio-Rad) rinsed with methanol before use, using a blotting chamber at a current of 300 mA for 2 hour. Membrane was blocked with 5 % non-fat dry milk in TBST buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05 % Tween 20, for 1 hour. Blocked blots were probed with mouse antibody to alkaline phosphatase, or rabbit antibody to β-galactosidase (Biogenesis) in the same buffer for 1 hour. Washed membranes with TBST buffer 3

times, and reprobed them with a polyclonal goat anti-mouse or anti-rabbit alkaline phosphatase conjugate (Leinco Technologies or Santa Cruz Biotechnology) in TBST buffer with 5 % non-fat dry milk. Washed 3 times and developed the blots in BCIP-NBT substrate buffer (5-bromo-4-chloro-3-indolylphosphate - nitroblue tetrazolium in AP butter) (Harlow and Lane, 1988).

Chapter 3. Results

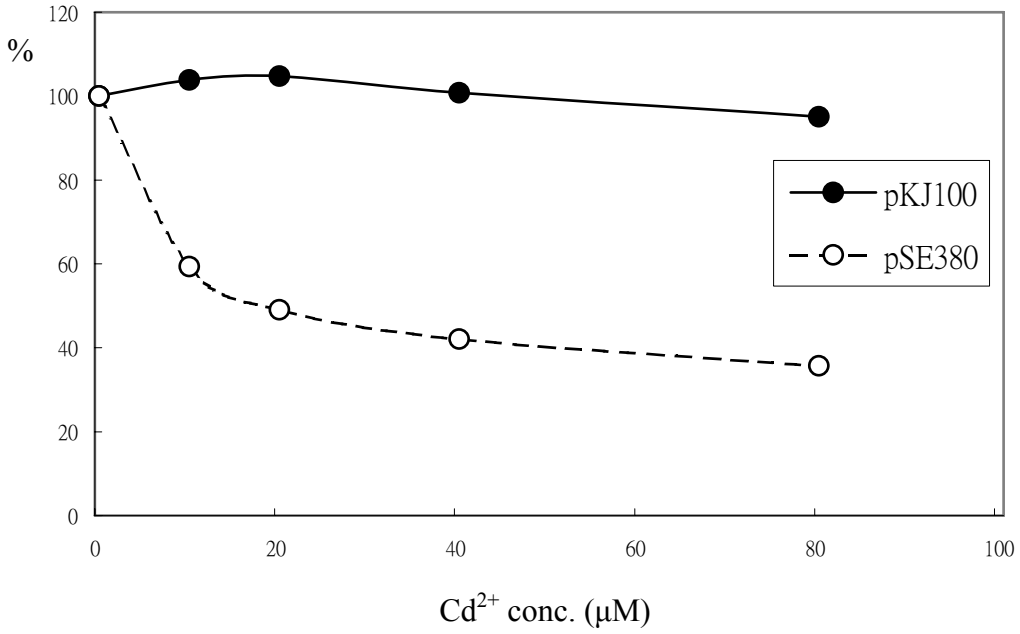
3.1 Determine the cadmium resistance of cells harboring pKJ100 plasmid

In order to determine the cadmium resistance of those cells harboring plasmid pKJ100, in which the full-length *cadA* gene was placed under control by a *trc* promoter, cells were grown in the presence of different concentrations of cadmium. The survivals of these cells were measured their optical densities at a wavelength of 600 nm spectrophotometrically after a 6-hr of incubations. As shown in the Fig. 6, cells contain plasmid pKJ100 grew well in the presence of higher concentration of Cd^{2+}; however those cells without the pKJ100 plasmids display cadmium sensitivities (at least 10 times less in resistance). Suggesting that CadA is functionally expressed in this system and implying the fact that the CadA protein is naturally folded in bacterial cell membrane.

3.2 Computer hydropathy analysis

In this thesis study, our major goal is to investigate the CadA topography. Using the method of Kyte and Doolittle (1982) and computer program of TMpred (Hofmann and Stoffel, 1993), a predicted CadA topography was drawn based on the hydrophathy profile of the 727 amino acid sequences of CadA (Fig. 2a, 2b). And there are six transmembrane segments (TMs) in CadA protein were identified and a CadA protein model was proposed (Silver and Walderhaung 1992). In this postulated

a.



b.

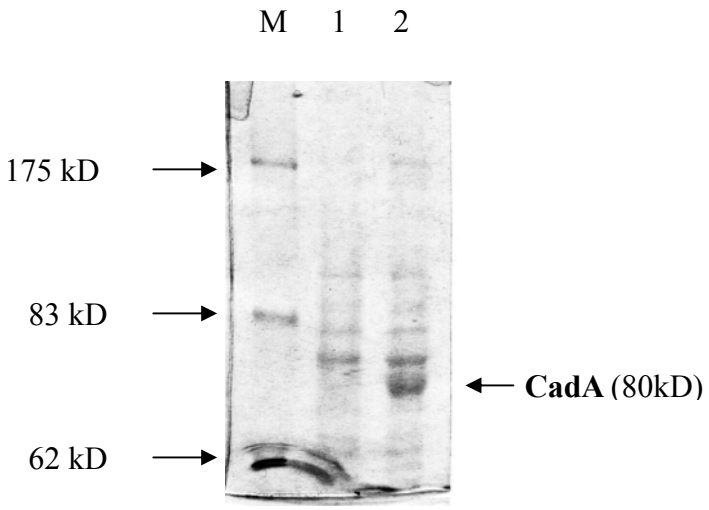


Fig. 6. Cadmium resistance assays and SDS-PAGE analysis of wild-type CadA in pKJ100. (A) Cadmium resistance assays were performed using *E. coli* strain RW3110 harboring pKJ100, wild-type *cadA* gene, or the parental plasmid, pSE380, to grow in the presence of different concentrations of cadmium as described. The survival rates were calculated by dividing the OD_{600} of the final reading in culture with each given cadmium concentration by the OD_{600} measured from culture without the cadmium addition, and shown as percentage (%) in the figure. The average absorbances of the cultures without cadmium addition were 1.82 and 1.94, respectively. The SD values in these results were all less than 8%. **(B)** CadA protein was visualized in SDS 7%-polyacrylamide gel electrophoresis by comparing the membrane proteins prepared from cells harboring pKJ100 and pSE380 plasmids. M, M.W. markers; 1, cells containing pSE380 plasmid; 2, cells harboring pKJ100 plasmid.

CadA topography, 6 highly hydrophobic stretches of amino acid residues of the highly possible membrane-spanning domains including amino acid sequences at 106-126, 130-150, 336-356, 364-384, 626-646 and 686-706 were suggested (Silver and Walderhaung 1992). However, from hydropathy profile of CadA, there might be another 3 TMs, which are located at the amino acid positioned 160-179, 180-196 and that of amended from the latest domain, 677-697 and 699-719, should be included in the CadA topography. The possibility of the presence of additional TMs in CadA was examined in this study. Experiments were designed to prepare CadA fusions with the reporter enzymes which was placed in each possible loop structure and facing either the periplasm or the cytoplasm location. Furthermore, additional CadA fusions were prepared in the putative transmembrane domains and in the large cytoplasmic portions of the protein in order to clarify the CadA topography.

3.3 Isolation of *cadA-phoA* and *cadA-lacZ* fusions

To study the membrane topography of CadA, *phoA* and *lacZ* genes were used as enzyme reporters and were separately fused at 22 different sites on CadA using PCR cloning method as described in the "Experimental Procedures". The 44 *cadA* fusion plasmids were transformed in *E. coli* JM109 cells and the successful clones were selected in agar plates in the presence of BCIP or X-gal. The *phoA* fusions at the CadA amino acid positions 130, 181, 356 and 703 were isolated from the blue colonies on the BCIP plates. Suggesting that these clones contain the translational alkaline phosphatase (AP) on their

periplasmic locations of the cells. Other *cadA-phoA* fusions were isolated from those pale colonies and confirmed by DNA sequence determinations, which indicating that the AP in these fusions were located within the cytoplasmic side in their harboring cells (data not shown).

To isolate the *cadA-lacZ* fusions, we grew cells in the agar plates with X-gal. Most of the successful clones display a blue-color colony formation and were picked up for DNA sequence determination to confirm the fusions. These fusions implied their cytoplasmic location of the CadA junction region with the LacZ moieties. However, fusions at the CadA positions 130, 356, 388 and 703 produced pale colonies, suggesting that these later clones contain periplasmic locations of LacZ moieties of the fusions (data not shown).

The nucleotide sequences of the *cadA-phoA* and *cadA-lacZ* fusion junctions are listed in Table II.

