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Structural and functional study of multidrug transporters

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Structural and functional study of multidrug transporters

by

Feng Long

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# TABLE OF CONTENTS

Abstract ........................................................................................................................................... iii

Chapter 1. General Introduction ........................................................................................................ 1
  Bacterial multidrug resistance ........................................................................................................1
  Multidrug efflux pumps of the MATE family ............................................................................ 1
  Multidrug efflux pumps of the RND family ............................................................................ 4
  Organization of the dissertation .............................................................................................. 7
  References .................................................................................................................................. 7

Chapter 2. Functional cloning and characterization of the multidrug efflux pumps NorM from *Neisseria gonorrhoeae* and YdhE from *Escherichia coli* .................................................. 16
  Abstract .................................................................................................................................... 17
  Introduction ................................................................................................................................. 18
  Materials and Methods ............................................................................................................... 20
  Results and Discussion .............................................................................................................. 26
  References .................................................................................................................................. 36
  Tables ......................................................................................................................................... 42
  Figures and Captions .................................................................................................................. 44

Chapter 3. Crystal structures of the CusA heavy-metal efflux pump suggest a methioninemEDIATED transport mechanism ........................................................................................................ 54
  References .................................................................................................................................. 70
  Figures and Captions .................................................................................................................. 76
  Supplemental Materials .............................................................................................................. 83
    Methods .................................................................................................................................. 83
    References ................................................................................................................................. 92
  Supplemental Tables ................................................................................................................... 94
  Supplemental Figures and Captions .......................................................................................... 98

Chapter 4. General Conclusions and Future Directions .................................................................. 120
  References ................................................................................................................................. 123
  Acknowledgements .................................................................................................................... 125
ABSTRACT

Active efflux of antimicrobial agents is one of the most important strategies that bacteria use to ensure their survival in a toxic extracellular environment. Our interest is to elucidate the structural and functional relationships of bacterial multidrug efflux transporters that give rise to antibiotic resistance. In this dissertation, I focus on studying two specific groups, the multidrug and toxic compound extrusion (MATE) family and the resistance-nodulation-division (RND) family, of multidrug efflux transporters. The functions of the two MATE efflux pumps, NorM of Neisseria gonorrhoeae and YdhE of Escherichia coli, are characterized using a variety of biochemical assays, including drug susceptibility and transport assays. Direct measurements of efflux allow us to confirm that NorM behaves as a Na⁺-dependent transporter. The capacity of NorM and YdhE to recognize structurally divergent compounds is also examined using steady-state fluorescence polarization assays. The results suggest that NorM and YdhE bind these antimicrobials with dissociation constants ($K_D$s) in the micromolar range.

In addition, I present the crystal structures of the CusA transporter, the only heavy-metal efflux (HME) pump of the RND family in E. coli, in the absence and presence of Cu(I) and Ag(I) ions. These are the first structures of any efflux pumps belonging to the HME-RND family. Surprisingly, the binding of Cu(I) and Ag(I) triggers significant conformational changes in both the periplasmic and transmembrane domains of the pump. Based on the crystal structures and biochemical studies, I put forward my hypothesis that CusA is capable of actively picking up metal ions from the cytoplasm as well as the periplasmic space, utilizing the methionine pairs/clusters in the pump to bind and export metal ions. A stepwise shuttle mechanism is probably employed by the pump to extrude these heavy metals.
CHAPTER 1. General Introduction

Bacterial multidrug resistance

Since the discovery of penicillin in 1928, antibiotics have had been used to cure many potentially lethal bacterial infections. As a consequence of the widespread use of these antibiotics, an increasing number of pathogens are becoming resistance to multiple antibiotics. One of the most serious threats to human health was posed with the emergence of vancomycin resistance in methicillin-resistant Staphylococcus aureus (MRSA) (1). To date it has been found to be more difficult to treat bacterial infections with standard therapies. Bacteria can work against the action of antibiotics through various strategies (2). These include altering or modifying the target, enzymatic inactivating the drug, preventing an antibiotic from entering the bacterial cell, and actively transporting an antibiotic out of the cell. Except for the intrinsic resistance to given antibacterial drugs, bacteria can resist antibiotics in a broad range by acquiring resistance genes from other species (3).

Bacterial multidrug efflux transporters are currently categorized in five different families: the major facilitator (MF) superfamily (4-6), the resistance nodulation cell division (RND) family (7), the small multidrug resistance (SMR) family (8), the ATP binding cassette (ABC) family (9) and the multidrug and toxic compound extrusion (MATE) family (10). The ABC family transporters take the free energy generated from ATP hydrolysis to expel toxic substances out of cells. Efflux transporters in the other families, however, utilize the transmembrane electrochemical gradient of protons or sodium ions to extrude these harmful substrates from cells. In this dissertation, I mainly focus on the study of representative transporters belonging to the RND and MATE families.
Multidrug transporters have been identified in both mammalian and bacterial cells. An understanding of how these efflux transporters work would allow us to find better approaches for treating bacterial infections and cancer. The key to understand how these efflux pumps operate involves the determination of the structures of representative transporters and the elucidation of the conformational changes that accompany with drug translocation across the cell membrane.

Multidrug efflux pumps of the MATE family

The MATE family is the most recently classified family with only about twenty transporters having been identified so far. MATE transporters are typically composed of 12 putative transmembrane domains, and have been found in all major kingdoms (Eukarya, Archaea, and Eubacteria). NorM of *Vibrio parahaemolyticus*, the first characterized MATE transporter, is a Na⁺/drug antiporter that confers resistance to dyes, fluoroquinolones and aminoglycosides (11). Subsequently, other MATE efflux pumps have been identified in many pathogenic bacteria, including *Acinetobacter baumannii* (12), *Brucella melitensis* (13), *Clostridium difficile* (14), *Haemophilus influenza* (15), *Neisseria gonorrhoeae* (16), *Neisseria meningitides* (16), *Pseudomonas aeruginosa* (17), *Staphylococcus aureus* (18), and *Vibrio cholera* (19-21). The main substrates for these MATE transporters are cationic and aromatic compounds. Until now, little knowledge has been obtained regarding the structures and fundamental mechanisms that give rise to multidrug resistance mediated by this novel MATE family of transporters. Nonetheless, the presence of these multidrug efflux pumps in pathogens highlights the potential clinical significance of this transporter family. In this
dissertation, I target the NorM pump of *N. gonorrhoeae* and the YdhE transporter of *Escherichia coli*, and characterize the functions of these two MATE-type proteins.

*N. gonorrhoeae*, a gram-negative diplococcus, is found only in humans and causes one of the most common sexually transmitted diseases. This pathogen is progressively shown to be resistant to multiple antibiotics, including penicillin, tetracycline, erythromycin, and ciprofloxacin. In April 2007, the Centers for Diseases Control and Prevention (CDC) officially added gonorrhea to the list of antibiotic resistant ‘super bugs’ (22). In the USA, it is estimated that 700,000-800,000 new cases occur each year, with a cost of about $1.1 billion annually for this infection (23). While we are aware of the growing public health concern resulted from the antimicrobial resistance developed in *N. gonorrhoeae*, four multidrug transporters have been identified in *N. gonorrhoeae*. These transporters are MtrD (RND) (24-26), FarB (MF) (27), MacB (ABC) (28) and NorM (MATE). The gonococcal NorM appears to recognize and export a number of cationic toxic compounds, including ethidium bromide, acriflavin, 2-N-methylellipticinium, and ciprofloxacin (16).

The *E. coli* YdhE multidrug transporter is homologous to *N. gonorrhoeae* NorM (11, 29, 30). These two MATE transporters share 34% identity and 70% similarity. Both YdhE and NorM are membrane proteins, consisting of 457 and 459 amino acid residues, respectively. Our goal is to understand the structural and functional relationship of these MATE transporters using x-ray crystallography and different biochemical methods. Particularly, I investigate the active molecular functions of *N. gonorrhoeae* NorM and *E. coli* YdhE by conducting heterogenous drug susceptibility test, *in vivo* transport assay, and a novel fluorescence polarization assay. The information has allowed us to understand the
fundamental rules that govern drug recognition and extrusion in the MATE family of multidrug efflux pumps.

**Multidrug efflux pumps of the RND family**

The RND-type transporters play a major role in mediating clinically relevant antibiotic resistance in Gram-negative bacteria. These transporters can work against a broad range of structural unrelated toxic compounds. For example, *E. coli* AcrB is capable of extruding a variety of antibiotics, detergents, dyes and simple organic solvents (31). A typical RND efflux system consists of three fundamental components: an inner membrane RND transporter, a periplasmic membrane fusion protein (MFP) and an outer membrane protein (OMP) channel. The resulting tripartite complex spans both the inner and outer membranes and exports these toxic substrates directly out of the bacterial cell (32, 33). The energy source of the process comes from proton import, which is utilized by the inner membrane RND transporter.

The RND family can be further divided into two sub-families, the hydrophobic and amphiphilic efflux RND (HAE-RND) and heavy-metal efflux RND (HME-RND) sub-families. As a Gram-negative bacterium, *E. coli* possesses seven RND transporters. Six of these efflux pumps, AcrB (31, 34-41), AcrD (42), AcrF (43), MdtB (44, 45), MdtC (44, 45) and YhiV (46, 47), are categorized as the HAE-RND sub-family. These six transporters are responsible for the intrinsic tolerance of antibiotics and toxic organic compounds. The rest of the transporter CusA belongs to the HME-RND family and is found to be capable of exporting copper (I) and silver (I) ions out of *E. coli* cell (48, 49).
Among all known RND transporters, the X-ray structures of *E. coli* AcrB (35, 36, 38, 39) and *Pseudomonas aeruginosa* MexA (50) have been determined. Based on the crystal structures, the functional unit of these efflux pumps is a homotrimer. In each monomer of the AcrB pump, there are 12 transmembrane (TM) helices spanning the entire cytoplasmic membrane, and two large periplasmic loops protruding approximately 80Å into the periplasm. A large cavity with a diameter of approximate 35Å is formed in the transmembrane region within the trimer. The large periplasmic domain of the pump mainly comprises two distinct loops located between TM1 and 2, and TM7 and 8 (51). It has been well characterized that the AcrB transporter interacts with TolC (OMP) and AcrA (MFP) to form a functional tripartite RND efflux complex (52). The crystal structures of these three individual components are all available (53-55). A proposed assembled model of the tripartite RND efflux complex, based on cross-linking and computation method, has suggested that this efflux system is in the form of AcrB3-AcrA3-TolC3 (56). A pathway for transporting substrates in the pump has been shown through a tunnel formed by the periplasmic domain using computational calculation (40, 50). However, the fundamental mechanisms involved in the assembly and drug transport of this tripartite system are still not clear and confirmative.

Towards our goal to elucidate the structures and fundamental mechanisms that give rise to the recognition and extrusion of toxic compounds in RND efflux transporters, we focus on studying the *E. coli* CusCBA HME-RND efflux system. This study has provided us with the understanding of heavy-metal tolerance in bacteria.

As an alternative clinical treatment of bacterial infections, both silver and copper exhibit antimicrobial activities against a broad range of microorganisms at low concentrations. Although copper is required by most microorganisms in trace amount to
maintain their lives, excess amount of copper is extremely toxic (57). It has been reported that silver and copper compounds effectively eliminate *Legionella* in drinking water pipelines (58). Silver compounds are comparatively more often used for clinical purposes. Colloidal silver was first approved for wound management by the US Food and Drug Administration (FDA) in the 1920s (59). To date, silver compounds are still widely used in the treatment of open wounds due to the limited prescription of antibiotics. Because of the widespread use of silver and copper as microcidal agents, concerns about the emergence of bacterial resistance are being raised (59, 60).

The Cus system is encoded by a single operon *cusCFBA*, which is located on the *E. coli* chromosome (48, 49, 61). The transcription of this operon is regulated by a two-component system, consisting of a membrane kinase sensor (CusS) and a transcriptional regulatory responder (CusR) (62). CusB and CusC are the MFP and OMP proteins, respectively. Presumably, in cooperation with the CusA efflux pump, they form a tripartite complex that spans both the inner and outer membranes of *E. coli*, resembling the AcrAB-TolC system. Distinct from most RND efflux complexes, the Cus system has an additional component - the small periplasmic protein CusF (63-65). The crystal structure of CusF depicts that this protein forms a five-stranded β-barrel with the conserved residues H36, W44, M47 and M49 making up the Cu(I)/Ag(I) binding site. It has been proposed that CusF functions as a metallochaperone that delivers Cu(I) or Ag(I) in the periplasm to the CusCBA efflux machinery. The x-ray crystal structure of CusB has been solved recently in our lab (66). The multiple Cu(I)/Ag(I) binding sites were discovered at the potential hinge regions between the individual domains. One of the objectives of this dissertation is to understand the structure and function of the CusA inner membrane transporter, which is the most
important component of the Cus transport system. I have determined the crystal structures of CusA in the absence and presence of Cu(I) and Ag(I). Together with results from site-directed mutagenesis and different biochemical assays, I put forward to propose a molecular mechanism that governs the heavy-metal export in bacteria. It is expected that this information will provide us with important clues for the understanding of other HAE-RND efflux pumps.

Organization of the dissertation

Chapter 1 gives the general introduction of multidrug resistance in bacteria and of multidrug efflux pumps. Two transporter families, including MATE and RND, are described in details. The rationale of the dissertation is also addressed in this chapter. Chapter 2 is a manuscript, published in Antimicrobial Agents and Chemotherapy in 2008, which characterizes two MATE transporters, N. gonorrhoeae NorM and E. coli YdhE. Chapter 3 is a manuscript, submitted to the journal Nature, which describes the crystal structures of the HME-RND transporter CusA of E. coli in the absence and presence of Cu(I) and Ag(I). In this chapter, a variety of functional studies were also performed to elucidate the molecular mechanisms of the efflux pump. Chapter 4 summarizes the results and provides the overall conclusions of the dissertation.

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CHAPTER 2. Functional cloning and characterization of the multidrug efflux pumps NorM from *Neisseria gonorrhoeae* and YdhE from *Escherichia coli*


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Running title: MATE efflux pumps

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Abstract

Active efflux of antimicrobial agents is one of the most important adapted strategies that bacteria use to defend against antimicrobial factors that are present in their environment. The NorM protein of Neisseria gonorrhoeae and YdhE of Escherichia coli have been proposed to be multidrug efflux pumps that belong to the multidrug and toxic compound extrusion (MATE) family. In order to determine their antimicrobial export capability, we cloned, expressed, and purified these two efflux proteins, and characterized their functions both in vivo and in vitro. E. coli expressing norM or ydhE showed elevated (two-fold or greater) resistance to several antimicrobial agents, including fluoroquinolones, ethidium bromide, rhodamine 6G, acriflavin, crystal violet, barerine, doxorubicin, novobiocin, enoxacin, and tetraphenylphosphonium chloride. When expressed in E. coli, both transporters reduced ethidium bromide and norfloxacin accumulation through a mechanism requiring the proton motive force and direct measurements of efflux confirmed that NorM behaves as a Na⁺-dependent transporter. The capacity of NorM and YdhE to recognize structurally divergent compounds was confirmed using steady-state fluorescence polarization assays, and the results revealed that these transporters bind antimicrobials with dissociation constants (K_D) in the micromolar region.
Introduction

*Neisseria gonorrhoeae* is a gram-negative diplococcus, which is only found in humans and causes the sexually transmitted disease gonorrhea. Since it is a strictly human pathogen and can colonize male and female genital mucosal surfaces and other sites, it has developed mechanisms to overcome host antimicrobial systems that are important in innate host defense. One important mechanism that *N. gonorrhoeae* uses to subvert antimicrobial agents is the expression of multidrug efflux pumps that recognize and actively export a wide variety of toxic (often structurally unrelated) compounds, including antibacterial peptides, long-chain fatty acids, and several clinically useful antibiotics, from the bacterial cell (17, 34, 36, 37).

Recently, four efflux pumps have been identified in *N. gonorrhoeae*. One such pump is the MtrD inner membrane protein (21) that exists as a component of the tripartite resistance nodulation cell division (RND) transporter (42). MtrD interacts with a periplasmic membrane fusion protein, MtrC, and an outer membrane channel, MtrE, to mediate the export of hydrophobic antimicrobial agents, including antibiotics, nonionic detergents, certain antibacterial peptides, bile salts, and gonadal steroidal hormones (7, 9, 10, 37). The FarB efflux pump (17), belonging to the major facilitator (MF) family (8, 22), recognizes antibacterial long-chain fatty acids and exports them out of the cell in conjunction with a membrane fusion protein FarA and the MtrE outer membrane protein channel (17). The MacB transporter was recently described (33) and it belongs to the ATP binding cassette (ABC) transporter family (13). It is poorly expressed in wild type gonococci due to a natural mutation in its promoter, but can recognize and export certain macrolide antibiotics. Finally,
*N. gonorrhoeae* contains the NorM efflux pump (34), which is a member of the multidrug and toxic compound extrusion (MATE) family of efflux transporters (5, 25, 34). The *N. gonorrhoeae* NorM transporter is homologous to the NorM efflux pump of *Vibrio parahaemolyticus* (25), the YdhE protein of *Escherichia coli* (2, 25, 43), VmrA of *V. parahaemolyticus* (6), and *Vibrio cholerae* VcrM (1, 27). As a multidrug efflux pump, the gonococcal NorM appears to recognize a number of cationic toxic compounds, such as ethidium bromide, acriflavin, 2-N-methylellipticinium, and ciprofloxacin (34). The capacity of NorM to export ciprofloxacin was suggested to be of clinical relevance in the development of fluoroquinolone resistance in *N. gonorrhoeae* especially when isolates have other mutations that raise the minimal inhibitory concentration (MIC) to a level near that seen in treatment failures.

The MATE family transporters characteristically possess 12 putative transmembrane domains, and have been reported in all three kingdoms of life (Eukarya, Archaea, and Eubacteria) (5). Phylogenetic tree analysis suggests that this family possesses three distinct clusters: (i) the bacterial multidrug efflux pumps, including *N. gonorrhoeae* NorM and *E. coli* YdhE; (ii) the eukaryotic efflux proteins found in fungi and plants, such as Erc1; and (iii) a branch containing *E. coli* DinF (5). Among these three clusters, the putative NorM branch exhibits overall minimal identity and similarity of 26 and 47%.

In order to directly test the capacity of the *N. gonorrhoeae* NorM pump to recognize putative substrates, including ciprofloxacin that was, until recently, widely used in the U.S. to treat gonococcal infections (15), we expressed the gonococcal *norM* gene in *E. coli* and investigated its function using drug susceptibility and transport assays. We also studied the homologous protein YdhE in *E. coli*, and compared its function with that of *N. gonorrhoeae*
NorM. Using a sensitive fluorescence polarization assay (12, 18), we characterized the interactions of these two transporters with various drugs. The results revealed that NorM and YdhE bind a variety of structurally dissimilar agents in the micromolar range.

Materials and Methods

Cloning of norM and ydhE

The norM ORF from genomic DNA of N. gonorrhoeae strain FA19 was amplified by PCR using the primers 5’-GAGGAATAATAATGCTGCTCGACCTCGACCCGC-3’ and 5’-GTTAAAAGCTCAATGGTGATGGTGATGATGGCGGGATGCTCGTTGCAGAATGACGGCGCTCGAATACGCGC-3’ to generate a product that would encode a NorM recombinant protein with a 6xHis tag at the C-terminus (34). The corresponding PCR product was extracted from the agarose gel and cloned into pBAD-TOPO as described by the manufacturer (Invitrogen). To remove the V5 epitope and polyhistidine region of the vector, the plasmid was digested with PmeI and then self-ligated to form the expression vector, pBADΩnorM. The recombinant plasmid was transformed into DH5α cells and transformants were selected on LB agar plates containing 100 μg/ml ampicillin. The presence of the correct norM sequence in the plasmid construct was verified by DNA sequencing.

Similarly, the ORF of ydhE from E. coli K12 genomic DNA was amplified by PCR and TA-cloned into pBAD-TOPO using the primers 5’-
GAGGAATAATAATGCGAGAATATCAGTG-3’ and 5’-
GTTAAAAGCTCAATGGTGATGGTGATGATGGCAGAATGCGGCGCTCGGACGGCGCTCGAATACGCGC-3’ to
generate a PCR product that would encode a recombinant protein containing a 6xHis tag at the C-terminus. The recombinant plasmid (pBADΩydhE) was transformed into DH5α cells as described above, and the presence of the correct insert in a representative plasmid transformant was verified by DNA sequencing.

**Drug susceptibility assays**

The MICs to various antimicrobial agents of *E. coli* strains (inoculum, 500 cells/ml) harboring pBADΩnorM, pBADΩydhE, or the pBAD vector were determined by the twofold dilution method using LB agar containing the desired antimicrobial agent (28). Bacterial growth was recorded after 18 to 24 h of incubation at 37°C. Each assay was repeated at least four times to ensure the reproducibility of the results.

**Drug accumulation assays**

Assays of norfloxacin and ethidium bromide accumulations were performed as described previously (20, 24, 25). In brief, *E. coli* AG100AX carrying pBADΩnorM, pBADΩydhE, or pBAD were grown in LB broth with 0.01% L-arabinose at 37°C to OD₆₀₀ of 1.0, harvested, washed with buffer containing 0.1 M Tris-HCl (pH 7.0) three times, and suspended in the same buffer to OD₆₀₀ of 1.0. Norfloxacin was then added to a final concentration of 100 μM. 1 ml samples were taken at intervals, centrifuged at 10,000 rpm for 30 s at 4°C, and washed once with the same buffer. After 15 min, carbonyl cyanide *m*-
chlorophenylhydrazone (CCCP) was added to a final concentration of 100 \( \mu \)M to disrupt the proton gradient across the membrane. Then, 15 min later, CCCP was removed by centrifugation at 10,000 rpm for 30 s, and cells were resuspended with buffer containing 0.1 M Tris-HCl (pH 7.0) and 0.4% glucose. Each pellet was resuspended in 1 ml of 100 mM glycine-HCl (pH 3.0), shaken vigorously for 1 h at room temperature to release the fluorescent content, and then centrifuged at 15,000 rpm for 10 min at room temperature. The fluorescence of supernatants was measured at \( \lambda_{ex} \) and \( \lambda_{em} \) of 277 and 448 nm, respectively, using a PerkinElmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The amount of maximum fluorescence was normalized to 100%.

For ethidium bromide accumulation, cells were prepared similarly as above. A final concentration of 20 \( \mu \)g/ml of ethidium bromide was added to the cell suspension to initiate the assay. 1 ml samples were taken at different time points. After 15 min of incubation, CCCP was added to a final concentration of 100 \( \mu \)M. Then, 15 min later, CCCP was removed by centrifugation at 10,000 rpm for 30 s, and cells were resuspended with buffer containing 0.1 M Tris-HCl (pH 7.0) and 0.4% glucose. The fluorescence of samples was measured at \( \lambda_{ex} \) and \( \lambda_{em} \) of 500 and 580 nm, respectively (3). The amount of maximum fluorescence was normalized to 100%.

**Accumulation of ethidium bromide in the presence of Na\(^+\)**

*E. coli* AG100AX containing pBAD\(\Omega\)norM or pBAD were grown in LB broth with 0.01% L-arabinose at 37°C to \( \text{OD}_{600} \) of 1.0, harvested by centrifugation, washed three times
with buffer containing 0.1 M Tris-HCl (pH 7.0), and suspended in the same buffer to OD$_{600}$ of 1.0. Ethidium bromide was added to a final concentration of 20 µg/ml to the cell suspension, followed by the addition of 100 mM NaCl or KCl. At each time point, a 1 ml sample was removed, centrifuged at 10,000 rpm for 30 s at 4°C, and washed once with the same buffer. The fluorescence signal was measured at λ$_{ex}$ and λ$_{em}$ of 500 and 580 nm, respectively.

**Efflux of norfloxacin in the presence of Na$^+$**

For determining norfloxacin efflux, cells were grown in LB broth with 0.01% L-arabinose at 37°C to OD$_{600}$ of 1.0, harvested, and washed twice with buffer containing 0.1 M Tris-HCl (pH 7.0). To load cells with norfloxacin, bacteria were incubated in the same buffer supplemented with 100 µM norfloxacin and 20 µM CCCP at 37°C for 30 min as described previously (31). Cells were then pelleted, washed twice, and resuspended with the same buffer to OD$_{600}$ of 2.0. 1 ml samples were taken at intervals, centrifuged at 10,000 rpm for 30 s at 4°C, and washed once with the same buffer. After 10 min, NaCl or KCl was added to a final concentration of 100 µM. Fluorescence measurement of norfloxacin was performed by a similar procedure as described above.

**Purification of the transporters**
The *N. gonorrhoeae* NorM protein containing a 6xHis tag at the C-terminus was overproduced in *E. coli* TOP10 cells (Invitrogen) possessing pBADΩnorM. Cells were grown in 12 L of LB medium with 100 µg/ml ampicillin at 37°C. When the OD₆₀₀ reached 0.5, the culture was treated with 0.2% L-arabinose to induce norM expression and harvested within 3 h. The collected bacteria were resuspended as described (44) in low salt buffer containing 100 mM sodium phosphate (pH 7.2), 10 % glycerol, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF), and then disrupted with a French pressure cell. The membrane fraction was collected and washed twice with high salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10 % glycerol, 1 mM EDTA and 1 mM phenylmethanesulfonyl fluoride (PMSF), and once with 20 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM PMSF. The membrane protein was then solubilized in 1 % (w/v) *n*-dodecyl-β-D-maltoside (DDM). Insoluble material was removed by ultracentrifugation at 370,000 x g. The extracted protein was loaded into a Ni²⁺-affinity column, washed with buffer containing 20 mM HEPES-NaOH (pH 7.5), 50 mM imidazole and 0.02% DDM, and eluted with buffer consisting of 20 mM HEPES-NaOH (pH 7.5), 400 mM imidazole and 0.02% DDM. The eluted protein was then concentrated to 5 mg/ml and loaded into a G-200 sizing columns pre-incubated with 20 mM HEPES-NaOH (pH 7.5) and 0.02% DDM for further purification. The YdhE protein that contains a 6xHis tag at the C-terminus was overproduced in *E. coli* TOP10 pBADΩydhE and purified as described above for NorM. The purity (>90%) of the NorM and YdhE proteins were judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue.
Fluorescence polarization assays

Fluorescence polarization assays (12, 18) were used to determine the drug binding affinities of NorM and YdhE, respectively. The experiments were done using a ligand binding solution containing 20 mM HEPES-NaOH (pH 7.5), 0.02% DDM, and 1 μM ligand (rhodamine 6G, ethidium, proflavin, ciprofloxacin, or norfloxacin). The protein solution consisting of NorM or YdhE in 20 mM HEPES-NaOH (pH 7.5), 0.02% DDM, and 1 μM ligand was titrated into the ligand binding solution until the polarization (P) was unchanged. As this is a steady-state approach, fluorescence polarization measurement was taken after a 5 min incubation for each corresponding concentration of the protein and drug to ensure that the binding has reached equilibrium. It should be noted that the detergent concentration was kept constant at all times to eliminate the change in polarization generated by drug-DDM micelle interaction. All measurements were performed at 25°C using a PerkinElmer LS55 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier. The excitation wavelengths were 527, 483, 447, 330, and 277 nm, respectively, for rhodamine 6G, ethidium, proflavin, ciprofloxacin, and norfloxacin. Fluorescence polarization signals (in ΔP) were measured at emission wavelengths of 550, 620, 508, 415, and 448 nm, respectively, for these ligands. Each titration point recorded was an average of 15 measurements. Data were analyzed using the equation, \( P = \left\{ \left( P_{\text{bound}} - P_{\text{free}} \right)[\text{protein}] / \left( K_D + [\text{protein}] \right) \right\} + P_{\text{free}} \), where P is the polarization measured at a given total protein concentration, \( P_{\text{free}} \) is the initial polarization of free ligand, \( P_{\text{bound}} \) is the maximum polarization of specifically bound ligand, and [protein] is the protein concentration. The titration experiments were repeated for three times to obtain the average \( K_D \) value. Curve fitting was accomplished using the program ORIGIN (30).
Results and Discussion

Drug specificity of NorM and YdhE in vivo

To determine if the *N. gonorrhoeae* NorM multidrug transporter is functionally expressed in *E. coli*, and to compare its activity to a second member (YdhE) of the MATE family of efflux pumps, we transformed the *E. coli* AG100AX, an AcrAB-deficient strain (29) with the empty pBAD vector, pBAD\(\omega\)norM, or pBAD\(\omega\)ydhE. We then tested the susceptibility of the representative transformants bearing these plasmids to a panel of 24 structurally divergent antimicrobials (Table 1). These antimicrobials were selected based on whether or not they are putative substrates of MATE family exporters in the NorM subclass. It is important to stress that in many instances expression of drug efflux pumps, including members of the MATE superfamily (5), can have only modest changes (two-fold) in bacterial susceptibility to certain antimicrobials while more significant changes in susceptibility to other agents can be observed in the same system. Indeed, in our drug testing experiments we detected a range of changes in the susceptibility of *E. coli* cells producing either NorM or YdhE.