3.4 Determination of the first transmembrane segment

The N-terminal first 130 amino acids were determined their locations using immunoblotting procedure. Two plasmids, pL109P and pL130P, in which a PhoA reporter protein was fused at the amino acid positions 109 and 130 respectively, were prepared and were transformed into *E. coli* strain LMG194. Cells harboring either pL109P or pL130P plasmid were grown and their cell components were fractionated. Using immunoblotting method, only those either cytosolic fractions prepared from cells containing pL109P or membrane fraction prepared from cells with pL130P plasmid were found immunoreactive band when probed with anti- *E. coli* alkaline phosphatase antibody. As shown in the Fig. 7,

Table III Nucleotide sequence of the *cadA-phoA* and *cadA-lacZ* fusion junction. The fusion site, *BamH*I, is underlined. Left of the fusion sites are the sequences of *cadA* PCR fragments. Right of them are ether *phoA* or *lacZ* sequences. The lowercase letters represent altered nucleotides on *cadA*.

Plasmid	*CadA-phoA* fusion junction
pL109P	TTG CTG gaT cCC CGG GTA L L D P R V
pL130P	AAC CTg Gat cCC CGG GTA N L D P R V
pL155P	AAT TTG gat CcC CGG GTA N L D P R V
pE181P	GCA GAG Gat cCC CGG GTA A E D P R V
pR204P	TCA AGg gAt cCC CGG GTA S R D P R V
pV230P	CAT GTG GAt ccC CGG GTA H V D P R V
pV255P	ATT GTG gAT ccC CGG GTA I V D P R V
pA283P	TTT GCg GaT cCC CGG GTA F A D P R V
pV326P	TTC GTg GAT ccC CGG GTA F V D P R V
pW356P	AGT TGG GAT cCC CGG GTA S W D P R V
pG388P	ATT GGg gAT cCC CGG GTA I G D P R V
pF414P	GCA TTg GAT ccC CGG GTA A L D P R V
pI445P	TCT ATg gaT cCC CGG GTA S M D P R V
pV477P	CAA GTG GAt ccC CGG GTA Q V D P R V
pN509P	TTA AAg GaT cCC CGG GTA L K D P R V
pV542P	GGC GTg gaT cCC CGG GTA G V D P R V
pQ577P	AAT CAg GaT cCC CGG GTA N Q D P R V
pE611P	TCG GAG gAT ccC CGG GTA S E D P R V
pI646P	GCA ATg GAt cCC CGG GTA A M D P R V
pF681P	ACT TTg GaT ccC CGG GTA T L D P R V
pA703P	ATA GCG gaT CcC CGG GTA I A D P R V
pD726P	GTG AAG GAT ccC CGG GTA V K D P R V

Plasmid	*CadA-lacZ* fusion junction
pL109L	TTG CTG gaT cCC GTC GTT L L D P V V
pL130L	AAC CTg Gat cCC GTC GTT N L D P V V
pL155L	AAT TTG gat CcC GTC GTT N L D P V V
pE181L	GCA GAG Gat cCC GTC GTT A E D P V V
pR204L	TCA AGg gAt cCC GTC GTT S R D P V V
pV230L	CAT GTG GAt ccC GTC GTT H V D P V V
pV255L	ATT GTG gAT ccC GTC GTT I V D P V V
pA283L	TTT GCg GaT cCC GTC GTT F A D P V V
pV326L	TTC GTg GAT ccC GTC GTT F V D P V V
pW356L	AGT TGG GAT cCC GTC GTT S W D P V V
pG388L	ATT GGg gAT cCC GTC GTT I G D P V V
pF414L	GCA TTg GAT ccC GTC GTT A L D P V V
pI445L	TCT ATg gaT cCC GTC GTT S M D P V V
pV477L	CAA GTG GAt ccC GTC GTT Q V D P V V
pN509L	TTA AAg GaT cCC GTC GTT L K D P V V
pV542L	GGC GTg gaT cCC GTC GTT G V D P V V
pQ577L	AAT CAg GaT cCC GTC GTT N Q D P V V
pE611L	TCG GAG gAT ccC GTC GTT S E D P V V
pI646L	GCA ATg GAt cCC GTC GTT A M D P V V
pF681L	ACT TTg GaT ccC GTC GTT T L D P V V
pA703L	ATA GCG gaT CcC GTC GTT I A D P V V
pD726L	GTG AAG GAT ccC GTC GTT V K D P V V

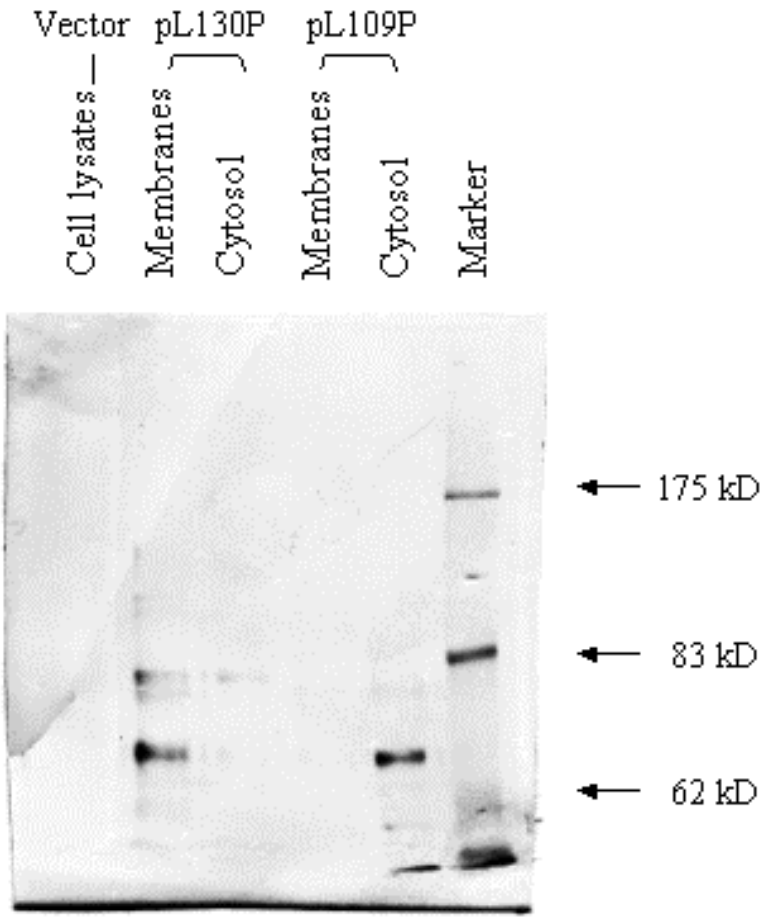


Fig. 7. Determination of the first transmembrane segment of CadA by western blotting. Cultures of *E. coli* strain LMG194 bearing the first and second *cadA-phoA* fusion plasmids, pL109P and pL103P, were induced by 0.1 mM IPTG for 2 hr. Cells were fractionated into cytosol and membranes as described. Samples for SDS-PAGE were the whole cell lysates of control cells, and the cytosol and membranes of the fusion plasmid-bore cells. The samples were analyzed on a 7 % polyacrylamide gel followed by western blotting with an antibody to alkaline phosphatase. The hybrid proteins that could be detected on the blot were in the cytosol of pL109P-bore cells and the membranes of the L130P-bore cells.

immunoreactive bands with a molecular weight of 63.4 kDa and 65.7 kDa were observed in cytosolic fraction of pL109P clone and membrane fraction of pL130P clone, respectively. The proximal molecular weights calculated from the blot were close to the predicted hybrid proteins and these data suggested that the N-terminal first 109 amino acids were located within the cytoplasmic portion of the cell, and the first TM occurred approximately between CadA amino acid sequences 109 to 130.

3.5 Enzyme activity analysis of *cadA-phoA* and *cadA-lacZ* fusions

Other than the blue-white selection of the colony formation to determine the locations of the reporter enzymes, we have also performed enzymatic assays to measure the enzyme activities of those fusion proteins. Two reporter genes, *phoA* and *lacZ*, were used for this topology study, and each of the reporter genes was fused at twenty-two different locations throughout the *cadA* gene to generate either as *cadA-phoA* or *cadA-lacZ* fusions. These genes are transformed into *E. coli* LMG194 cells, for expression of *cadA-phoA* gene fusions, and *E. coli* MC1000 cells, for expression of *cadA-lacZ* gene fusions. Either membrane or cytosolic fraction prepared from cells harboring these fusion genes was assayed for their alkaline phosphatase or β-galactosidase activities. As shown in the Table IV, two classes of enzymatic activities were found among those cells with alkaline phosphatase fusions. Including those fusions of L130, E181, W356, and A703 display a high alkaline phosphatase activity, and the activities were ranging from 51 to 200 AP activity units (Table IVa). Suggesting that the expressed reporter enzymes from these fusions were located in the periplasmic region of the cells. On

Table IV. Fusion enzyme activities. (a.) Alkaline phosphatase activity of *E. coli* strain LMG194 expressing *cadA-phoA* fusions. **(b.)** β-galactosidase activity of *E. coli* strain MC1000 harboring *cadA-lacZ* fusions. *Activity = ($\triangle A_{420}$ • 1000) / (min • ml • A_{600}), 23ºC. Standard deviations (SD) are given between parentheses.

a.

Fusion plasmid	Alkaline phosphatase*	
pSE380	4	(0)
pL109P	4	(0)
pL130P	200	(21)
pL155P	19	(2)
pE181P	105	(7)
pR204P	7	(3)
pV230P	0	(0)
pV255P	0	(0)
pA283P	1	(1)
pV326P	3	(2)
pW356P	51	(7)
pG388P	4	(2)
pF414P	0	(0)
pI445P	0	(0)
pV477P	0	(0)
pN509P	3	(3)
pV542P	3	(3)
pQ577P	0	(0)
pE611P	3	(3)
pI646P	1	(1)
pF681P	0	(0)
pA703P	93	(6)
pD726P	0	(0)

b.

Fusion plasmid	β-galactosidase*	
pSE380	0	(0)
pL109L	27	(4)
pL130L	0	(0)
pL155L	48	(8)
pE181L	28	(9)
pR204L	12	(4)
pV230L	15	(2)
pV255L	18	(1)
pA283L	25	(7)
pV326L	15	(1)
pW356L	2	(1)
pG388L	4	(1)
pF414L	50	(20)
pI445L	35	(12)
pV477L	84	(5)
pN509L	92	(15)
pV542L	93	(31)
pQ577L	52	(10)
pE611L	76	(15)
pI646L	94	(5)
pF681L	32	(2)
pA703L	1	(1)
pD726L	54	(5)

the other hand, eighteen of these fusions, which include L109, L155, R204, V230, V255, A283, V326, G388, F414, I445, V477, N509, V542, Q577, E611, I646, F681, and D726 showed no enzymatic activities at all. Indicating that the expressed alkaline phosphatase from these fusions were at the cytoplasmic side of the cells.

Totally twenty-two *cadA-lacZ* fusions at the same positions as those of *phoA* fusions were prepared in *E. coli* MC1000 cells in this study. And the expressed β-galactosidase activities from these fusions were also investigated. In these β-galactosidase activity assays, we found that most of those fusions with a low alkaline phosphatase activity in *cadA-phoA* clones displayed high β-galactosidase activities when fused with *lacZ* gene, except for the G388 fusion, and their activities were ranging from 12 to 94 units of β-galactosidase activity (Table IVb). As we predicted, those periplasmic located fusions demonstrated above have a low β-galactosidase activity with an exception of the fusion at the E181 position of CadA. The reasonable explanation for the observation is that the expressed β-galactosidases resulted in *cadA-lacZ* fusion at the G388 position of CadA might be due to the closely location of the enzyme to the membrane and the high β-galactosidase activity found in E181 fusion might be due to its incomplete transmembrane. Other than these two fusions, the β-galactosidase activity assays are in agreement with those found in the PhoA enzyme activity assays (Fig. 8).

3.6 Determine the protein productions of CadA fusion

In order to determine the production of those CadA fusion proteins used in this study, Western blot analysis of those fusion proteins were

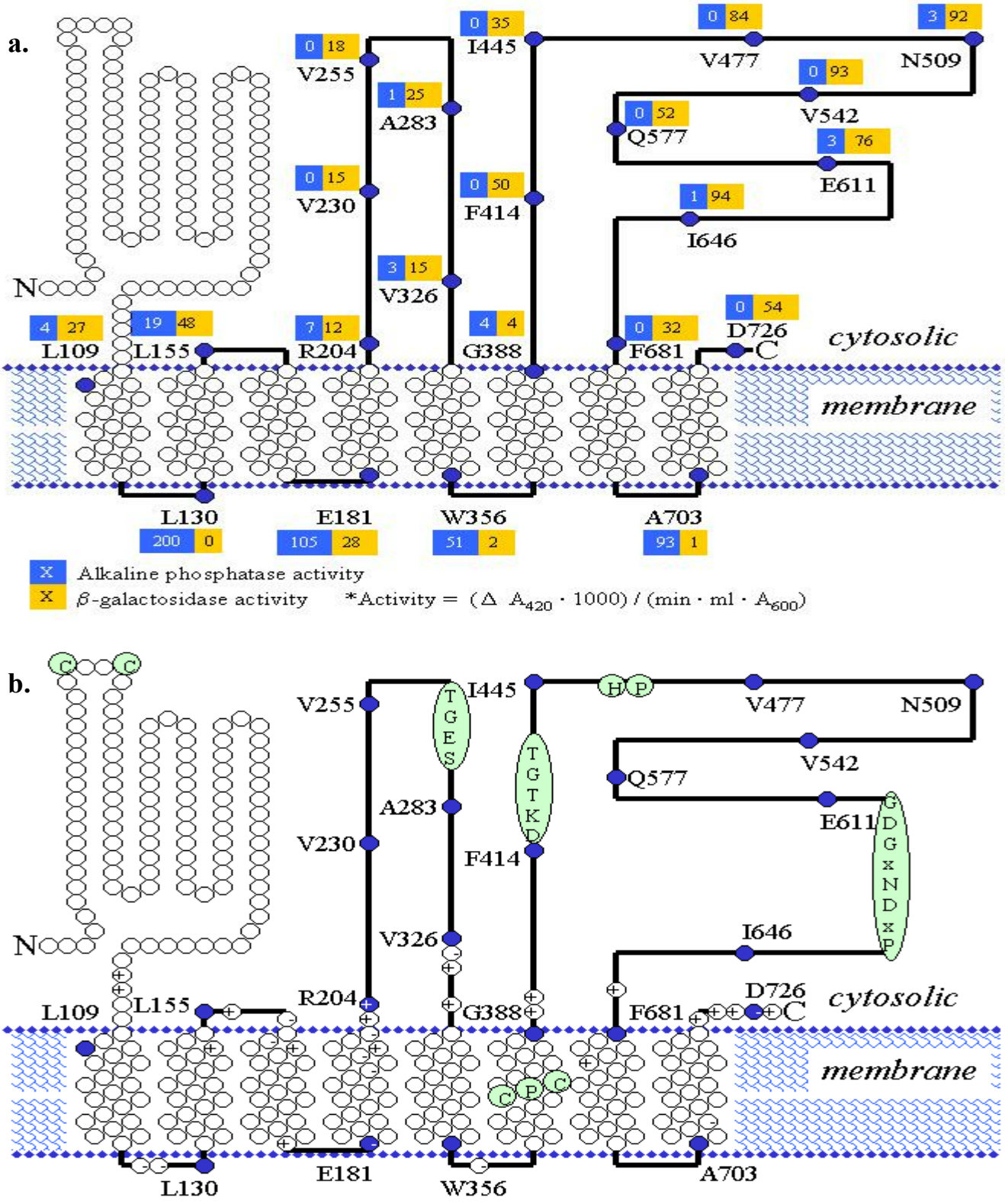


Fig. 8. Topological structure of the CadA protein. (a.) A model with enzyme activities is shown. Alkaline phosphatase activity is shown in a dark rectangle, while the β-galactosidase activity is shown in a light rectangle **(b.)** Shown with conserved motifs belonged to CPx-ATPases, the model indicates these motifs by light-colored circles and ellipses. The locations of the *phoA* and *lacZ* fusion sites are indicated as filled circles, and expressed by a one-letter code and the residue number. The fusion sites are fairly distributed on the CadA protein, except the first 109 residues, which were verified else by western blot (Fig. 8). The first 109 residues as well as the amino acids in the 8 certain transmembrane segments are shown by open circles. Charged amino acids around the transmembrane segments are also marked here.

performed. Either membrane or cytosolic fractions prepared from cells harboring plasmids with either *cadA-phoA* or *cadA-lacZ* fusions were grown and induced in the presence of 0.1 mM IPTG as described. For all forty-four fused protein preparations were examined using monoclonal antibody against either alkaline phosphatase or β-galactosidase. As shown in fig. 9, all fusion proteins of both CadA-alkaline phosphatase (Fig. 9a) and CadA-β-galactosidase (Fig. 9b) were detected using the immunoblotting analysis. The proximal molecular weights calculated from the blot were close to the predicted fusion proteins, and the molecular weights of the fusion proteins are listed in Table V.