Of the 24 antimicrobials tested, the AG100AX cells expressing the *N. gonorrhoeae* NorM were less sensitive (two- to eight-fold) than AG100AX cells containing pBAD to a subset of antibiotics (norfloxacin, doxurubicin, novobiocin, lomefloxacin, enoxacin and ciprofloxacin), dyes (rhodamine 6G, ethidium bromide, acriflavine and crystal violet) and quaternary ammonium compounds (berberine, benzalkonium and tetraphenylphosphonium chloride). The YdhE-producing cells displayed a similar drug susceptibility profile (with the
exception of lomefloxacin) as that of the NorM-producing *E. coli* cells, suggesting that these two MATE transporters are functionally similar *in vivo*.

Like YdhE of *E. coli*, our drug susceptibility experiments showed that NorM functions as a multidrug efflux pump when expressed in *E. coli*. The MICs to 14 of the 24 antimicrobials (Table 1) were observed to increase by two- to eight-fold in NorM-producing AG100AX bacteria, which lacks the AcrB efflux pump. Similar differences (data not presented) in antimicrobial susceptibility were obtained in AcrB⁺ *E. coli* TOP10 cells that were used to obtain recombinant NorM. In that the MICs of norfloxacin, ciprofloxacin, enoxacin, lomefloxacin were raised in cells expressing NorM or YdhE, our results suggest that like other MATE transporters, such as BexA of *B. thetaiotaomicron* (23), VcmA of *Vibrio cholerae* (16), NorM of *Vibrio cholerae* (38) and AbeM of *Acinetobacter baumannii* (41), fluoroquinolones might be a common substrate for MATE transporters in the NorM cluster.

**NorM and YdhE reduce drug accumulation in bacteria**

The observed (Table 1) capacity of NorM and YdhE to reduce the susceptibility of *E. coli* to norfloxacin, ciprofloxacin and ethidium bromide is consistent with the drug recognition profile of other members of the MATE efflux transporter family. To confirm the drug susceptibility testing results, we measured the accumulation of norfloxacin and ethidium bromide in AG100AX cells that carried pBADΩ*norM*, pBADΩ*ydhE*, or pBAD. The results showed lower levels of norfloxacin (Figure 1a) or ethidum bromide (Figure 1b) accumulation
in AG100AX cells producing NorM or YdhE compared to control cells harboring the empty pBAD vector.

Members of the MATE family use energy from the proton motive force (PMF) during export and loss of the PMF inactivates pump activity resulting in enhanced accumulation of substrates, which translates to increased susceptibility to the agents normally recognized by the transporter. Accordingly, we also measured norfloxacin and ethidium bromide accumulation after addition of CCCP, a de-coupler of the membrane proton gradient. After the addition of CCCP into the assay solution, the accumulation of norfloxacin (Figure 1a) and ethidium bromide (Figure 1b) increased drastically in the NorM- and YdhE- expressing cells, with the levels of accumulation being nearly the same in the three different strains within 5 min. It should be noted that the effect of CCCP is reversible simply by removing CCCP and adding glucose to re-energize AG100AX cells. The experiments were performed at least three times. Statistical comparisons were assessed by Student’s t test, and P values of < 0.05 were considered statistically significant differences (Figure 1a and b).

NorM expressed in E. coli behaves as a Na⁺ ion-dependent transporter

It has been suggested but not proven that NorM of N. gonorrhoeae is a Na⁺-drug antiporter (34). Thus, we investigated the accumulation of ethidium bromide in isogenic strains AG100AX/pBADΔnorM and AG100AX/pBAD in the presence of Na⁺ or K⁺. In AG100AX/pBADΔnorM cells, a lower level of ethidium bromide accumulation was observed in the presence of 100 mM NaCl when compared to that in the presence of 100 mM
KCl (Figure 2a). In contrast, we did not observe a difference in ethidium bromide accumulation in AG100AX/pBAD regardless of the presence of NaCl or KCl (Figure 2b). Furthermore, in the presence of NaCl the accumulation level of ethidium bromide in the NorM-producing strain was lower than that in AG100AX/pBAD.

We next measured the efflux of norfloxacin that had accumulated in strains AG100AX/pBADΔnorM or AG100AX/pBAD, respectively, in the presence of Na\(^+\) or K\(^+\). Cells were first loaded with norfloxacin, thereafter, 100 mM of either NaCl or KCl was added to test the effect on drug efflux. As shown in Figure 3a, the addition of 100 mM NaCl to AG100AX/pBADΔnorM cells resulted in greater efflux of the drug from cells compared to that observed in the presence of KCl or without added salt. This effect was NorM-dependent because the addition of either NaCl (100 mM) or KCl (100 mM) to AG100AX/pBAD cells did not impact norfloxacin efflux (Figure 3b). Taken together with the ethidium bromide accumulation results, our observations support the earlier proposal (34) that the gonococcal NorM protein is a Na\(^+\)-dependent transporter.

In order to directly test whether efflux of an antimicrobial by NorM is Na\(^+\)-dependent, we then studied the extrusion of norfloxacin that has been accumulated in AG100AX/pBADΔnorM in the presence of CCCP. Cells were first loaded with norfloxacin. Thereafter, different concentrations of NaCl (50-200 mM) were added to test the effect on drug efflux. All these tests were done in the presence of 20 μM CCCP. If NorM requires proton ion as a coupler, then the accumulated level of norfloxacin in cell should not be reduced under these conditions. However, if the coupling ion is Na\(^+\), we should be able to observe norfloxacin efflux in a [Na\(^+\)]-dependent manner. Figure 4 depicts the effect of Na\(^+\)
on the extrusion of norfloxacin. The experiment suggests that efflux activity was stimulated by Na\(^+\) ion. The addition of NaCl resulted in a significant change in norfloxacin efflux.

Previous study in *Pseudomonas aeruginosa* PmpM, an H\(^+\)-dependent transporter in the MATE family, indicated that Na\(^+\) ion has no effect on drug transport (11). Thus, it is highly likely that Na\(^+\) is the coupling cation for NorM.

Our drug accumulation and efflux assays confirm the earlier report which suggested that NorM of *N. gonorrhoeae* is a Na\(^+\)/drug antiporter (34). First, Na\(^+\) was found to enhance the ability of *E. coli* producing NorM to maintain a lower accumulation level of ethidium bromide as compared to those cells not producing NorM, even in the presence of Na\(^+\) (Figure 2). Hence, drug export mediated by NorM of *N. gonorrhoeae* is driven by Na\(^+\) gradient. This export mechanism is also influenced by the integrity of the PMF of the cytoplasmic membrane since CCCP, a proton conductor, could significantly enhance the accumulation level of ethidium bromide in NorM-producing bacteria. These accumulation assays provide, however, indirect evidence for efflux. Accordingly, direct support for efflux was obtained in experiments that measured norfloxacin export by NorM-producing *E. coli*. The observed Na\(^+\)-dependent capacity of NorM to mediate export of norfloxacin (Figures 3 and 4) provides direct evidence for this model.

**Binding affinities of different drugs *in vitro***

The *in vivo* antimicrobial susceptibility testing (Table 1) and accumulation/efflux studies (Figures 1-4) suggested that NorM and YdhE recognize and export structurally
diverse compounds. There is, however, little known about the detail of substrate binding by these MATE transporters. In order to directly test the capacity of these proteins to bind such diverse compounds, we used a fluorescence polarization assay to quantify the binding affinities of several compounds to NorM and YdhE \textit{in vitro}. This technique has been found to be sensitive and precise enough, and allow us for the first time, to detect ligand binding of the \textit{E. coli} AcrB multidrug efflux pump (an RND transporter) in a detergent environment (40). As an example of the results obtained by this protocol, Figure 5a illustrates the binding isotherm of NorM in the presence of rhodamine 6G. As presented in the figure, a simple hyperbolic curve was observed for the binding of rhodamine 6G with a dissociation constant, $K_D$, of 3.4 ± 0.2 \(\mu\)M. A Hill plot of the data yielded a Hill coefficient of 1.01 ± 0.03 (Figure 5b), suggesting a simple drug binding process with a stoichiometry of one NorM monomer per rhodamine 6G ligand. When the data were presented as a Scatchard plot (not shown), it indicated a similar $K_D$ of 3.6 ± 0.1 \(\mu\)M with no sign of heterogeneity in binding sites. In addition, the titration experiments indicated that YdhE binds rhodamine 6G with a $K_D$ of 3.0 ± 0.2 \(\mu\)M (Figure 6a). This value is comparable with that of NorM, suggesting that these two transporters share similar affinity for this ligand. The Hill (Figure 6b) and Scatchard (not shown) plots of the data indicated that the transporter employs a simple binding stoichiometry of 1:1 monomeric YdhE-to-rhodamine 6G molar ratio.

Fluorescence polarization assays were also employed to elucidate the interaction of a variety of drugs with the NorM and YdhE transporters. The titration experiments indicated that NorM binds ethidium bromide, proflavin, ciprofloxacin, and norfloxacin with dissociation constants of $12.3 \pm 1.3 \muM$, $33.6 \pm 1.9 \muM$, $121.3 \pm 15.7 \muM$, and $105.6 \pm 9.8$
µM, respectively (Table 2). These values are in the same range with those of YdhE, which binds ethidium bromide, proflavin, ciprofloxacin and norfloxacin with K\textsubscript{D} values of 9.8 ± 0.9 µM, 22.1 ± 0.9 µM, 90.9 ± 12.4 µM, and 98.4 ± 16.2 µM, respectively (Table 2). This binding was not impacted by detergent since similar dissociation constants for the rhodamine 6G-YdhE complex were obtained in reaction mixtures that included 0.2%, 0.02% and 0.0075% DDM.

To verify the validity of the fluorescence polarization assay, we studied the binding affinity of rhodamine 6G with the purified, detergent-solubilized YdhE efflux pump using equilibrium dialysis. The dialysis data suggest a K\textsubscript{D} of 3.0 ± 0.1 µM, with a stoichiometry of 1:1 monomeric YdhE-to-ligand molar ratio, for the binding of YdhE with rhodamine 6G (see Suppl. Fig. S1). This result is in good agreement with that observed from fluorescence polarization in which the K\textsubscript{D} of YdhE-rhodamine 6G is 3.0 µM with a 1:1 binding stoichiometry. Apparently, NorM and YdhE bind these drugs with similar affinities. These values are similar to the K\textsubscript{D} values for tetrphenylphosphonium binding in MdfA, a MF transporter, and EmrE, a SMR transporter, determined by radiolabeled binding assays (19, 26), as well as the most recently determined dissociation constants for AcrB, an RND transporter, with four different ligands using fluorescence polarization assays (40).

The results from fluorescence polarization and equilibrium dialysis assays suggest that NorM and YdhE use a binding stoichiometry of 1:1 monomeric transporter-to-ligand ratio. Thus, it is very likely that these transporters employ a simple drug binding process with no cooperativity. There is, however, a possibility that drug molecules may bind to more than one site in the proteins, with similar affinities, that may not be able to be distinguished...
using these techniques. Further experiments utilizing different approaches may need to confirm this drug binding stoichiometry.

**pH dependence of drug binding to NorM**

We performed fluorescence polarization experiments to determine the binding of rhodamine 6G to NorM at different pH values. Figure 7 illustrates the decrease in $K_D$ values for rhodamine 6G as the pH increases from 5.8 to 8.5. Within this pH range, the $K_D$ value decreases by about 2.6 $\mu$M. This change is quite modest when compared with the effect of proton on the PMF-dependent efflux pumps, *E. coli* AcrB (40) and EmrE (26). The binding affinities for drugs in these two transporters were strikingly dependent on pH. In the case of AcrB, within the range of pH from 5.5 to 8.4, fluorescence polarization assay suggests that the change in $K_D$ for AcrB-rhodamine 6G reached 10.3 $\mu$M. As NorM shows a very different pH-dependent profile compared with those of the PMF-dependent pumps AcrB and EmrE, it is very likely that NorM does not use H$^+$ as an energy-coupling ion. Since NorM tends to bind positively charged drugs, we expect that its drug-binding pocket should consist of at least one acidic residue. Indeed, the protein sequence of NorM suggests that this transporter consists of two negatively charged residues, Glu 261 and Asp 377, in the transmembrane region. These acidic residues potentially could form the multidrug binding site. Thus, the modest pH effect on NorM could be accounted for the protonation state of the acid residue(s) in the binding pocket.
**Na\(^+\) dependence of drug binding to NorM**

As *in vivo* studies of drug accumulation and efflux suggest, NorM is a Na\(^+\)-dependent transporter, the presence of Na\(^+\) ions may affect the drug binding affinity *in vitro*. We therefore investigated the Na\(^+\) dependence of drug binding using fluorescence polarization. As shown in Figure 8, the $K_D$ for rhodamine 6G is Na\(^+\) dependent when the concentration of Na\(^+\) is in the sub-micromolar range. In this region, the dissociation constant for rhodamine 6G was a simple hyperbolic function of the Na\(^+\) concentration. The curve showed saturability over 0.01 to 100 mM. Like the pH effect, the change in $K_D$ for rhodamine 6G due to the presence of Na\(^+\) ion is quite small. Within 0 to 100 mM Na\(^+\), the change in $K_D$ only reached 2.5 \(\mu\)M (Figure 8). We also added various concentrations of NaCl ranging from 100 to 400 mM (data not shown) and observed no significant change in the $K_D$.

**Competition binding of different drugs in YdhE**

To confirm that the drug molecules bind specifically in these transporters, we performed competition experiments in which tetrathenylphosphonium chloride (TPP) was titrated into a solution containing the preformed YdhE-rhodamine 6G complex. In this case, TPP was chosen as a second ligand to knock off the bound rhodamine 6G from YdhE. The absorption spectra of TPP (from 200 to 600 nm) showed that this molecule absorbs light at the wavelengths of 224.9, 268.0 and 275.9 nm. At $\lambda = 527$ nm, which is the excitation wavelength for rhodamine 6G, the energy is too low to excite TPP. Thus, TPP was treated as a non-fluorescent ligand in the “knock off” experiments. The data revealed that TPP was
able to bind YdhE and replace the bound rhodamine 6G molecule from the protein as demonstrated by the release of rhodamine 6G observed from the reduction of polarization (Figure 9). This binding assay provides direct evidence that TPP interferes with the binding of rhodamine 6G, possibly by a direct competitive binding process for the same binding site. Alternatively, the release of rhodamine 6G by TPP may be due to an allosteric interaction between distinct binding sites of these two ligands. Regardless, the titrations demonstrate that rhodamine 6G is bound specifically in the YdhE transporter.

We have determined the binding affinities of five different drugs to the purified, detergent-solubilized NorM and YdhE proteins, respectively, using fluorescence polarization assays. We note that fluorescence polarization has been widely used for studying protein-DNA interaction (12, 14, 18), and protein-ligand interaction in transcriptional regulators (4, 35, 39). To our knowledge, this is the first attempt using this methodology to investigate interaction between MATE proteins and their transported substrates. This approach also allows us, for the first time, to quantify the strength of transporter-drug interaction among the MATE family of transporters.

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References


30. ORIGIN Ver. 7.5. OriginLab Corporation, Northampton, Massachusetts, USA.


### Table 1. Drug susceptibility.

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<th>AG100AX/pBADΩ*&lt;i&gt;norM&lt;/i&gt;</th>
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Table 2. Dissociation constants and Hill coefficients of NorM and YdhE with five different transported drugs.

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<th>YdhE</th>
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Figures and Captions

Figure 1. NorM and YdhE reduce accumulation of norfloxacin and ethidium bromide in *E. coli* cells. (a) Accumulation of norfloxacin in cells transformed by NorM (AG100AX/pBAD\_norM), YdhE (AG100AX/pBAD\_ydhE), or empty vector (AG100AX/pBAD). CCCP was added to the suspensions (first arrow) at a final concentration of 100 μM. After 15 min, glucose was added (second arrow) at a final concentration of 0.4%. (b) Accumulation of ethidium bromide in the same strains. CCCP was added to the suspensions (first arrow) at a final concentration of 100 μM. After 15 min, glucose was added (second arrow) at a final concentration of 0.4%. “*” indicates values of AG100AX/pBAD\_norM and AG100AX/pBAD\_ydhE cells that are significantly different from the control (AG100AX/pBAD) values ($P < 0.05$).
Figure 2. Sodium ion reduces accumulation of ethidium bromide in cells transformed by NorM. (a) Accumulation of ethidium bromide in AG100AX/pBADnorM after the addition of 100 mM NaCl or KCl. (b) Accumulation of ethidium bromide in AG100AX/pBAD (carrying the empty vector) after the addition of 100 mM NaCl or KCl. “*” indicates values of ethidium fluorescence intensity in the presence of 100 mM NaCl that are significantly different from those in the presence of 100 mM KCl ($P < 0.01$).
Figure 3. Sodium ion enhances norfloxacin efflux via NorM. (a) Norfloxacin efflux from AG100AX/pBADΔnorM cells carrying the norM gene from N. gonorrhoeae. (b) Norfloxacin efflux from AG100AX/pBAD cells carrying the empty vector. NaCl or KCl was added to the suspensions (arrow) at a final concentration of 100 mM. "*" indicates values of norfloxacin fluorescence in the presence of 100 mM NaCl that are significantly different from those in the presence of 100 mM KCl ($P < 0.05$).
Figure 4. Effect of Na\(^+\) concentration on norfloxacin extrusion via NorM. Norfloxacin efflux from AG100AX/pBADΔ\(\omega\)norM cells carrying the \(\text{norM}\) gene in the presence of 0 to 200 mM NaCl. “*” indicates values of norfloxacin fluorescence in the presence of 50, 100 or 200 mM NaCl that are significantly different from those without NaCl \((P < 0.003)\), as well as values of norfloxacin fluorescence in the presence of 100 or 200 mM NaCl that are significantly different from those in the presence of 50 mM NaCl \((P < 0.01)\).
Figure 5. Representative fluorescence polarization of NorM in 0.02% DDM with rhodamine 6G. (a) Binding isotherm of NorM with rhodamine 6G, showing a $K_D$ of $3.4 \pm 0.2 \mu M$, in buffer containing 20 mM Tris (pH 7.5) and 0.02% DDM. (b) Hill plot of the data obtained for rhodamine 6G binding to NorM. $\alpha$ corresponds to the fraction of bound rhodamine 6G. The plot gives a slope of $1.01 \pm 0.03$, indicating a simple binding process with no cooperativity. The interception of the plot provides a $K_D$ of $3.6 \pm 0.1 \mu M$ for the rhodamine 6G binding.
Figure 6. Representative fluorescence polarization of YdhE in 0.02% DDM with rhodamine 6G. (a) Binding isotherm of YdhE with rhodamine 6G, showing a $K_D$ of 3.0 ± 0.2 µM, in buffer containing 20 mM Tris (pH 7.5) and 0.02% DDM. (b) Hill plot of the data obtained for rhodamine 6G binding to YdhE. $\alpha$ corresponds to the fraction of bound rhodamine 6G. The plot gives a slope of 1.00 ± 0.04, indicating a simple binding process with no cooperativity. The interception of the plot provides a $K_D$ of 3.6 ± 0.2 µM for the rhodamine 6G binding.
Figure 7. Effect of pH on the $K_D$ of rhodamine 6G binding to NorM. The resulting $K_{DS}$ were plotted against pH.
Figure 8. Effect of Na\(^+\) concentration on the K\(_D\) of rhodamine 6G binding to NorM. The resulting K\(_D\)s were plotted against NaCl concentration.
Figure 9. YdhE binding competition experiment between rhodamine 6G and tetraphenylphosphonium chloride (TPP). YdhE (5 μM) was pre-incubated with rhodamine 6G (1 μM) for two hours before titration. The change in fluorescence polarization signals (ΔFP) of rhodamine 6G was measured at an emission wavelength of 550 nm. TPP was non-fluorescent in the experimental conditions. The decrease in ΔFP showed that the bound rhodamine 6G was knocked off by TPP.
Figure S1. Scatchard plot of equilibrium dialysis data for YdhE binding to rhodamine 6G. The YdhE-rhodamine 6G dialysis experiments were carried out with 2-5 µM monomeric YdhE dialyzed against 3-5 µM rhodamine 6G. The slope of the plot provides a $K_D$ of $3.0 \pm 0.1$, and the interception ($1.0 \pm 0.2$) gives a stoichiometry of 1:1 monomeric YdhE-to-R6G molar ratio.
CHAPTER 3. Crystal structures of the CusA heavy-metal efflux pump suggest a methionine-mediated transport mechanism

A manuscript submitted to *Nature*

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Gram-negative bacteria, such as *Escherichia coli*, frequently utilize tripartite efflux complexes in the resistance-nodulation-division (RND) family to expel diverse toxic compounds from the cell.\textsuperscript{1,2} One such efflux system CusCBA is responsible for extruding biocidal Cu(I) and Ag(I) ions.\textsuperscript{3,4} As no structural information is available for the heavy-metal efflux (HME) subfamily of the RND efflux pumps, we here describe the crystal structures of the inner membrane transporter CusA in the absence and presence of bound Cu(I) or Ag(I). The CusA structures reported here provide important new structural information about the HME subfamily of RND efflux pumps. The structures suggest that the metal binding sites, formed by a three-methionine cluster, are located within the cleft region of the periplasmic domain. Intriguingly, this cleft is closed in the apo-CusA form but open in the CusA-Cu(I) and CusA-Ag(I) structures, which directly suggests a plausible pathway for ion export. The binding of Cu(I) and Ag(I) triggers significant conformational changes in both the periplasmic and transmembrane domains. The crystal structure indicates that CusA has, in addition to the three-methionine metal binding site, five methionine pairs - three located in the transmembrane region and two in the periplasmic domain. Genetic analysis and transport assays suggest that CusA is capable of actively picking up metal ions from the cytosol, utilizing five of these methionine pairs/clusters to bind and export metal ions. We propose a stepwise shuttle mechanism for transport between these sites.

Silver is a heavy metal with a relatively high toxicity in prokaryotes. Ionic silver exhibits antimicrobial activity against a broad range of microorganisms, and has long been used as an effective broad antimicrobial agent against pathogens.\textsuperscript{5,6} Copper, although
required in trace amounts for bacterial growth, is highly toxic even at low concentrations.\textsuperscript{7} Thus, both silver and copper are well-known bactericides, and their biocidal effects have been used for centuries. In Gram-negative bacteria, efflux systems of the resistance-nodulation-division (RND) superfamily play major roles in the intrinsic and acquired tolerance of antibiotics and toxic compounds, including silver and copper ions.\textsuperscript{1,2} The Gram-negative bacterium \textit{Escherichia coli} harbors seven different RND efflux transporters. These transporters can be categorized into two distinct sub-families, the hydrophobic and amphiphilic efflux RND (HAE-RND) and heavy-metal efflux RND (HME-RND) families.\textsuperscript{1,2} Six of these transporters, such as AcrB, AcrD, AcrF, MdtB, MdtC and YhiV, are multidrug efflux pumps, which belong to the HAE-RND protein family.\textsuperscript{1} In addition to these multidrug efflux pumps, \textit{E. coli} contains one HME-RND efflux transporter, CusA, which specifically recognizes and confers resistance to Ag(I) and Cu(I) ions.\textsuperscript{3,4} The two sub-families of these RND transporters share relatively low protein sequence homology. For example, sequence alignment shows that CusA and AcrB have only 19\% identity.

As a RND transporter, CusA works in conjunction with a periplasmic component, belonging to the membrane fusion protein family, and an outer membrane channel to form a functional protein complex. CusA is a large proton motive force-dependent inner membrane efflux pump comprised of 1,047 amino acids.\textsuperscript{3,4} CusC, however, is a 457 amino acid protein that forms an outer membrane channel.\textsuperscript{3,4} The membrane fusion protein CusB (379 amino acids) contacts both the inner membrane CusA and outer membrane CusC proteins.\textsuperscript{3,4} Presumably, the three components of this HME-RND system form a tripartite efflux complex CusCBA, which spans both the inner and outer membranes of \textit{E. coli} to export Ag(I) and
Cu(I) directly out of the cell. Heavy-metal efflux by the CusCBA complex is driven by proton import, and this process is catalyzed through the inner membrane transporter CusA.

At present, only two crystal structures of RND-type efflux pumps are available. These efflux pumps, belonging to the HAE-RND subfamily, are the *E. coli* AcrB\(^{8-12}\) and *Pseudomonas aeruginosa* MexB\(^{13}\) multidrug transporters. Their structures suggest that both AcrB and MexB span the entire width of the inner membrane and protrude approximately 70 Å into the periplasm. Along with the models of these two HAE-RND transporters, the crystal structures of the other components of these tripartite complex systems have also been determined. These include the outer membrane channels, *E. coli* TolC\(^{14}\) and *P. aeruginosa* OprM,\(^{15}\) as well as the periplasmic membrane fusion proteins, *E. coli* AcrA\(^{16}\) and *P. aeruginosa* MexA.\(^{17-19}\)

Currently, no structural information has yet been available for any HME-RND type efflux pump. Several attempts at crystallizing the CusA efflux pump have not succeeded, probably because of the flexible nature of this pump.\(^{20,21}\) Presumably, the three components of this HME-RND system form a tripartite complex that resembles the AcrAB-TolC complex. Different from the HAE-RND family, members of the HME-RND family are highly substrate-specific, with the ability to differentiate between monovalent and divalent ions. To elucidate the mechanism used by the CusCBA efflux system for Cu(I)/Ag(I) recognition and extrusion, we have previously reported the crystal structure of the full-length CusB membrane fusion protein.\(^{22}\) Now we describe the crystal structure of the HME-RND transporter CusA, both in the absence and presence of Cu(I) or Ag(I). The structures
strongly suggest that CusA relies upon methionine residues to bind and export the metal ions, as has been proposed.

We cloned, expressed and purified the full-length CusA protein containing a 6xHis tag at the N-terminus. We obtained crystals of the *E. coli* CusA heavy-metal efflux pump following an extensive screening for crystallization conditions with different detergents. We then used multiple-isomorphous replacement with anomalous scattering (MIRAS) to determine the three-dimensional structure. The diffraction data can be indexed to the space group *R*32. Data collection and refinement statistics are summarized in Tables S1 and S2. The resulting experimental electron density maps (Fig. S1) reveal that the asymmetric unit consists of one protomer. The native crystal structure of CusA has been determined to a resolution of 3.52 Å (Table S1). Currently, 97.9% of the residues (residues 5-504 and 516-1040) are included in our final model. The final structure is refined to *R*<sub>work</sub> and *R*<sub>free</sub> of 23.7% and 27.9%, respectively.