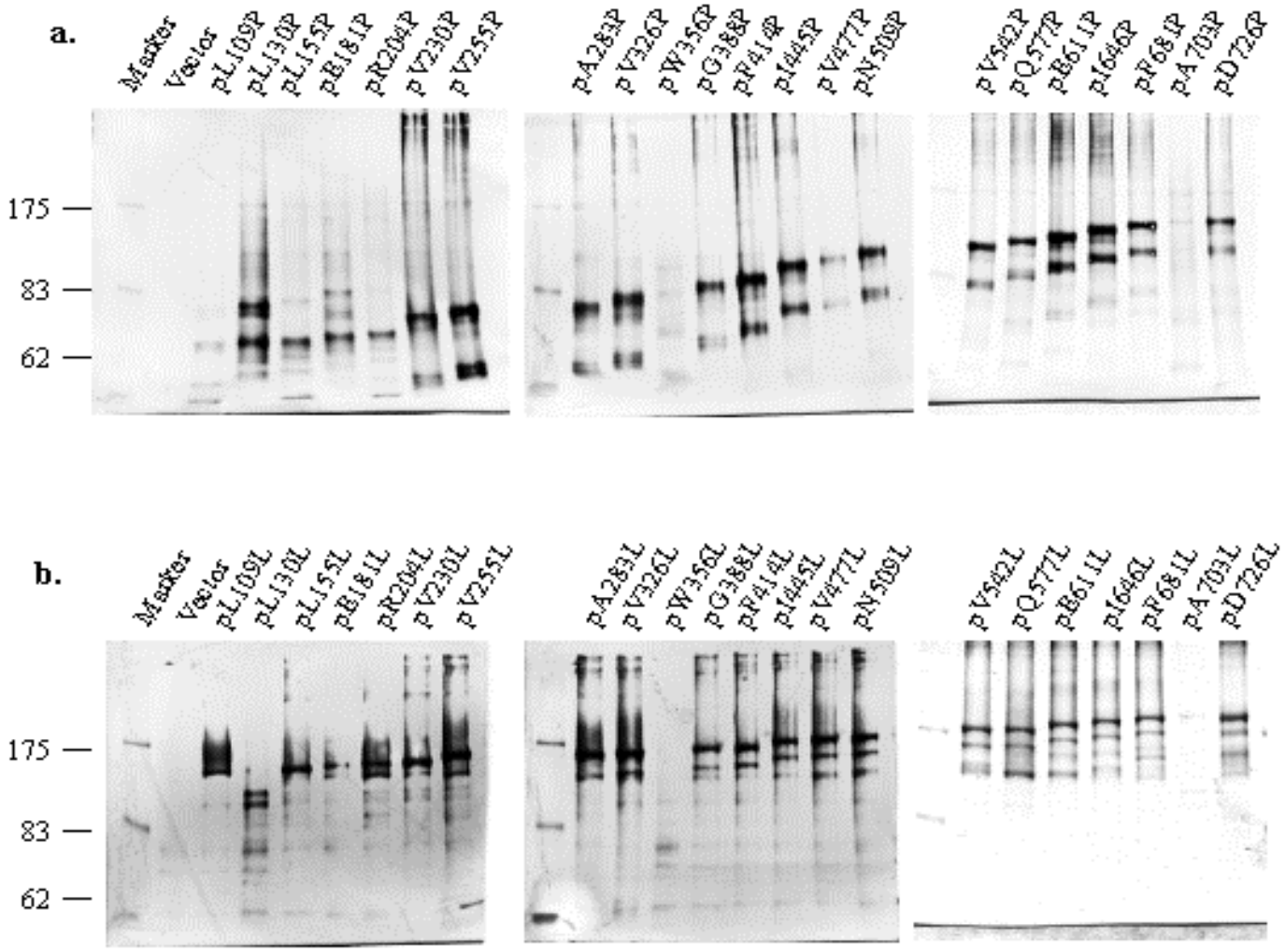


Fig. 9. Western blots of cell extracts of various fusions. (a.) *E. coli* strain LMG194 expressing various *cadA-phoA* fusions. **(b.)** *E. coli* strain MC1000 expressing various *cadA-lacZ* fusions. Samples for SDS-PAGE were the whole cell lysates of control cells, the cytosol of pL109P- and pL109L-bore cells, and the membranes of other cloned cells. The solubilized samples were analyzed on 7 % polyacrylamide gels, and immunoblotting with antibodies to alkaline phosphatase and β-galactosidase, respectively.

Table V. Molecular weights of the fusion proteins. (a.) CadA-alkaline phosphatase fusions. **(b.)** CadA-β-galactosidase fusions.

a.

Fusion protein	Molecular weight (kD)
L109P	63.4
L130P	65.7
L155P	68.4
E181P	71.3
R204P	73.8
V230P	76.7
V255P	79.4
A283P	82.5
V326P	87.3
W356P	90.6
G388P	94.1
F414P	96.9
I445P	100.3
V477P	103.9
N509P	107.4
V542P	111.0
Q577P	114.9
E611P	118.6
I646P	122.5
F681P	126.3
A703P	128.7
D726P	131.3

b.

Fusion protein	Molecular weight (kD)
L109L	128.3
L130L	130.6
L155L	133.4
E181L	136.2
R204L	138.7
V230L	141.6
V255L	144.4
A283L	147.4
V326L	152.2
W356L	155.5
G388L	159.0
F414L	161.8
I445L	165.3
V477L	168.8
N509L	172.3
V542L	175.9
Q577L	179.8
E611L	183.5
I646L	187.4
F681L	191.2
A703L	193.6
D726L	196.2

Chapter 4. Discussion

To understand the structure of membrane transport proteins and their orientation across the membrane is necessary for determining the molecular mechanism responsible for the phenomenon in a membrane transport system. There are many techniques available for the membrane topology determination, including chemical labeling, immunodetection, *in vitro* transcription/translation, and reporter gene fusions. In this thesis study, the structure of the CadA protein was examined experimentally using the genetic approach to construct various *cadA* gene fusions with the genes encoding either alkaline phosphatase (phoA) or β-galactosidase (lacZ) enzymes at different locations of the protein. This approach of using gene fusions to elucidate the architecture of membrane proteins has been applied in many protein topographical studies (Manoil, 1988 and 1991; Lee & Manoil, 1996).

Two reporter gene fusions were used in this study separately. The results of the two types of fusions were all consistent to each other and were used to propose the topological model as shown in Fig. 7. In this model, 8 transmembrane segments (TMs) were identified, along with 3 cytoplasmic loops, and 4 periplasmic loops were also found. In this model, the N-terminal domain of approximately 100 amino acid residues and the C-terminal domain of CadA protein were shown to be located in the cytoplasmic side. Taken together with the hydropathy data (using SOAP and TMpred method to predict) and positive-inside rule (von Heijne, 1992), we proposed that the TMs of CadA protein should be amended at the amino acid positions ranging from 105-123 (TM1), 131-151 (TM2), 164-192 (TM3+TM4), 332-356 (TM5), 363-391 (TM6),

677-697 (TM7), and 699-719 (TM8).

On the other hand, our data have demonstrated a difference in TM3 and TM4 when compared it to that of the Helicobacter CadA (Melchers *et al.*, 1996, 1999). For example, the amino acid sequence of 164-192, including about 30 amino acids was originally predicted to be as a low hydrophobic domain, and seems unable to traverse the lipid bilayer by separating into two independent TMs (Fig. 2). Our data have shown that fusion enzyme activities on the fusion site E181, at the middle of the region, were asymmetric in this study. As we have predicted that enzyme activity of *phoA* fusions at E181, pE181P fusion, showed a high alkaline phosphatase activity (Table IVa), suggesting that the amino acid is at the periplasmic side as that of Helicobacter CadA (Melchers *et al.*, 1999). However, using *lacZ* fusion at the position, the pE181L clone, exhibited an unexpected high β-galactosidase activity which result disagrees with the data from *phoA* fusion at the same position and the Helicobacter CadA (Melchers *et al.*, 1999; Table IV). The difference could be explained that the periplasm-tended alkaline phosphatase is much easily to translocate the fusion site, E181, out to the periplasm; however, the larger cytoplasm-tended β-galactosidase would be kept in its cytoplasmic location due to the short hydrophobic region between the amino acid positions of 164 and 181 can not make the enzyme translocation. Therefore, we believe that the region including the amino acid sequences from 164 to 192 are associated with the cytoplasmic membrane, but not traverses across the membrane. There is another possible explanation that the region is arranged around the hydrophilic channel of the transporter, so it can be separated into two TMs in spite of its low hydrophobicity.

In general, our CadA topological results are similar to that of the

previously reported *Helicobacter pylori* homologous (Melchers *et al.*, 1996, 1999). In these two CadA models, we found that both CadAs include 8 TMs and spanning on the membrane in a similar distribution of those common motifs (Fig. 8b). The conserved phosphatase domain (TGES motif) special to P-type ATPases is located between TM4 and TM5, while the signatures of phosphorylation (DKTGT motif) and ATP binding (GDGXNDXP motif) are positioned between TM6 and TM7. The conserved CPC motif found in all CPx-type ATPases is located within the TM6, as it was previously predicted (Solioz and Vulpe, 1996) and has demonstrated by the Helicobacterial CadA (Melchers *et al.*, 1996, 1999).