Overall, the crystal structure of CusA is quite distinct from those of the HAE-RND pumps, AcrB and MexB. Superimposition of the structure of CusA with the structure of AcrB (pdb code:1IWG)<sup>8</sup> results in a high RMSD of 11.4 Å for 1,003 C<sub>α</sub> atoms, suggesting highly significant differences between these two transporters. CusA exists as a homotrimer, and each subunit of CusA consists of 12 transmembrane helices (TM1-TM12) and a large periplasmic domain formed by two periplasmic loops between TM1 and TM2, and TM7 and TM8, respectively (Fig. 1 and Fig. S2). In the transmembrane region, the relative locations of TM1-TM6 are related to those of TM7-TM12 by pseudo-twofold symmetry. These TM helices are arranged in such a way that TM4 and TM10 form the center of the core and are
surrounded by the other TM helices. Differently from AcrB and MexB, four helices, TM4, TM5, TM10 and TM11, extend into the cytoplasm, forming the cytoplasmic domain of the pump. Two other helices, TM2 and TM8, protrude into the periplasm and contribute part of the periplasmic domain. It is important to note that TM2, TM4 and TM5 of the N-terminal half correspond to TM8, TM10 and TM11 of the C-terminal half, respectively, in the pseudo-twofold symmetry.

Like AcrB and MexB, the periplasmic domain of CusA can be divided into six subdomains, PN1, PN2, PC1, PC2, DN and DC (Fig. 1). Sub-domains PN1, PN2, PC1 and PC2 form the pore domain, with PN1 making up the central pore and stabilizing the trimeric organization. Sub-domains DN and DC, however, contribute to form the docking domain, presumed to be interacting with the outer membrane channel CusC. The trimeric CusA structure suggests that sub-domains PN2, PC1 and PC2 are located at the outermost core of the periplasmic domain, facing the periplasm. In AcrB, sub-domains PC1 and PC2 form a large external cleft and are presumed to create the entrance for drugs from the periplasm. However, the apo-CusA structure shows that the gap between PC1 and PC2 is completely closed (Fig. 1b). In vitro cross-linking coupled with mass spectrometry suggested that sub-domain PN2 of CusA should interact with the N-terminus of the CusB membrane fusion protein.\textsuperscript{22} This result agrees with the cross-linking experiment where the N and C-termini of AcrA was found to contact PN2 and PC1 of AcrB.\textsuperscript{19} Thus, sub-domains PN2 and PC1 of CusA most likely form the binding surface for the membrane fusion protein CusB.

Perhaps the most interesting secondary structural feature appears in the cleft region of the periplasmic domain. Residues located on the left side of the wall (Fig. 2), formed by one
α-helix (residues 690-706) and three β-sheets (residues 681-687, 711-716 and 821-827), appear to tilt into the cleft to close the opening. Surprisingly, residues 665-675, located at the bottom of the cleft, form an α-helix. This structural feature, not found in AcrB and MexB, likely governs the specificity of the CusA pump. The α-helix orients horizontally and roughly divides the transmembrane and periplasmic domains into two compartments.

Located just above this horizontal helix, we find three proximal methionine residues, M573, M623 and M672, presumably creating a three-methionine specific binding site for Cu(I) and Ag(I) ions.\textsuperscript{23,24} Notably M672 is also one of the residues within the horizontal helix. Site-directed mutagenesis had suggested that these three methionine residues are essential for mediating copper resistance.\textsuperscript{4} Indeed, a strong peak at the copper edge was observed at the center of these three methionines in our CusA-Cu(I) crystal derivative, indicating the binding of a Cu(I) ion in a three-sulfur binding site (see below). The location of this Cu(I) binding site is coincident with the multidrug binding site in AcrB at the periplasmic cleft.\textsuperscript{25,26}

Intriguingly, the overall structure of the CusA-Cu(I) complex is quite distinct from that of apo-CusA (Fig. 2). Superimposition of these two structures gives an overall RMSD of 3.9 Å (for 1,006 C\textsuperscript{α} atoms). As mentioned above, the bound Cu(I) ion was found to coordinate residues M573, M623 and M672. These three methionines specifically form a typical three-methionine coordination site for binding Cu(I)/Ag(I).\textsuperscript{23,24} The signal for this bound copper is strong with a large peak height of 44 σ. Binding of Cu(I) initiates significant conformational changes in the periplasmic as well as transmembrane domains of CusA. Perhaps, the most noticeable difference between the apo and ion-bound structures appears in the PC2 region (Fig. 2 and Fig. S3). Cu(I) binding leads to a 30° swing of the
entire PC2 sub-domain. This motion shifts PC2 away from the PC1 sub-domain. The hinge for this rotational movement appears to be at the junction between sub-domains PC2 and DC with residues G721 and P810 forming the hinge. As a consequence, the gap between PC1 and PC2 appears to open up after binding this metal ion. This gap presumably creates an entrance for metal ions from the periplasmic space. The horizontal helix, residues 665-675, located inside the cleft also makes a substantial movement. The C-terminal end of this helix is found to tilt upward by 21° in the Cu(I)-bound structure with respect to the apo form (Fig. 2c). This tilting motion allows M672 to move closer to M573 and M623 to complete the three-methionine coordination site. Coupled with this movement, TM8 also shifts in position to a more vertical orientation while retaining its α-helical structure. Overall, the N-terminal end of TM8 is found to shift away from the core by 10 Å after Cu(I) binding.

For the CusA-Ag(I) complex, an anomalous difference Fourier peak (38 σ) was found at the center of residues M573, M623 and M672, indicating that the bound Ag⁺ ion is coordinated by these three methionines (Fig. 2d). The overall conformational changes triggered by Cu(I) and by Ag(I) binding are nearly identical. Superimposition of the CusA-Cu(I) and CusA-Ag(I) structures gives an overall RMSD of 1.0 Å (for 1,021 Cαs). Based on the crystal structures, the horizontal helix in the cleft directly interacts with the N-terminal end of TM8. The movement of TM8 may relate directly to transmembrane signaling and could initiate the translocation of a proton across the membrane. Indeed, in the AcrB pump, there is evidence that proton translocation is coupled to the conformational change in TM8.¹²,²⁷ The change in conformation takes place in TM8 by reeling in some random coil residues to extend the helix.¹² In addition, when individual residues of the proton-relay
network was changed to alanine, disrupting the hydrogen bonds in the system, it was reported that TM8 of AcrB becomes longer and extends into the periplasmic domain. In contrast to AcrB, the helical structure of the N-terminus of TM8 in CusA is retained after metal binding. Instead, the N-terminal end of TM8 moves outward, together with the movement of the PC2 sub-domain. According to the crystal structures of the apo and bound-CusA, we confirm prior speculation that the structure of apo-AcrB is actually the detergent-bound form.

Intriguingly, it appears that the binding of Cu(I) or Ag(I) also triggers significant conformational changes in the other transmembrane helices of the pump. In addition to the movement of TM8, all other transmembrane helices except TM2 shift horizontally by as much as 4 Å, mimicking the motion of TM8. Further, TM1, TM3 and TM6 readjust in an approximately 3 Å upward shift with respect to the inner membrane surface. The net result is that all three of these transmembrane helices move towards the periplasm by one turn. Mutagenesis studies of the transmembrane domain of CusA indicated the conserved charged residue D405 of TM4 to be essential for transporter function. Based on the crystal structure, this acidic residue interacts with E939 and K984 (Fig. S4). It is possible that these three charged residues participate in forming the proton-relay network in the transmembrane region of the pump.

Coupled with the above conformational changes, the sub-domain PN1 at the periplasmic domain was also found to undergo substantial movement. Overall, PN1 also shifts upward by 3 Å. This moves the central pore helix upward by one turn upon metal binding. It should be noted that the binding of metal ions does not significantly affect the conformation of the sub-domains DN, DC, PN2 or the transmembrane helix TM2. PN2 has
been found to interact with the N-terminus of the CusB membrane fusion protein. Thus, we expect that PN2 and TM2 could move in the presence of CusB, to accommodate the binding of this membrane fusion protein. If TM8 were responsible for signaling the binding of metal ions, it would suggest that TM2 is important for detecting the presence of the membrane fusion component CusB.

Flexible docking was then carried out, where flexibility between the domains of CusB was accounted for by docking Domains 1 and 2 of CusB separately to the structure of apo-CusA using 3-D dock, followed by energy minimization with the CHARMM27 force field and steered molecular dynamics to restrain CusB to fit the docked fragments. The resulting CusBA structure suggests that Domains 1 and 2 of the CusB membrane fusion protein contact CusA at the interface between PN2 and PC1 (Fig. S5).

The full-length CusA includes 34 methionine residues with 18 of them located in the transmembrane domain. Of the 18 methionines, six are paired up to form three distinct methionine pairs (Fig. S1b). These methionine pairs are M410 of TM4 and M501 of TM6; M403 of TM4 and M486 of TM6; and M391 of TM4 and M1009 of TM12 (Fig. 3, Fig. S6 and Fig. S7). Copper tolerance proteins, such as CusF, CueR and Atx1, have been found to utilize two-methionine or two-cysteine binding pockets to carry their Cu(I)/Ag(I) cargos. Thus, these methionine pairs could potentially form binding sites for Ag(I) and Cu(I) in the transmembrane region of the pump. If this is the case, then CusA could transport these metal ions from the cytoplasm along these methionine pairs. In the periplasmic domain of CusA, we have also found two pairs of methionines (M271-M755, and M738-M792) in addition to the three-methionine metal binding site (Fig. 3). In view of the crystal structures,
these six methionine pairs/clusters are deemed to be important for binding and transport of metal ions.

We then determined whether the CusA protomer forms a channel by using the program CAVER (http://loschmidt.chemi.muni.cz/caver) and found that each protomer of CusA forms a channel spanning the entire transmembrane region up to the bottom of the periplasmic funnel (Fig. S8). Intriguingly, the channel includes four methionine pairs, three (M410-M501, M403-M486, M391-M1009) from the transmembrane region and one (M271-M755) from the periplasmic domain, as well as the three-methionine binding site formed by M573, M623 and M672 (Fig. 3). Taken together these five methionine pairs/clusters are likely to form a relay network facilitating metal ion transport. Remarkably, this channel spans almost the entire length of each protomer, from the transmembrane domain through to the bottom of the periplasmic funnel region, and is likely to represent a real path for transporting the metal ion from both the cytoplasm and periplasm to the periplasmic funnel for extrusion. It should be noted that the pair M738-M792, located at the top of the funnel, is not located directly within this channel.

We made an *E. coli* knock-out strain BL21(DE3)ΔcueOΔcusA that lacks both the cueO and cusA genes. We mutated M573, M623 and M672, which are members of the binding site triad inside the cleft of the periplasmic domain, into isoleucines (M573I, M623I and M672I). We then expressed these three mutant transporters in BL21(DE3)ΔcueOΔcusA, and tested their ability to confer copper and silver resistance *in vivo*. We found that the CusA mutants, M573I, M623I and M672I are unable to relieve the copper or silver sensitivity of strain BL21(DE3)ΔcueOΔcusA (Tables S3 and S4), thus agreeing well with the work of
Franke et al.\textsuperscript{4} in which M573, M623 and M672 were shown to be essential for the function of CusA.

We mutated M410 into an isoleucine (M410I) to disrupt the pair formed by M410 and M501 at the bottom of the transmembrane and also replaced M486 and M391, which pair with M403 and M1009, with isoleucines (M486I and M391I). Expression of these three mutants in BL21(DE3)\textsuperscript{Δ}cueO\textsuperscript{Δ}cusA showed a significant decrease in the level of copper and silver tolerances when compared with cells expressing wild-type CusA. In addition, we introduced M755I in the periplasmic domain. Again, when expressed in BL21(DE3)\textsuperscript{Δ}cueO\textsuperscript{Δ}cusA, this mutant transporter (M755I) showed a decrease in copper/silver tolerance in comparison with cells harboring the wild-type transporter. We then made two mutant transporters, M738I and M792I, in which these two methionines form a pair at the top of the periplasmic funnel. Unlike the other mutations, cells expressing these mutant transporters do not elevate the sensitivity level for copper or silver compared with cells expressing the wild-type pump (Tables S3 and S4). Together, these results strongly support five of these methionine pairs/clusters engaging in the methionine-residue ion relay channel.

Based on the crystal structure of CusA, it is expected that the charged residues D405, E939 and K984 are important for the proton-relay network of the pump, and these in turn were replaced with alanines (D405A, E939A and K984A) to disrupt the hydrogen-bonded network. Indeed, cells expressing this mutant were unable to tolerate copper and silver, demonstrating that these three charged residues are essential for the transporter’s functioning.

To investigate whether CusA can transport metal ions from the cytoplasm, we reconstituted the purified CusA protein into liposomes containing the fluorescent indicator
Phen Green SK (PGSK) in the intravesicular space (Fig. S9). The intravesicular and extravesicular pHs of these proteoliposomes were 6.6 and 7.0, respectively. We then determined whether these proteoliposomes can capture metal ions from the extravesicular medium with a stopped-flow transport assay. (The charged metal ions are unlikely to diffuse spontaneously across membrane bilayers.) When Ag$^+$ ions were added into the extravesicular medium, we detected the quenching of the fluorescence signal, suggesting the uptake of Ag$^+$ into the intravesicular space (Fig. 4a). The uptake into proteoliposomes is presumably due to the CusA active transport activity. It should be noted that no decrease in fluorescence signal was detected in the absence of a pH gradient (i.e. no differential pH) between the inside and outside of the proteoliposomes, or if the intravesicular pH is higher than the extravesicular pH (i.e. reverse pH gradient) (Fig. S10).