On the other hand, there are some differences between these two CadAs. The staphylococcal CadA differs from the Helicobacter CadA in one of its highly hydrophobic regions between amino acid positions 626 and 646 in Staphylococcal CadA, but not found in its Helicobacter counterpart. This region was predicted as a putative TM by computer analysis but has not found in Helicobacter CadA (Fig. 2). Although our data did not support the presence of this putative TM, this hydrophobic region, on the other hand, might associate to the membrane and participate the CadA function. Another difference is the high hydrophilic region found in the N-terminal of CadA protein, just before the TM1 and between amino acid positions 81 and 102. Within this region, the staphylococcal CadA contains more charged amino acids than that of Helicobacter CadA (Fig. 4). The differences in the arrangement of the charged amino acids were found in other regions of these two enzymes. For example, there are two lysine residues found in TM3 in staphylococcal CadA, however, no lysine or other positively charged amino acid are found in this domain of Helicobacter CadA. Four

positively and one negatively charged amino acids are found in C-terminal end of staphylococcal CadA while only two positively charged amino acids are found in that of Helicobacter CadA.

An unexpected result was found in this topological study, that is, the neither chimeras G388P nor G388L displayed fusion enzyme activity (Table IV). The possible reason for that may be due to the circumstance that the reporter enzymes were fused immediately downstream of the TM VI. It was known that *phoA* and *lacZ* fusions tend to introduce biases into membrane protein topology analysis, especially when positively charged residues in the amino acid sequence downstream of the TM are absent in the fusion protein (Frank *et al.*, 1999). Similarly, the reporter enzymes in these two clones were possibly embedded into the membrane because the lysine anchor at the amino acid position 392 was absent in these chimeras. Putting all these information together, a detail CadA topography is thus to establish (Fig. 8).

Furthermore, we have demonstrated the presence of these chimera proteins in our study. Since there might be a possibility that no enzyme activity measured is due to no production in these chimera, therefore, it will be very important to clarify if these chimera proteins are produced in the study. As shown in Fig. 9, proteins prepared from those chimera clones were subject to SDS-PAGE analysis and the bands of the target proteins were visualized by using immunoblotting analysis. Demonstrating that all these chimera proteins were produced in each particular clones, however, some with a small amount of protein production, and some are larger (Fig. 9). It was noted that the periplasmic fusions with these reporter enzymes expressed less hybrid proteins than those of cytoplasmic fusions. This observation has also been previously

reported when the fusions with higher alkaline phosphatase activities are sometime toxic to cells, especially the fusions at the third periplasmic location (Allard and Bertrand, 1992). Similarly, the chimera protein with β-galactosidase is also toxic when secreted to the periplasm of *E. coli* (Snyder and Silhavy, 1995). Some other studies shown that transmembrane-directed proteolysis of a membrane-spanning fusion protein causes periplasmic cleavage by a bacteriophage assembly protein (Guy-Caffey and Webster, 1993), a newly finding of a proteolytic transmembrane signaling pathway, the β-lactamase regulatory system, might be also involved in the periplasmic cleavage (Zhang *et al.*, 2001). Therefore, it would not be surprised that these periplasmic fusions generate proteins slowly, but degrade them rapidly.

In some of these chimera proteins, bands of lower molecular weights were observed and these might be due to the degradation of these fusion proteins. As it has been suggested that an *E. coli* FtsH could initiates proteolysis reaction to rapidly degrade fusion proteins if they were not tightly folded (Kihara *et al.*, 1999). Therefore, along with those predicted fusion protein bands can be seen by Western blotting analysis, some degraded protein bands in each chimera sample preparation were also observed (Fig. 9). These data strongly demonstrated that all the chimera protein were made and their unable to detect either alkaline phosphatase or β-galactosidase enzyme activity in certain constructs were not due to the problem of protein production, but the enzyme orientation on the membrane instead. Among the chimera proteins analyzed in this study, we found that proteins produced by L130P, E181P and L109L clones displayed a higher molecular weight than they should be expected (Fig. 9). This latter observation could be explained as that the chimera

proteins maintaining native conformations and binding less SDS in the electrophoresis buffer as previously described (Guan *et al.*, 1999). The mobility retardation might also result from the formation of disulfide bonds between the alkaline phosphatase (Derman and Beckwith, 1991).

As mentioned previously, more detail characterizations would be necessary to provide a close look into the common signatures in CadA-related CPx-type ATPases, which including the metal binding property, phosphatase activity and aspartyl kinase activity of the enzyme. Based on the hydropathy analysis (Fig. 2), and the membrane topological model of CadA presented here (Fig. 8), we discover some other features within the CadA protein and these structural specialties might be important for CadA functions. For examples, does the highly charged C-terminal end of this protein represent a functional domain? Is the high hydrophobic region behind the putative ATP binding site significant for the function? To understand the exact mechanism involving these charged amino acids and other structures of CadA for its activity will require more data to put into the puzzle.

References

Aguiar JM. Guzman E. Martinez JL. Heavy metals, chlorine and antibiotic resistance in *Escherichia coli* isolates from ambulatory patients. Journal of Chemotherapy. 2(4):238-40, 1990

Allard JD. Bertrand KP. Membrane topology of the pBR322 tetracycline resistance protein. TetA-phoA gene fusions and implications for the mechanism of TetA membrane insertion. The Journal of Biological Chemistry. 267(25):17809-19, 1992

Bamberg K. and Sachs G. Topological analysis of H+,K(+)-ATPase using in vitro translation. The Journal of Biological Chemistry. 269: 16909-16919. 1994

Baranano DE. Wolosker H. Bae BI. Barrow RK. Snyder SH. Ferris CD. A mammalian iron ATPase induced by iron. The Journal of Biological Chemistry. 275(20):15166-73, 2000

Bayle D. Wangler S. Weitzenegger T. Steinhilber W. Volz J. Przybylski M. Schafer KP. Sachs G. Melchers K. Properties of the P-type ATPases encoded by the copAP operons of *Helicobacter pylori* and *Helicobacter felis*. Journal of Bacteriology. 180(2):317-29, 1998

Beard SJ. Hashim R. Membrillo-Hernandez J. Hughes MN. Poole RK. Zinc(II) tolerance in Escherichia coli K-12: evidence that the zntA gene (o732) encodes a cation transport ATPase. Molecular Microbiology. 25(5):883-91, 1997

Binet MR. Poole RK. Cd(II), Pb(II) and Zn(II) ions regulate expression of the metal-transporting P-type ATPase ZntA in Escherichia coli. FEBS Letters. 473(1):67-70, 2000

Bull PC. Thomas GR. Rommens JM. Forbes JR. Cox DW. The Wilson disease gene is a putative copper transporting P-type ATPase similar to

the Menkes gene [published erratum appears in Nat Genet 1994 Feb;6(2):214]. Nature Genetics. 5(4):327-37, 1993

Chaouni LB. Etienne J. Greenland T. Vandenesch F. Nucleic acid sequence and affiliation of pLUG10, a novel cadmium resistance plasmid from *Staphylococcus lugdunensis*. Plasmid. 36(1):1-8, 1996

Chang CN. Kuang WJ. and Chen EY. Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. Gene 44 (1):121-125, 1986

Chelly J. Tumer Z. Tonnesen T. Petterson A. Ishikawa-Brush Y. Tommerup N. Horn N. Monaco AP. Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein [see comments]. Nature Genetics. 3(1):14-9, 1993

Chikramane SG. Dubin DT. PsiTn554: A *Staphylococcus aureus* chromosomal element encoding cadmium resistance determinants, and genes resembling the transposases genes of Tn554. Unpublished

Chopra I. Mechanism of plasmic-mediated resistance to cadmium in *Staphylococcus aureus*. Antimicrobial Agents & Chemotherapy. 7(1):8-14, 1975

Crupper SS. Worrell V. Stewart GC. Iandolo JJ. Cloning and expression of cadD, a new cadmium resistance gene of *Staphylococcus aureus*. Journal of Bacteriology. 181(13):4071-5, 1999

Derman AI. Beckwith J. *Escherichia coli* alkaline phosphatase fails to acquire disulfide bonds when retained in the cytoplasm. Journal of Bacteriology. 173(23):7719-22, 1991

Eckhardt M. Gotza B. Gerardy-Schahn R. Membrane topology of the mammalian CMP-sialic acid transporter. The Journal of Biological Chemistry. 274(13):8779-87, 1999

Ehrmann M. Bolek P. Mondigler M. Boyd D. Lange R. TnTIP and

TnTAP: mini-transposons for site-specific proteolysis in vivo. Proceedings of the National Academy of Sciences of the United States of America. 94(24):13111-5, 1997

Ellis J. Carlin A. Steffes C. Wu J. Liu J. Rosen BP. Topological analysis of the lysine-specific permease of *Escherichia coli*. Microbiology. 141 (Pt 8):1927-35, 1995

Engelman DM. Steitz TA. Goldman A. Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. [Review] [105 refs] Annual Review of Biophysics & Biophysical Chemistry. 15:321-53, 1986

Endo G. Silver S. CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. Journal of Bacteriology. 177(15):4437-41, 1995

Foster TJ. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. [Review] [444 refs] Microbiological Reviews. 47(3):361-409, 1983

Fowler AV. Zabin I. Amino acid sequence of beta-galactosidase. XI. Peptide ordering procedures and the complete sequence. The Journal of Biological Chemistry. 253 (15):5521-5525, 1978

Foye WO. Antimicrobial activities of mineral elements. In ED Weinberg, ed. Microorganisms and minerals. Microbiology series. Vol. 3. New York: Marcel Dekker, pp. 387, 1977

Franke CM. Tiemersma J. Venema G. Kok J. Membrane topology of the *lactococcal* bacteriocin ATP-binding cassette transporter protein LcnC. Involvement of LcnC in lactococcin a maturation. The Journal of Biological Chemistry. 274(13):8484-90, 1999

Froshauer S. Green GN. Boyd D. McGovern K. Beckwith J. Genetic

analysis of the membrane insertion and topology of MalF, a cytoplasmic membrane protein of *Escherichia coli*. Journal of Molecular Biology. 200(3):501-11, 1988

Fulkerson JF Jr. Mobley HL. Membrane topology of the NixA nickel transporter of *Helicobacter pylori*: two nickel transport-specific motifs within transmembrane helices II and III. Journal of Bacteriology. 182(6):1722-30, 2000

Guan L. Ehrmann M. Yoneyama H. Nakae T. Membrane topology of the xenobiotic-exporting subunit, mexB, of the mexA,B-oprM extrusion pump in *Pseudomonas aeruginosa*. The Journal of Biological Chemistry 274(15):10517-10522, 1999

Guy-Caffey JK. Webster RE. The membrane domain of a bacteriophage assembly protein. Transmembrane-directed proteolysis of a membrane-spanning fusion protein. The Journal of Biological Chemistry. 268(8):5488-95, 1993

Harlow E. and Lane D. Antibodies: A Laboratory Manual. 726 pages. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988

Hirata T. Fujihira E. Kimura-Someya T. Yamaguchi A. Membrane topology of the staphylococcal tetracycline efflux protein Tet(K) determined by antibacterial resistance gene fusion. Journal of Biochemistry. 124(6):1206-11, 1998

Hoffman DS. Wright A. Fusion of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proceedings of the National Academy of Sciences of the United States of America. 82:5107-11, 1985

Hofmann K. Stoffel W. TMbase - A database of membrane spanning proteins segments. Biological Chemistry Hoppe-Seyler 374:166. 1993

Holland EC. Drickamer K. Signal recognition particle mediates the insertion of a transmembrane protein which has a cytoplasmic NH2 terminus. The Journal of Biological Chemistry. 261(3):1286-92, 1986

Hustavova H. Obsitnikova K. Havranekova D. Influence of cadmium on resistance to antibiotics in salmonellae isolated from pigs. Folia Microbiologica. 40(3):274-8, 1995

Inbar O. Ron EZ. Induction of cadmium tolerance in *Escherichia coli* K-12. FEMS Microbiology Letters. 113(2):197-200, 1993

Ivey DM. Guffanti AA. Shen Z. Kudyan N. Krulwich T.A. The cadC gene product of alkaliphilic *Bacillus firmus* OF4 partially restores Na+ resistance to an Escherichia coli strain lacking an Na+/H+ antiporter (NhaA). Journal of Bacteriology. 174 (15):4878-4884, 1992

Jennings ML. Anderson MP. Monaghan R. Monoclonal antibodies against human erythrocyte band 3 protein. Localization of proteolytic cleavage sites and stilbenedisulfonate-binding lysine residues. The Journal of Biological Chemistry. 261(19):9002-10, 1986

Jennings ML. Topography of membrane proteins. Annul Review of Biochemistry. 58:999-1027, 1989

Kanamaru K. Kashiwagi S. Mizuno T. The cyanobacterium, *Synechococcus sp.* PCC7942, possesses two distinct genes encoding cation-transporting P-type ATPases. FEBS Letters. 330(1):99-104, 1993

Kihara A. Akiyama Y. Ito K. Dislocation of membrane proteins in FtsH-mediated proteolysis. EMBO Journal. 18(11):2970-81, 1999

Klein P. Kanehisa M. DeLisi C. The detection and classification of membrane-spanning proteins. Biochimica et Biophysica Acta. 815(3):468-76, 1985

Knowles FC. Benson AA. The biochemistry of arsenic. Trends in Biochemical Sciences. 8: 178-180, 1983

Kyte J. Doolittle R. F. A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology. 157(1):105-32, 1982

LaRossa RA. Smulski DR. Van Dyk TK. Interaction of lead nitrate and cadmium chloride with *Escherichia coli* K-12 and *Salmonella typhimurium* global regulatory mutants. Journal of Industrial Microbiology. 14(3-4):252-8, 1995

Lebrun M. Audurier A. Cossart P. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are similar to cadA and cadC of Staphylococcus aureus and are induced by cadmium. Journal of Bacteriology. 176 (10):3040-3048, 1994

Lee M. Manoil C. Molecular genetic analysis of membrane protein topology. P. 189-2012. *In* Konings WN. Kaback HR. and Lolkema JS. (ed.). Handbook of Biological Physics. Elsevier Science B. V. 1996

Lepke S. Passow H. Effects of incorporated trypsin on anion exchange and membrane proteins in human red blood cell ghosts. Biochimica et Biophysica Acta. 455(2):353-70, 1976

Long JC. Wang S. Vik SB. Membrane topology of subunit a of the F1F0 ATP synthase as determined by labeling of unique cysteine residues. The Journal of Biological Chemistry. 273(26):16235-40, 1998

Loo TW. Clarke DM. Covalent modification of human P-glycoprotein mutants containing a single cysteine in either nucleotide-binding fold abolishes drug-stimulated ATPase activity. The Journal of Biological Chemistry. 270(39):22957-61, 1995

Lutsenko S. Kaplan JH. Organization of P-type ATPases: significance of

structural diversity. [Review] [54 refs] Biochemistry. 34(48):15607-13, 1995

MacLennan DH. Brandl CJ. Korczak B. Green NM. Amino-acid sequence of a Ca2+ + Mg2+-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. Nature. 316(6030):696-700, 1985

Manoil C. Analysis of membrane protein topology using alkaline phosphatase and beta-galactosidase gene fusions. [Review] [27 refs] Methods in Cell Biology. 34:61-75, 1991

Manoil C. Beckwith J. A genetic approach to analyzing membrane protein topology. Science. 233(4771):1403-8, 1986

Manoil C. Beckwith J. Tn*phoA*: A transposon probe for protein export signals. Proceedings of the National Academy of Sciences of the United States of America. 82:8129-33, 1985

Manoil C. Boyd D. Beckwith J. Molecular genetic analysis of membrane protein topology. [Review] [39 refs] Trends in Genetics. 4(8):223-6, 1988

Manoil C. Mekalanos JJ. Beckwith J. Alkaline phosphatase fusions: sensors of subcellular location. [Review] [48 refs] Journal of Bacteriology. 172(2):515-8, 1990

Melchers K. Schuhmacher A. Buhmann A. Weitzenegger T. Belin D. Grau S. Ehrmann M. Membrane topology of CadA homologous P-type ATPase of *Helicobacter pylori* as determined by expression of phoA fusions in *Escherichia coli* and the positive inside rule. Research in Microbiology. 150(8):507-20, 1999

Melchers K. Weitzenegger T. Buhmann A. Steinhilber W. Sachs G. Schafer KP. Cloning and membrane topology of a P type ATPase from

Helicobacter pylori. The Journal of Biological Chemistry. 271(1): 446-57, 1996

Mercer JF. Livingston J. Hall B. Paynter JA. Begy C. Chandrasekharappa S. Lockhart P. Grimes A. Bhave M. Siemieniak D. et al. Isolation of a partial candidate gene for Menkes disease by positional cloning [see comments]. Nature Genetics. 3(1):20-5, 1993

Michaelis S. Inouye H. Oliver D. Beckwith J. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. Journal of Bacteriology. 154(1):366-74, 1983

Miller JH. Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972

Nies D. Mergeay M. Friedrich B. Schlegel HG. Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34. Journal of Bacteriology. 169(10):4865-8, 1987