In addition to the above experiment, we investigated the methionine residues that were shown to be important for copper and silver tolerances with the above transport assay. We expressed, purified and reconstituted the CusA mutants, M573I, M623I and M672I, into liposomes encapsulated with the same fluorescence indicator. Surprisingly, these mutant transporters do not take up Ag$^+$ from the extravesicular medium of the proteoliposomes (Fig. 4a). It is clear that even single point mutations M573I, M623I or M672I abolish the process of metal transport across the membrane. Thus, it is expected that this three-methionine site is requisite for both metal binding and export.

We then purified the mutants, M391I, M486I and M755I, and reconstituted them into liposomes. In these three cases, the fluorescence signals did not attenuate as indicated by the stopped-flow assay (Fig. 4a). When compared with the result from the wild-type CusA, this
suggests that the M391I, M486I and M755I mutant transporters are unable to actively transport Ag⁺ across the membrane.

Although all of the above experimental results strongly suggest that these five methionine pairs/clusters (Fig. 3) are important for metal transport, we cannot definitively rule out the possibility that these mutations may affect the structure of the pump and in that way impair metal binding. Nonetheless, our collective experiments provide direct compelling evidence that CusA is capable of taking up Ag⁺ from the cytoplasm.

The crystal structure of CusA suggests that the charged residues D405, E939 and K984 in the transmembrane domain may be important for proton translocation. When reconstituted into liposomes, mutant transporters (D405A, E939A and K984A) do not take up Ag⁺ into the intravesicular space (Fig. 4b), thus confirming the importance of D405, E939 and K984 for the function of the pump.

It is likely that CusA operates through an alternating-access mechanism.⁹⁻¹² Thus we superimposed a protomer of apo-CusA onto each protomer of the “asymmetric” AcrB (pdb code: 2DHH).⁹ Likewise, we also superimposed a protomer of CusA-Cu(I) onto those of the AcrB pump. These superpositions gave high overall RMSDs, each RMSD exceeding 11 Å. However, when the portion of the apo-CusA protomer that contains only sub-domains PC1, PC2, PN1 and PN2 was supimposed onto those of the “extrusion”, “binding” and “access” conformers of AcrB (2DHH),⁹ we obtained much smaller RMSDs of 4.7 Å, 5.6 Å and 5.5 Å. Similarly, superpositions of the periplasmic domain (only containing PC1, PC2, PN1 and PN2) of a protomer of CusA-Cu(I) onto those of the “extrusion”, “binding” and “access” conformers of AcrB (2DHH)⁹ gave RMSDs of 6.2 Å, 5.0 Å and 5.3 Å, respectively. These
superpositions suggest that the conformation of the apo-CusA protomer may correspond to the “extrusion” state, and that the CusA-Cu(I) protomer structure may represent the “binding” conformation of the pump. If this is the case, CusA may go through a cyclic conformational change, from the “access”, through to the “binding” and finally to the “extrusion” conformer, as suggested for the AcrB pump,\textsuperscript{10-12} to export copper/silver.

We also calculated the dynamics of the trimeric CusA pump using the elastic network model.\textsuperscript{34} The result indeed suggests that CusA functions through three coupled motions in which the periplasmic cleft formed by sub-domains PC1 and PC2, presumably acting as the periplasmic metal entry site, alternately open and close (Figs. S11 and S12).

There is strong evidence that the CusCBA system captures Cu(I) and Ag(I) ions from the periplasm. The periplasmic membrane fusion protein CusB is known to specifically bind Cu(I).\textsuperscript{35} It has also been demonstrated that the periplasmic chaperone protein CusF can directly transfer these metals to CusB.\textsuperscript{36} Indeed, the crystal structure of CusB indicates that this membrane fusion protein consists of multiple Cu(I) and Ag(I) binding sites.\textsuperscript{22} Thus, in addition to capturing metal ions from the periplasm, the CusA pump may also be able to take up these metals from the cytoplasm. In fact, it has been demonstrated that the divalent cation efflux pump CzcA is capable of catalyzing the transport of divalent cations, such as Zn\textsuperscript{2+}, from the cytosol.\textsuperscript{37} Further, the AcrD multidrug efflux pump has also been observed to capture aminoglycosides from both the periplasm and cytoplasm.\textsuperscript{38}

We hypothesize that CusA can pick up metal ions from both the cytoplasm and periplasm. We propose that this transporter utilizes methionine pairs/clusters to export Cu(I) and Ag(I). The periplasmic cleft of CusA presumably remains closed when there is no
Cu(I)/Ag(I). In the presence of Cu(I) or Ag(I) ions, the periplasmic cleft opens. Metal ions could enter the three-methionine binding site inside the cleft directly through the periplasmic cleft or via the methionine pairs within the transmembrane region (Fig. 3). Thus, transport of a metal ion within the membrane is likely to involve a stepwise process that shuttles the metal ion from one methionine pair to another along the pathway. The locations of these methionine pairs/clusters allow us to depict a direct pathway for metal transport and extrusion. The metal ion bound at the three-methionine binding site of the periplasmic cleft could then be released to the nearest methionine pair, formed by M277 and M755, following which the metal ion could then be released into the central funnel to reach the CusC channel for final extrusion. Interestingly, the proposed metal export pathway in the periplasmic domain, based on the location of the methionine pairs/clusters, is similar to the proposed drug export pathway in AcrB. Nonetheless, our mechanism for export of metal ions intermediated by binding to sequential methionine pairs/clusters is fully consistent with the body of evidence in all of the structural and mutagenesis studies reported here.

**Methods Summary**

Crystals were grown by sitting-drop vapor diffusion. Phasing, refinement methods and statistics are provided in the Methods.

**Author Information**

Atomic coordinates and structure factors have been deposited with the Protein Data Bank as codes 3KO7 (apo-CusA), 3KSS (CusA-Cu(I)) and 3KSO (CusA-Ag(I)).
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Figures and Captions

Fig. 1. Structure of the CusA efflux pump. (a) Ribbon diagram of the CusA homotrimer viewed in the membrane plane. Each subunit of CusA is labeled with a different color. Sub-domains DN, DC, PN2, PC1 and PC2 are labeled on the front protomer (green). The location of PN1 in this protomer is behind PN2, PC1 and PC2 (see text). (b) Top view of the CusA trimer. The six sub-domains are labeled yellow (DN), blue (DC), pink (PN1), orange (PN2), green (PC1) and red (PC2). In the apo-CusA structure, the cleft between PC1 and PC2 is closed.
Fig. 2. Comparison of the apo and metal bound structures of CusA. (a) Superposition of a monomer of apo-CusA (red) onto a monomer of Cu(I) bound-CusA (green). The bound Cu(I) is pink. The arrow represents a major swing of the PC2 sub-domain initiated by Cu(I) binding. (b) Conformational changes of the periplasmic domain of CusA. The conformation of each sub-domain of CusA before (left) and after (right) Cu(I) binding. The periplasmic cleft formed between PC1 and PC2 is opened after Cu(I) binding. The bound coppers in the CusA-Cu(I) structure are blue. M573, M623 and M672 forming a metal binding site at the periplasmic cleft are shown in stick form (yellow). (c) The changes in conformation of the horizontal helix and TM8, are shown in a superimposition of the structures of apo (red) and Cu(I)-bound (green) CusA. The bound Cu(I) is shown as a pink sphere. Anomalous map of the bound Cu(I), contoured at 8σ, is in blue. M573, M623 and M672 are shown as sticks. (d) The Ag(I) binding site. The bound Ag(I) is shown as a gray sphere. Anomalous map of the bound Ag(I), contoured at 10σ, is in red.
Fig. 3. Proposed metal transport pathway of the CusA efflux pump. (a) The five methionine pairs/clusters of the apo-CusA transporter form a pathway for metal export. These five methionines are shown as spheres (C, green; O, red; N, blue; S, orange). The carbon atoms
of the pair M738-M792, located at the top of the periplasmic funnel, are in blue. This pair does not engage directly in the methionine-residue relay network. (b) The five methionine pairs/clusters of the Cu(I) bound-CusA transporter form a pathway for metal export. These five methionines are given as spheres (C, pink; O, red; N, blue; S, orange). The carbon atoms of the pair M738-M792, located at the top of the periplasmic funnel, are in orange. This pair is not involved in the methionine-residue relay network. The pair M271-M755 is located at the bottom of the periplasmic funnel where the metal ion could then be released for final extrusion. The paths for metal transport through the periplasmic cleft and transmembrane region are illustrated with black curves.
Fig. 4. Stopped-flow transport assay of reconstituted CusA with extravesicular Ag⁺ ion. (a) Mutants of the methionine-residue relay network. The decrease in fluorescence signal of
PGSK mediated by proteoliposomes of wild-type CusA indicates the transport of Ag\(^+\) across the membrane. The stopped-flow traces are the cumulative average of four successive recordings (wild-type CusA, black curve; M486I mutant, green curve; M391I mutant, brown curve; M573I mutant, red curve; M623I, purple curve; M672I, orange curve; M755I, cyan curve; control liposome, dark blue curve). (b) Mutants of the proton relay network. The stopped-flow traces are the cumulative average of four successive recordings (D405A mutant, dark blue curve; E939A mutant, red curve; K984A mutant, green curve). The wild-type CusA and control liposomes are shown in part (a).
Supplemental Materials

Methods

Cloning, expression and purification of CusA

Briefly, the full-length CusA membrane protein containing a 6xHis tag at the N-terminus was overproduced in *E. coli* BL21(DE3)ΔacrB cells, which harbors a deletion in the chromosomal *acrB* gene, possessing pET15bΩcusA. Cells were grown in 12 L of LB medium with 100 μg/ml ampicillin at 37°C. When the OD₆₀₀ reached 0.5, the culture was treated with 1 mM IPTG to induce *cusA* expression, and cells were harvested within 3 h. The collected bacteria were resuspended in low salt buffer containing 100 mM sodium phosphate (pH 7.2), 10 % glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethanesulfonyl fluoride (PMSF), and then disrupted with a French pressure cell. The membrane fraction was collected and washed twice with high salt buffer containing 20 mM sodium phosphate (pH 7.2), 2 M KCl, 10 % glycerol, 1 mM EDTA and 1 mM PMSF, and once with 20 mM HEPES-NaOH buffer (pH 7.5) containing 1 mM PMSF. The membrane protein was then solubilized in 1 % (w/v) Cymal-6. Insoluble material was removed by ultracentrifugation at 100,000 x g. The extracted protein was purified with a Ni²⁺-affinity column. The purity of the CusA protein (>95%) was judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue. The purified protein was then dialyzed and concentrated to 20 mg/ml in a buffer containing 20 mM Na-HEPES (pH 7.5) and 0.05% CYMAL-6.

For 6xHis SeMet-CusA protein expression, a 10 ml LB broth overnight culture containing *E. coli* BL21(DE3)ΔacrB/pET15bΩcusA cells was transferred into 120 ml of LB
broth containing 100 µg/ml ampicillin and grown at 37°C. When the OD_{600} value reached 1.2, cells were harvested by centrifugation at 6000 rev/min for 10 min, and then washed two times with 20 ml of M9 minimal salts solution. The cells were re-suspended in 120 ml of M9 media and then transferred into a 12 L pre-warmed M9 solution containing 100 µg/ml ampicillin. The cell culture was incubated at 37°C with shaking. When the OD_{600} reached 0.4, 100 mg/l of lysine, phenylalanine and threonine, 50 mg/l isoleucine, leucine and valine, and 60 mg/l of L-selenomethionine were added. The culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) after 15 min. Cells were then harvested within 3 h after induction. The procedures for purifying the 6xHis SeMet-CusA were identical to those of the native protein.

**Crystallization of CusA**

Crystals of the 6xHis CusA were obtained using sitting-drop vapor diffusion. The CusA crystals were grown at room temperature in 24-well plates with the following procedures. A 2 µl protein solution containing 20 mg/ml CusA protein in 20 mM Na-HEPES (pH 7.5) and 0.05% (w/v) CYMAL-6 was mixed with a 2 µl of reservoir solution containing 10% PEG 3350, 0.1 M Na-MES (pH 6.5), 0.4 M (NH₄)₂SO₄, 1% JM 600 and 10% glycerol. The resultant mixture was equilibrated against 500 µl of the reservoir solution. The crystallization conditions for SeMet-CusA were the same as those for the native CusA protein. Crystals of CusA grew to a full size in the drops within a month. Typically, the dimensions of the crystals were 0.2 mm x 0.2 mm x 0.2 mm. Cryoprotection was achieved
by raising the glycerol concentration stepwise to 30% with a 5% increment in each step.

Crystals of the gold or tantalum derivatives were prepared by incubating the crystals of apo-CusA in solution containing 10% PEG 3350, 0.1 M Na-MES (pH 6.5), 0.4 M (NH$_4$)$_2$SO$_4$, 1% JM 600, 10% glycerol, 0.05% (w/v) CYMAL-6, and 1 mM KAuCl$_4$ or 5 mM Ta$_6$Br$_{12}$Br for 1.5 hours at 25ºC.

The CusA-Cu(I) complex crystals were prepared by incubating crystals of apo-CusA in solution containing 10% PEG 3350, 0.1 M Na-MES (pH 6.5), 0.4 M (NH$_4$)$_2$SO$_4$, 1% JM 600, 10% glycerol, 2 mM [Cu(CH$_3$CN)$_4$]PF$_6$, 2 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.05% (w/v) CYMAL-6 for 1 hour at 25ºC. Crystals of the CusA-Ag(I) complex were prepared using a similar method in solution containing 10% PEG 3350, 0.1 M Na-MES (pH 6.5), 0.4 M (NH$_4$)$_2$SO$_4$, 10% glycerol, 1 mM AgNO$_3$ and 0.05% (w/v) CYMAL-6 for 1 hour at 25ºC.

**Data collection, structural determination and refinement**

All diffraction data were collected at 100K at beamline 24ID-C located at the Advanced Photon Source, using an ADSC Quantum 315 CCD-based detector. Diffraction data were processed using DENZO and scaled using SCALEPACK.$^{39}$ The crystals belong to space group $R32$ (Tables S1 and S2). Based on the molecular weight of CusA (115.72 kDa), a single molecule per asymmetric unit with a solvent content of 67.5% is expected. Two heavy-atom derivatives (gold and Ta$_6$Br$_{12}$ cluster) and the selenomethionyl-substituted (SeMet) crystal were isomorphous with the native crystal (Table S1). Ten heavy-atom sites
for the gold derivative were identified using SHELXC and SHELXD\textsuperscript{40} as implemented in the HKL2MAP package.\textsuperscript{41} These heavy-atom sites were refined using the program MLPHARE.\textsuperscript{42,43} The resulting phases were subjected to density modification by RESOLVE\textsuperscript{44} using the native structure factor amplitudes. Density modified phases were good enough to allow us to visualize the secondary structural features of the molecule. These phases were then used to locate three Ta\textsubscript{6} clusters and 31 selenium sites using the corresponding data sets. The final experimental phases were generated using 10 Au, three Ta\textsubscript{6} and 33 Se sites. The Ta\textsubscript{6} sites were treated as super-atoms containing all six tantalum atoms at a given site. These phases were then subjected to density modification and phase extension to 3.52 Å-resolution using the program RESOLVE.\textsuperscript{44} The resulting phases were of excellent quality that enabled tracing of most of the molecule. The full-length CusA protein consists of 34 methionine residues. The SeMet data not only augmented the experimental phases, but also helped in tracing the molecules by anomalous difference Fourier maps where we could ascertain the proper registry of SeMet residues. After tracing the initial model manually using the program Coot,\textsuperscript{45} the model was refined against the native data at 3.52 Å-resolution using TLS refinement techniques adopting a single TLS body as implemented in PHENIX\textsuperscript{46} leaving 5\% of reflections in Free-R set. Iterations of refinement using PHENIX\textsuperscript{46} and CNS\textsuperscript{47} and model building in Coot\textsuperscript{45} lead to the current model, which consists of 1,025 residues with excellent geometrical characteristics (Table S1).

While the structure of CusA-Cu(I) complex could be determined using molecular replacement, the structure resulted in a relatively high refinement R-factor of 48.7\%. Upon inspection of the electron density maps, it was found that several regions, especially the PC2
sub-domain, of CusA-Cu(I) have undergone significant conformational changes as discussed in the main text. To aid modeling of these conformational changes in the least biased manner, SAD phasing using the program PHASER was employed to obtain experimental phases in addition to the phases from the structural model of apo-CusA that has sub-domains PC2 and PN1 removed. Phases were then improved using RESOLVE. Even though the data resolution is low (4.10 Å), the phases were of sufficient quality that allowed for modeling of the conformational change (Fig. S3) using the program COOT. At a later stage, higher resolution (3.88 Å) data of the CusA-Cu(I) were collected (Table S2). To reduce radiation damage, the data was collected away from the copper absorption edge, at 1.033 Å, where Cu still has ~2.4 anomalous electrons. The model was refined using PHENIX and CNS.

Since the X-ray absorption edge of Ag was not reachable at the synchrotron beamline (L-edges below 4 KeV and K-edge above 25 KeV), the data were collected at the copper edge where Ag has an anomalous contribution of ~3.5e⁻. The structure of the CusA-Ag(I) complex was phased using molecular replacement by using CusA-Cu(I) as the search model. Structural refinement was then performed using PHENIX and CNS by refining the model against the 4.35 Å-resolution data from the CusA-Ag(I) crystal (Table S2).

Docking structures of CusB onto CusA

Initial rigid-body docking of the crystal structures of CusA and CusB did not reveal any feasible complexes that satisfied the CusA-K150/CusB-K95 crosslinking constraint, indicating that docking may likely require a conformational change in one or both molecules.
As previously mentioned in this paper it has been hypothesized that the PN2 and PC1 sub-domains may move to accommodate CusB. For CusA, the normal modes from the Anisotropic-Network-Models (ANMs)\textsuperscript{34} were calculated, but did not reveal any appropriate structures. For CusB, ANM revealed significant hinge motions between Domains 1 and 2. And, following this lead these two domains were docked individually onto CusA. Steered molecular dynamics simulations were then used to drive the CusB into a docked form agreeing with the individually docked fragments, thus allowing for the flexibility of the PC1, PN2 and TM2 domains of CusA.

**Data-driven docking of Domains 1 and 2 of CusB**

Rigid-body docking was performed using the 3D-dock software suite,\textsuperscript{28} which utilizes the Katchalski-Katzir algorithm.\textsuperscript{49} For all runs, 10,000 conformations were generated and filtered using the following data-driven constraints: transmembrane helices were masked from docking interactions; and side-chain amines of CusA-K150 and CusB-K95 were within 25 Å\textsuperscript{22}; CusB was oriented so that its domains were free to interact with CusC and its N-terminus was directed towards the inner membrane.

**Steered Molecular Dynamics and Energy minimization**

An initial conformation was calculated by aligning CusB with the two individually docked fragments. To relax CusB from this conformation, we utilized steered molecular dynamics to guide the center of mass of domains 1 and 2 into the corresponding docked
position. Energy minimization was employed to alleviate steric clashes. During the simulation CusA was kept rigid with the exception of TM2, PN2, and PC1, which make up most of the CusB binding region. All calculations were performed using NAMD\textsuperscript{50} and the CHARMM27 force field.\textsuperscript{29}

**Double knocked-out strain and susceptibility assays**

The double knocked-out \textit{E. coli} strain BL21(DE3)ΔcueOΔcusA was produced from the BL21(DE3) strain using an RED disruption system as described by Datsenko and Wanner.\textsuperscript{51} The ΔcueO::kan cassette, which was used to replace the chromosomal cueO gene, was produced by PCR, and then introduced into pKD46/BL21(DE3) by electroporation. The knocked-out BL21(DE3)ΔcueO::kan strain was selected on LB plate containing 30 µg/ml kanamycin, and verified by PCR. The kanamycin resistant gene was then released to generate the BL21(DE3)ΔcueO knocked-out strain. The deletion of cusA from BL21(DE3)ΔcueO was done using similar procedures as described above to generate the final BL21(DE3)ΔcueOΔcusA double knocked-out strain.

The susceptibility to copper of \textit{E. coli} BL21(DE3)ΔcueOΔcusA harboring pET15bΩcusA expressing the wild-type or mutant transporters, or the pET15b empty vector was tested on agar plates. Cells were grown in Luria Broth (LB) medium with 100 µg/ml ampicillin at 37 °C. When the OD\textsubscript{600} reached 0.5, the cultures were induced with 0.5 mM IPTG and harvested in two hours after induction. The minimum growth inhibitory concentrations (MICs) to copper of \textit{E. coli} BL21(DE3)ΔcueOΔcusA (incoculum, 500 cells/ml)
harboring these vectors were then determined using LB agar containing 50 μg/ml ampicillin, 0.1 mM IPTG and different concentrations of CuSO₄ (0.25 to 2.75 mM in steps of 0.25 mM).

For the susceptibility to silver of *E. coli* BL21(DE3)ΔcueOΔcusA harboring these vectors, cells were grown in Mueller Hinton (MH) medium containing 100 μg/ml ampicillin with the same procedures. The MICs to silver of *E. coli* BL21(DE3)ΔcueOΔcusA (incoculum, 500 cells/ml) harboring these vectors were then performed in MH agar containing 50 μg/ml ampicillin, 0.1 mM IPTG and different concentrations of AgNO₃ (5.0 to 50.0 μM in steps of 2.5 μM). The expression level of each mutant in BL21(DE3)ΔcueOΔcusA is similar to that of the wild-type transporter as indicated by Western blot analysis (Fig. S13). Bacterial growth in the LB or MH agar was recorded after 24 h of incubation at 37°C. Each assay was repeated at least four times to ensure the reproducibility of the results.

**Reconstitution and stop-flow transport assay**

The purified wild-type CusA, M391I, M486I, M573I, M623I, M672I, M722I, D405A, E939A or K984A was reconstituted into liposomes made of *E. coli* polar lipid and egg-yolk phosphatidylcholine (Avanti Polar Lipids) in a molar ratio of 3:1. Briefly, 50 μg of the purified protein (wild-type CusA or its mutant) was added to 4.5 mg of lipids dispersed in the reconstitution buffer containing 20 mM HEPES-KOH, pH 6.6. The protein samples were completely incorporated into liposomes as judged using 10% SDS-PAGE stained with Coomassie Brilliant Blue. Control liposomes were prepared following the same procedure
without adding protein.  200 μM fluorescence indicator Phen Green SK (PGSK) (Invitrogen) was encapsulated by two freeze-thaw cycles, followed by gel filtration to remove the untrapped dye. Transport experiments were performed at 25°C on a stopped-flow apparatus (Hi-Tech Scientific) connected to a spectrofluorometer (PerkinElmer LS55). Proteoliposomes and a transport assay buffer (20 mM HEPES-KOH pH 7.0 and 1 mM AgNO₃) were loaded into a two separate syringes of equal volume. Transport reactions were initiated by pushing 400 μl fresh reactants at a 1:1 ratio through the 90 μl mixing cell at a flow rate of 2 ml/s. Stopped-flow traces were the cumulative average of four successive recordings at 530 nm with the excitation wavelength at 480 nm.

**Dynamics Simulations**

The observed conformational changes between the Cu(I) bound and the apo forms, displayed in Fig. 2, are shown to be intrinsically favored by the new structure through the use of normal mode analysis (Fig. S11). A coarse-grained elastic network model³⁴ was investigated that is composed of the Cα atom coordinates and a portion of coarse-grained lipid bilayer between the trans-membrane domains. The opening and closing of the space between PC1 and PC2 coupled with a shift of TM8 is reproduced by this simple model (Fig. S12). The model does not show simultaneous opening and closing of all three periplasmic heavy metal entrance sites, but rather has a more complex opening/closing motion in which each pair of two adjacent is alternately open and closed. All three combinations of these coupled motions are obtained by using just two low energy motions.
References


Supplemental Tables

Table S1. Data collection, phasing and structural refinement statistics of apo-CusA.

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<th>Native CusA</th>
<th>Derivatives</th>
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<td>ADSQ Q315</td>
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<tr>
<td>Detector type/source</td>
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<td>APS-24IDC</td>
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<td>Space group</td>
<td>R₃2</td>
<td>R₃2</td>
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<td>a = b = 178.42, c = 285.75</td>
<td>a = b = 179.31, c = 287.10</td>
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<td>Resolution (Å)</td>
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<td>(4.50-4.31)</td>
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<td>Redundancy</td>
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<td>3.2 (2.8)</td>
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<td>Completeness (%)</td>
<td>94.2 (95.8)</td>
<td>100 (100)</td>
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<tr>
<td>Rₛₘᵢᵣ (%)</td>
<td>5.7 (43.5)</td>
<td>10.2 (35.9)</td>
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<td>Average I/σ(I)</td>
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<td>MIR Phasing</td>
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<td>1.09/1.23</td>
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<td>0.76/0.71</td>
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<tr>
<td>Rₜₒₒₜ (%)</td>
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<td></td>
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<tr>
<td>Rᵣᵣᵣ (%)</td>
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<td>Protein atoms</td>
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<td>rms deviation from ideal Bond lengths (Å)</td>
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<td>most favored</td>
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<td>additional allowed</td>
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<td>generously allowed</td>
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Table S2. Data collection, phasing and structural refinement statistics of the CusA-Cu(I) and CusA-Ag(I) complexes.

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<tr>
<th>Data Collection</th>
<th>Cu(I) (peak)</th>
<th>Cu(I)</th>
<th>Ag(I)</th>
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<tr>
<td>Detector type/source</td>
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<td>ADSQ Q315</td>
<td>ADSQ Q315</td>
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<td>APS-24IDC</td>
<td>APS-24IDC</td>
<td>APS-24IDC</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>1.0333</td>
<td>1.3779</td>
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<td>Space group</td>
<td>R32</td>
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<td>Cell constants (Å)</td>
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<td>179.99, c = 287.98</td>
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<td>Resolution (Å)</td>
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<td>50-3.88</td>
<td>50-4.35</td>
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<tr>
<td>Total reflections</td>
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<td>346,057</td>
<td>258,908</td>
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<td>Unique reflections</td>
<td>14,211</td>
<td>17,119</td>
<td>15,666</td>
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<td>Redundancy</td>
<td>2.5 (2.5)</td>
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<tr>
<td>Completeness (%)</td>
<td>96.6 (98.7)</td>
<td>99.5 (98.8)</td>
<td>99.7 (99.9)</td>
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<td>Rsym(%)</td>
<td>8.9 (42.4)</td>
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<td>9.2 (39.6)</td>
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<td>Average I/σ(I)</td>
<td>11.1 (1.8)</td>
<td>10.5 (2.1)</td>
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Phasing

| Figure of merit | 0.50/0.43 |
| Figure of merit (acentric/centric) | |

Refinement

| Resolution (Å) | 50-3.88 | 50-4.35 |
| Rwork (%) | 26.1 | 27.1 |
| Rfree (%) | 29.6 | 31.2 |
| Protein residues built | 1,015 | 1,014 |
| Protein atoms | 7,802 | 7,782 |
| rms deviation from ideal | 0.005 | 0.006 |
| Bond lengths (Å) | |
| Bond angles (°) | 0.995 | 1.252 |

Ramachandran

| most favored | 85.1 | 84.5 |
| additional allowed | 12.1 | 12.3 |
| generously allowed | 1.5 | 1.8 |
| disallowed | 1.2 | 1.4 |
Table S3. MICs of copper for different CusA mutants expressed in *E. coli*  

BL21(DE3)ΔcueOΔcusA

<table>
<thead>
<tr>
<th>Gene in BL21(DE3)ΔcueOΔcusA</th>
<th>MIC (mM) of CuSO₄</th>
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<td>Empty vector</td>
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<tr>
<td>cusA (wild-type)</td>
<td>2.25</td>
</tr>
<tr>
<td>cusA (M573I)</td>
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</tr>
<tr>
<td>cusA (M623I)</td>
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</tr>
<tr>
<td>cusA (M672I)</td>
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<tr>
<td>cusA (M391I)</td>
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<tr>
<td>cusA (M410I)</td>
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<tr>
<td>cusA (M486I)</td>
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<tr>
<td>cusA (M755I)</td>
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<td>cusA (M738I)</td>
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<td>cusA (M792I)</td>
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<td>cusA (D405A)</td>
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<td>cusA (E939A)</td>
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<tr>
<td>cusA (K984A)</td>
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<tr>
<td>cusA (E412A)</td>
<td>1.00ᵃ</td>
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<tr>
<td>cusA (S453A)</td>
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<tr>
<td>cusA (C353A)</td>
<td>2.25ᵇ</td>
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<tr>
<td>cusA (C375A)</td>
<td>2.25ᵇ</td>
</tr>
</tbody>
</table>

ᵃResidues E412 and S453, located in the vicinity of the triad formed by D405, E939 and K984, may also involve in the proton-relay network.