Nies DH. Silver S. Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus*. Journal of Bacteriology. 171(2):896-900, 1989

Nies DH. Nies A. Chu L. Silver S. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proceedings of the National Academy of Sciences of the United States of America. 86(19):7351-5, 1989

Nies DH. Resistance to cadmium, cobalt, zinc, and nickel in microbes. [Review] [94 refs] Plasmid. 27(1):17-28, 1992

Novick RP. Murphy E. Gryczan TJ. Baron E. Edelman I. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid. 2(1):109-29, 1979

Novick RP. Roth C. Plasmid-linked resistance to inorganic salts in

Staphylococcus aureus. Journal of Bacteriology. 95(4):1335-42, 1968

Nucifora G. Chu L. Misra TK. Silver S. Cadmium resistance from *Staphylococcus aureus* plasmid pI258 cadA gene results from a cadmium-efflux ATPase. Proceedings of the National Academy of Sciences of the United States of America. 86(10):3544-8, 1989

Odermatt A. Suter H. Krapf R. Solioz M. An ATPase operon involved in copper resistance by *Enterococcus hirae*. Annals of the New York Academy of Sciences. 671:484-6, 1992

Ogawa H. Haga T. Toyoshima C. Soluble P-type ATPase from an archaeon, *Methanococcus jannaschii*. FEBS Letters. 471(1):99-102, 2000

Ouchane S. Kaplan S. Topological analysis of the membrane-localized redox-responsive sensor kinase PrrB from *Rhodobacter sphaeroides* 2.4.1. The Journal of Biological Chemistry. 274(24):17290-96, 1999

Pedersen PL. Carafoli E. Ion motive ATPase. I. Ubiquity, properties, and significance to cell function. Trends in Biochemical Sciences. 12: 146-150, 1987a

Pedersen P. L. Carafoli E. Ion motive ATPase. II. Eneergy coupling and work output. Trends in Biochemical Sciences. 12: 186-189, 1897b

Perry RD. Silver S. Cadmium and manganese transport in *Staphylococcus aureus* membrane vesicles. Journal of Bacteriology. 150(2):973-6, 1982

Phung L.T. Ajlani G. Haselkorn R. P-type ATPase from cyanobacterium *Synechococcus* 7942 related to the human Menkes and Wilson disease gene products. Proceedings of the National Academy of Sciences of the United States of America. 91:9651-9654, 1994

Rensing C. Mitra B. Rosen B. P. The zntA gene of *Escherichia coli*

encodes a Zn(II)-translocating P-type ATPase. Proceedings of the National Academy of Sciences of the United States of America. 94(26):14326-31, 1997

Rensing C. Sun Y. Mitra B. Rosen BP. Pb(II)-translocating P-type ATPases. The Journal of Biological Chemistry. 273(49):32614-7, 1998

Rensing C. Ghosh M. Rosen BP. Families of soft-metal-ion-transporting ATPases. [Review] [63 refs] Journal of Bacteriology. 181(19):5891-7, 1999

Rensing C. Fan B. Sharma R. Mitra B. Rosen BP. CopA: An *Escherichia coli* Cu(I)-translocating P-type ATPase. Proceedings of the National Academy of Sciences of the United States of America. 97(2):652-6, 2000

Sarsero JP. Pittard AJ. Membrane topology analysis of *Escherichia coli* K-12 Mtr permease by alkaline phosphatase and beta-galactosidase fusions. Journal of Bacteriology. 177(2):297-306, 1995

Serrano R. Kielland-Brandt MC. Fink GR. Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ /K+), K+- and Ca2+-ATPases. Nature. 319(6055):689-93, 1986

Shull GE. Schwartz A. Lingrel JB. Amino-acid sequence of the catalytic subunit of the (Na+ + K+)ATPase deduced from a complementary DNA. Nature. 316(6030):691-5, 1985

Silver S. Nucifora L. Chu L. Misra TK. Bacterial resistance ATPases: primary pumps for exporting toxic cations and anions. Trends in Biochemical Sciences. 14:76-80, 1996

Silver S. Walderhaug M. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. [Review] [257 refs] Microbiological Reviews. 56(1):195-228, 1992

Sipos L. von Heijne G. Predicting the topology of eukaryotic membrane proteins. European Journal of Biochemistry. 213(3):1333-40, 1993

Slauch JM. Silhavy TJ. Genetic fusions as experimental tools. [Review] [106 refs] Methods in Enzymology. 204:213-48, 1991

Smith K. Novick RP. Genetic studies on plasmid-linked cadmium resistance in *Staphylococcus aureus*. Journal of Bacteriology. 112(2):761-72, 1972

Snyder WB. Silhavy TJ. Beta-galactosidase is inactivated by intermolecular disulfide bonds and is toxic when secreted to the periplasm of *Escherichia coli*. Journal of Bacteriology. 177(4):953-63, 1995

Solioz M. Odermatt A. Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. The Journal of Biological Chemistry. 270(16):9217-21, 1995

Solioz M. Vulpe C. CPx-type ATPases: a class of P-type ATPases that pump heavy metals [see comments]. Trends in Biochemical Sciences. 21(7):237-41, 1996

Steck TL. Ramos B. Strapazon E. Proteolytic dissection of band 3, the predominant transmembrane polypeptide of the human erythrocyte membrane. Biochemistry. 15(5):1153-61, 1976

Tanzi RE. Petrukhin K. Chernov I. Pellequer JL. Wasco W. Ross B. Romano DM. Parano E. Pavone L. Brzustowicz LM. et al. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nature Genetics. 5(4):344-50, 1993

Tsai KJ. Linet AL. Formation of a phosphorylated enzyme intermediate by the cadA Cd(2+)-ATPase. Archives of Biochemistry & Biophysics. 305(2):267-70, 1993

Tsai KJ. Yoon KP. Lynn AR. ATP-dependent cadmium transport by the cadA cadmium resistance determinant in everted membrane vesicles of *Bacillus subtilis*. Journal of Bacteriology. 174(1):116-21, 1992

Tynecka Z. Gos Z. Zajac J. Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus*. Journal of Bacteriology. 147(2):305-12, 1981

Ueno S. Kaieda N. Koyama N. Characterization of a P-type Na+-ATPase of a facultatively anaerobic alkaliphile, *Exiguobacterium aurantiacum*. The Journal of Biological Chemistry. 275(19):14537-40, 2000

Varcamonti M. Marasco R. De Felice M. Sacco M. Membrane topology analysis of the *Bacillus subtilis* BofA protein involved in pro-sigma K processing. Microbiology. 143 (Pt 4):1053-8, 1997

von Heijne G. Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. Nature. 341(6241):456-8, 1989

von Heijne G. Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. Journal of Molecular Biology. 225(2):487-94, 1992

Vulpe C. Levinson B. Whitney S. Packman S. Gitschier J. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase [published erratum appears in Nat Genet 1993 Mar;3(3):273] [see comments]. Nature Genetics. 3(1):7-13, 1993

Wanner BL. Phosphorus assimilation and control of the phosphate regulon, p. 1357-1381. *In* Neidhardt FC. (editor in chief), *Escherichia coli* and *Salmonella*: cellular and molecular biology (2^{nd} ed.). American Society for Microbiology, Washington, D. C. 1996

Weiss AA. Silver S. Kinscherf TG. Cation transport alteration associated

with plasmid-determined resistance to cadmium in *Staphylococcus aureus*. Antimicrobial Agents & Chemotherapy. 14(6):856-65, 1978

Weissman Z. Berdicevsky I. Cavari BZ. Kornitzer D. The high copper tolerance of Candida albicans is mediated by a P-type ATPase. Proceedings of the National Academy of Sciences of the United States of America. 97(7):3520-5, 2000

Wu J. Rosen BP. The ArsR protein is a trans-acting regulatory protein. Molecular Microbiology. 5(6):1331-6, 1991

Wu J. Tisa LS. Rosen BP. Membrane topology of the ArsB protein, the membrane subunit of an anion-translocating ATPase. Journal of Biological Chemistry. 267(18):12570-6, 1992

Yamaguchi Y. Heiny ME. Gitlin JD. Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. Biochemical & Biophysical Research Communications. 197(1):271-7, 1993

Yoon KP. Silver S. A second gene in the *Staphylococcus aureus* cadA cadmium resistance determinant of plasmid pI258. Journal of Bacteriology. 173(23):7636-42, 1991

Zhang HZ. Hackbarth CJ. Chansky KM. Chambers HF. A proteolytic transmembrane signaling pathway and resistance to β-lactams in *Staphylococci.* Science. 291, 1962-65, 2001