ᵇThe CusA pump contains only two cysteine residues, C353 and C375, which may not involve in copper resistance.
Table S4. MICs of silver for different CusA mutants expressed in *E. coli* BL21(DE3)ΔcueOΔcusA

<table>
<thead>
<tr>
<th>Gene in BL21(DE3)ΔcueOΔcusA</th>
<th>MIC (µM) of AgNO₃</th>
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<td><em>cusA</em> (M672I)</td>
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<td>10.0</td>
</tr>
<tr>
<td><em>cusA</em> (M410I)</td>
<td>17.5</td>
</tr>
<tr>
<td><em>cusA</em> (M486I)</td>
<td>17.5</td>
</tr>
<tr>
<td><em>cusA</em> (M755I)</td>
<td>12.5</td>
</tr>
<tr>
<td><em>cusA</em> (M738I)</td>
<td>30.0</td>
</tr>
<tr>
<td><em>cusA</em> (M792I)</td>
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</tr>
<tr>
<td><em>cusA</em> (D405A)</td>
<td>12.5</td>
</tr>
<tr>
<td><em>cusA</em> (E939A)</td>
<td>12.5</td>
</tr>
<tr>
<td><em>cusA</em> (K984A)</td>
<td>12.5</td>
</tr>
<tr>
<td><em>cusA</em> (E412A)</td>
<td>12.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>cusA</em> (S453A)</td>
<td>15.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Residues E412 and S453, located in the vicinity of the triad formed by D405, E939 and K984, may also involve in the proton-relay network.
Supplemental Figures and Captions

(a) [Image]

(b) [Image]
Fig. S1. Stereo view of the experimental electron density map at a resolution of 3.8 Å. (a) The electron density map contoured at 1.2 $\sigma$ is in blue. Each subunit of CusA consists of 34 methionines. 33 selenium sites are found with a single SeMet crystal. The anomalous maps of the selenium sites contoured at 4 $\sigma$ are in green. The C$\alpha$ traces of CusA are in red. (b) Anomalous maps of the 33 selenium sites (contoured at 4 $\sigma$). The selenium sites corresponding to the 11 methionines forming the methionine-residue relay network are in green. The rest of the 22 selenium sites are in purple. The C$\alpha$ traces of CusA are in red. (c) Representative section of the electron density in the second domain of CusA. The electron density (colored blue) is contoured at the 1.2 $\sigma$ level and superimposed with the final refined model (yellow, carbon; red, oxygen; blue, nitrogen).
Fig. S2. Sequence and topology of CusA and AcrB. Alignment of the amino acid sequences of CusA and AcrB were done using CLUSTAL W. *, identical residues; :, >60% homologous residues. Secondary structural elements are indicated: TM, transmembrane.
helix; Nα and Nβ, helix and strand, respectively, in the N-terminal half; Cα and Cβ, helix and strand, respectively, in the C-terminal half. The CusC or TolC docking domain is divided into two sub-domains, DN and DC; whereas the pore domain is divided into four sub-domains, PN1, PN2, PC1 and PC2. The sequence and topology of CusA are shown at the top, and those for the AcrB pump are shown at the bottom.
Fig. S3. Experimental electron density of the periplasmic domain of CusA. (a) The electron density map of apo-CusA (3.8 Å-resolution) contoured at 1.2 $\sigma$ is in blue. The C$\alpha$ traces of the periplasmic domain of apo-CusA are in red. (b) The electron density map of CusA-Cu(I) (4.1 Å-resolution) contoured at 1.2 $\sigma$ is in purple. The C$\alpha$ traces of the periplasmic domain of CusA-Cu(I) are in green. The bound copper is shown as a brown sphere.
Fig. S4. Ion pairs in the transmembrane domain viewed from the cytoplasmic side.

Residues D405 of TM4, E939 of TM10 and K984 of TM11 that form ion pairs, which may play an important role in proton translocation, are in yellow sticks. The six methionines, M391, M403, M410, M486, M501 and M1009, are shown as pink sticks.
Fig. S5. Docking of CusB to CusA. (a) Side view of the docked complex of CusBA. The three CusB protomers are shown in green ribbons. The trimeric CusA is in gray surfaces. Sub-domains PN2 and PC1 of CusA are in red and blue, respectively. Specific interaction is found to occur between Domain 2 of CusB and the groove formed between DN and DC sub-domains of CusA to further stabilize the complex. (b) Top view of the docked complex of CusBA.
Fig. S6. Stereo view of the methionine-residue relay network. (a) The transmembrane domain of a subunit of CusA viewed from the cytoplasmic side. The six methionines that form three pairs in the transmembrane region are in green sticks. (b) The periplasmic domain of a subunit of CusA viewed from the periplasmic side. The five methionines that form a triad and a pair in the periplasmic domain are in green sticks.
Fig. S7. Alignment of amino acid sequences of the HME-RND-type Cu(I) and/or Ag(I) efflux pumps using CLUSTAL W. *, identical residues; :, >60% homologous residues. The alignment suggests that the methionine residues forming the relay network are conserved among these 30 different pumps. Six of these methionines located at the transmembrane region are highlighted with gray bars.
Fig. S8. Channel in the CusA pump. (a) The channel formed by the front protomer of apo-CusA (red) leading through the transmembrane and periplasmic domains is in gray color. The 11 methionines forming the relay network are in spheres (green, carbon; red, oxygen; blue, nitrogen; orange, sulfur). The Ca atoms of the methionine pair, located at the top of the funnel, which does not involve in creating the channel are colored cyan. Two other CusA protomers behind the front protomer are shown as blue wires. (b) The channel formed by the front protomer of Cu(I) bound-CusA (green) leading through the transmembrane and periplasmic domains is in gray color. The 11 methionines forming the relay network are in spheres (pink, carbon; red, oxygen; blue, nitrogen; orange, sulfur). The Ca atoms of the methionine pair, located at the top of the funnel, which does not involve in creating the channel are colored cyan. Two other CusA protomers behind the front protomer are shown as blue wires. The funnel formed by sub-domains DN and DC is indicated with a dotted curve. For clarity, the channels formed by the other two protomers at the back are omitted. The calculations were done using the program CAVER (http://loschmidt.chemi.muni.cz/caver).
Fig. S9. A cartoon of a proteoliposome containing the CusA trimers. The CusA trimers are in green. The intravesicular space is loaded with the fluorescence indicator PGSK (blue star). Ag⁺ (red sphere) is then added into the extravesicular medium for metal transport.
Fig. S10. Stopped-flow transport assay of reconstituted wild-type CusA with extravesicular Ag\(^+\) ion at different intravesicular and extravesicular pHs. The stopped-flow traces are the cumulative average of four successive recordings (intravesicular pH = 6.6 and extravesicular pH = 7.0, black curve; intravesicular pH = extravesicular pH = 6.6, blue curve; intravesicular pH = extravesicular pH = 7.0, green curve; intravesicular pH = 7.0 and extravesicular pH = 6.6, pink curve; control liposome, red curve).
Fig. S11. The neighbor dependent opening closing motions. We show the effects of the strong internal motions on the CusA trimer. In the center is a diagram of the periplasmic domain looking downward, towards the inner membrane. We build an elastic network model using the formalism of Atilgan et al.\textsuperscript{34} to predict the natural motions of CusA. On each of three sides we show the effect of two of the structure’s natural motions. These motions describe coupled opening and closing of adjacent periplasmic metal entry sites. Black wedges are shown in open and closed form, indicating the approximate angle between the
edges of PC1 and PC2 in each motion. From these the alternating open/close motion is evident.

Fig. S12. The swinging motion of PC2 coupled to TM8, as computed with the same elastic network model used for Fig. S11. This motion is similar to the difference between the apo and bound crystal forms displayed in Fig. 2. An arrow indicates the motion of swinging from the red conformation to the green. For clarity PC2 and TM8 are shown in thicker lines than the remainder of the monomer.
Fig. 13. Expression level of the CusA pumps. An immunoblot against CusA of crude extracts from 50 μg dry cells of strain BL21(DE3)ΔcueOΔcusA expressing the CusA pumps (M755I, lane 1; M391I, lane 2; M410I, lane 3; M486I, lane 4; wild-type, lane 5; marker, lane 6; M573I, lane 7; M623I, lane 8; M672I, lane 9) is shown.
CHAPTER 4. General Conclusions and Future Directions

Multidrug resistance of common pathogens is one of the most serious issues regarding human health. To ensure their survival, bacteria have developed a mechanism that involves the expression of multidrug efflux transporters to subvert antibiotics and other toxic compounds. In this dissertation, several efflux pumps belonging to different transporter families, such as MATE and RND, were functionally and structurally characterized.

We have cloned, expressed and purified the *N. gonorrhoeae* NorM MATE-type transporter. The functions of NorM and its homologous protein *E. coli* YdhE were examined using drug susceptibility and transport assays. Both NorM and YdhE confer resistance to various antimicrobial agents in a similar but slightly different manner. Direct measurements of drug efflux in living cells indicated that NorM behaves as a Na\(^+\)/drug antiporter, which is consistent with the previous hypothesis that MATE transporters utilize Na\(^+\) as the coupling cation. Recently, a few efflux pumps, identified as MATE transporters, have been identified as H\(^+\)/substrate antiporters. These antiporters include PmpM of *P. aeruginosa* (1), AbeM of *A. baumannii* (2), and hMATE1 from human chromosome 17 (3). The selection of cations for energy coupling is definitely not by random. Thus, the question of how a bacterium selectively chooses a particular cation for better energy coupling remains to be elucidated. To this point, it is expected that the extracellular environment might somehow account for it (4). The functional information of more MATE transporters is needed to uncover this energy coupling mechanism.

The work of NorM of *V. parahaemolyticus* suggested that the three conserved anionic residues (Asp32, Glu251 and Asp367) in the predicted transmembrane regions are important
for the function of the pump (5). Our preliminary results (not shown) from drug
susceptibility assay also indicated that the corresponding mutants in *N. gonorrhoeae* NorM
(Asp41, Glu261 and Asp377) are defective in drug resistance. It is unclear how these
charged residues involve in the operation of the active pumps. Systematic and rational
mutations should be made to elucidate the molecular mechanism that NorM utilizes to expel
toxic compounds out of the cell. The crystal structure of NorM appears to be essential to
address this fundamental question. Until now, the structural information of the MATE
family transporters has not yet been available. Preliminary crystallization study of NorM in
our laboratory has shown that the best crystal diffracted to a resolution of 3.8 Å, thus
providing us with a strong foundation in optimizing the crystallization conditions and
hopefully having the crystal structure determined (6).

In this dissertation, we have determined the crystal structure of CusA, which serves as
the first three-dimensional structural model of the HME-RND efflux pumps. A major
conformational change was revealed by comparing the crystal structures with and without
Cu(I) and Ag(I). The metal binding site, located at the periplasmic domain, is formed by
three methionines (M573, M623 and M672). Based on the crystal structures, we proposed
that the metal ion is exported through a methionine-residue relay network which involves
five methionine pairs/clusters in the pump. We also performed metal transport assay using
proteoliposomes. The results suggest that these methionine pairs/clusters are essential for the
active efflux of silver ions. In addition, we have also identified three charged residues that
form the proton relay network of the pump. Sequence alignment among different RND
efflux pumps shows these charged residues, located at the transmembrane domain, are
conserved among the HME-RND efflux pumps.
It has been generally accepted that the RND-type transporters operate through an alternating-access mechanism that involves the asymmetric conformation of the trimeric pump (7, 8). Thus, CusA may go through a cyclic conformational change, from the “access”, through the “binding” and finally to the “extrusion” conformers of the trimeric pump to export metal ions. With the development of the single molecule technique, it is possible to capture the functional dynamics of CusA during metal export.

The study of AcrB tripartite system suggested that AcrB is likely to capture its substrates from the periplasm (9). It is not known if AcrB could pick up drug molecules from the cytoplasm. Our proposed metal export pathway suggests that CusA is capable of accepting the metal ions through both the cytoplasm and periplasm. This may imply that the RND pumps may be able to detoxify the interior of the cell by picking up toxic compounds both from the cytoplasmic and periplasmic spaces. The fact that substrate can be directly transported from the cytoplasm is also supported by the works of other RND transporters. For example, the C. metallidurans CzcA HME-RND transporter has been found to export Zn\(^{2+}\) ions through the cytoplasm (10). Further, these RND transporters are also found in Gram-positive bacteria (YerP, ActII3) (11, 12), mycobacteria (MmpL7) (13, 14), and human cells (NPC1) (15, 16), in which both of these cells possess only one single layer of cell membrane.

To understand how the RND pumps operate to mediate drug and toxic chemical resistance, the crystal structure of the tripartite efflux complex is essential. Currently, our laboratory is in the process of crystallizing the CusBA and CusCBA complexes. It is expected that the complex structures will allow us to uncover the mechanisms by which the RND transporters employ to export toxic substances.
References


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