私立中山醫學院醫學研究所碩士論文

口試問答紀錄

題目: 對金黃色葡萄球菌上抗鎘腺核甘三磷酸酶穿膜結構之研究

指導教授: 蔡淦仁 博士

口試委員: 林嬪嬪 博士　劉昭君 博士

研究生: 林詠峰 R88129

日期: 民國九十年六月二十二日 下午一點三十分

地點: 研究大樓 研五三教室

蔡博士: 你在論文中有提到 positive-inside rule，可否進一步解釋其原理？

研究生: Positive-inside rule 基本上是一個統計的結果，意思是說在一膜蛋白上帶正電荷的氨基酸傾向於留滯在細胞內。其原因應該是因為細胞內相較於細胞外為帶負電荷的環境，故帶正電荷的氨基酸易於留滯在細胞內。

劉博士: 圖四中，你在兩個基因的序列中間所標示的一個點和兩個點之間的差異為何？

研究生: 兩個點代表列序中的氨基酸完全相同，一個點代表這兩個氨基酸性質相近。亦即同為極性或非極性的氨基酸。

劉博士: 你的實驗中，調控基因表達的 promoter 是什麼？為何可以用 IPTG induce？

研究生: 我在實驗中所用的表達系統是 pSE380 載體。它的 promoter 很特別，是結合了 *trp* promoter 和 *lac* operator 來的，所以受 IPTG 調控。

劉博士: 它的調控比其它的 promoter 好嗎？

研究生: 可以說是的。我們曾經試過其它的 promoter，如 T7 和 pBAD 等，但基因表達的情形不盡理想。千挑萬選之後，我們發現這一個表達系統正符合我們的需要。

蔡博士: 其實以前我們一直偏好 T7 promoter，也因此我們花費了很多時間在這上面。最後找到了這一個，它的確有比較好的 control。

劉博士: 在表二中，你所用的 primer 相當多，可否標明其所對應在 DNA 序列上的位置？

研究生: 其實每個 primer 名字上面的數字部份，所代表的就是其對應在 *cadA* 基因上的位置。除了第一個 primer 是 forward，並辨識第一個 *cadA* 上的 codon 外，其餘的都是 backward，並辨識不同位置的 *cadA* 基因。畫線的地方代表了突變的位置。

劉博士: 在你論文第三十七頁的圖六 a 中，你給對照組的名字為 vector。到底是那一個 vector，可否標明一下？

研究生: 在這個實驗中我所用的 vector 都是 pSE380。我會把它修改過來

蔡博士: 另外在圖六 b 中，你用英文字母 V 和 K 來代表 vector 和 pKJ100。其實英文縮寫是不能亂用的，你倒不如用數字來表示還比較好。

研究生: 好的！我會修改。

劉博士: 還有在圖六 b 中，你只用了 SDS-PAGE 來呈現蛋白的表達。為何不用 western？

研究生: 因為我們還沒有 CadA 的 antibody，所以只能用 SDS-PAGE 來看蛋白的表達。

蔡博士: 膜蛋白本身既不容易純化，也不容易拿來製作抗體。

劉博士: 第四十頁的表三，你列出來的是 *cadA* 和 reporter gene 的 fusion site。但我仍然無法從表中看出真正 fusion 的情形。也許加一欄 product size，會讓整個結果更明瞭？

研究生: 的確！這些 fusion 含有各種不同長度的 CadA 片段，有必要秀出它們的大小。我會想辦法把它列進去。

劉博士: 你這些 fusion 的點是怎麼選的？有什麼特別的規則嗎？

研究生: 我是平均選取的。平均二十到三十個氨基酸選一點，然後也要考慮突變的好做與否。

蔡博士: 一般是建議二十個氨基酸要有一個 fusion，不過還算幸運的是詠峰的 data 一致性相當好。點選得不錯。

劉博士: 從你最後的結果看來，似乎 C-端的 fusion enzyme 活性比較好。這是不是有什麼特別的意義？

研究生: 基本上這跟 protein folding 的情形有關。reporter enzyme 的活性跟 fusion site 的關係，基本上並沒有定論。

劉博士: 你有沒有想過用 N-端來作 fusion？

研究生: Reporter gene 接在 N-端的話，只怕結果會只有一種。因為蛋白的表達是從 N-端開始的。

蔡博士: 沒錯！一般相信是這樣的。但也有人認為是整段蛋白作好之後才放到膜上的。不過不管如何，膜蛋白的穿膜基本上還是由 N-端決定的。

劉博士: 你的 western blot 用的 antibody 是 mono 的，還是 poly 的？

研究生: 一次抗體是用 mono 的，二次抗體則是用 poly 的。

劉博士: 這樣子，那你蛋白有定量嗎？不然為什麼從 western blot 上看起來蛋白的量似乎不太一致？

研究生: 我有定 total protein 的量。蛋白量看起來不一致的原因，就如同我先前講的，是因為細胞外的 fusion 對細胞本身有毒性的關係，所以這些細胞外的 fusion protein 的表達可能被某些程度地抑制了。

蔡博士: 這些 clone 的 protein 基本上被 degraded 非常快，這是沒有辦法的事情。

劉博士: 最後看到你的 references。標點符號的使用，以及一些格式上的編排不太一致，要注意一下！

研究生: 基本上我是從 medline 直接 copy 下來的。但也的確有一些例

外。我會再做全盤的審視。

林博士: 其實這也是其它研究生常犯的錯誤。這要特別留意。

林博士: 就你使用的這種 fusion 的方法，你自己覺得它有什麼缺點？

研究生: 在這個實驗裏面,我用的是片段的 CadA 蛋白 fuse 在 reporter enzyme 前面。其最大的缺點就是那麼大的一個 reporter 接在 target protein 後面。也許這已影響了原本蛋白的 folding。不過經過多年來無數人使用的經驗發現答案是不會。一般相信膜蛋白的 folding 只跟 N-端的序列有關。另外一個缺點就是它無法保有原蛋白的活性。

林博士: 還有什麼其它方法可用？

研究生: 當然還有很多方法可用。其它較常用的還有用 tag 或 epitope 的方式，這可以用 antibody 去作偵測。

蔡博士: 最新奇的方法是用 *in vitro* translation。就用一小段一小段的 DNA，在 *in vitro* 去作表達。但這也須要花費大筆金錢，而且它也欠缺 whole protein 真正的 folding。

林博士: 據我所知，你們原本是使用 His-tag，它是不是有什麼缺點？

研究生: 它最主要的缺點是太短了。只有六個 histidine，這很容易在 *E. coli* 裏面造成偽陽性的反應。

蔡博士: 也因為它太短，基本上並不容易作 fusion。而且它是帶正電，是不是會影響穿膜也是不可知的。

林博士: 除了 fusion 的方法以外，還有其它方法嗎？

研究生: 其它方法是有的，不過並不常用。

蔡博士: 有人用過 NMR 的方法去 scan 特定的氨基酸。你有沒有看過一篇一九九五年的 paper？

研究生: 不好意思，我不太清楚。

蔡博士: 他們是先把所有 cysteine 突變掉，再一次放一個 cysteine 上去。然後用 NMR 去 scan，一樣可以得到有或無的 data。不過這只能用在 cysteine 不扮演重角色的蛋白上面，不適合用在 CadA。

林博士: 可不可以告訴我們 ß-galactosidase 為什要在細胞內才有活性？

研究生: ß-galactosidase 必須要形成四合體才有活性。而只有在細胞內才有適當的環境讓它形成四合體，細胞外則沒有這樣的環境。

劉博士: 那 AP 呢？它又是怎麼一回事？

研究生: Alkaline phosphatase 的情形跟 ß-galactosidase 不太一樣。它活性的有無跟 disulfide bond 有關係。它必須要形成 disulfide bond 才會有活性。而它也只有在被傳送到細胞膜外面的過程中才能形成 disulfide bond，而發揮其活性。

林博士: 你為什麼會選用這些菌株來作表達 ？

研究生: 基本上我必須選用 reporter gene 已被 knock-out 的菌株來作表達，才能避免偽陽性的產生。而比較特別的部份是 *lacZ* fusion。我試過很多種菌株，包括 TOP10 和 BL21 等。最後發現只有 MC1000 表達得最好。

劉博士: 為何 N-端有一大段沒有 fusion 呢 ？

研究生: 事實上在剛才的 presentation 中我有講過。我強調說我用 western blot 的方法證明，第一百零九個氨基酸之前的片段被發現存在於 cytosol 中，而不是在 membrane 上面。這證明前一百零九個氨基酸裏面並沒有穿膜片段。多用了這個方法，我可以少作好幾個 fusion。

蔡博士: 我們總共作了四十幾個 fusion。多作一個 western blot，的確省事不少。

蔡博士: 作這麼多的 fusion 的確是一件辛苦的事。不過話說回來，你的 presentation 在 introduction 的部份仍欠缺完整的 logic。你必須要加強在佈局以及故事的連貫性上面。試著從聽眾的角度去敘述，而不是從你自己的想法。這是在你將來的 scientific career 中要去注意的。