Characterization of DrrAB Complex from Streptomyces Peucetius as A Multidrug Transporter

Wen Li

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CHARACTERIZATION OF DRRAB COMPLEX FROM *STREPTOMYCES PEUCETIUS* AS A MULTIDRUG TRANSPORTER

by

WEN LI

Under the Direction of Parjit Kaur, PhD

ABSTRACT

The soil bacterium *Streptomyces peucetius* produces two widely used anticancer antibiotics doxorubicin and daunorubicin. Present within the biosynthesis gene cluster in *S. peucetius* is the *drrAB* operon which codes for a dedicated ATP-binding cassette type transporter for the export of these two closely related antibiotics. DrrAB system was believed to be the single-drug transporter due to its dedicated nature; however, our study demonstrated under both *in vivo* and *in vitro* conditions that DrrAB system can transport not only doxorubicin but also Hoechst 33342 and ethidium bromide. Moreover, many other well-studied multi-drug resistance proteins substrates (including verapamil, vinblastine and rifampicin) inhibit DrrAB-mediated doxorubicin transport, indicating they are also substrates of DrrAB pump. Kinetic studies show
competitive inhibition of doxorubicin transport by Hoechst 33342 and rifampicin and non-competitive inhibition by verapamil, suggesting the possibility of more than one drug binding site in the DrrAB system. This is the first in-depth study of a drug resistance system from a producer organism, and it shows that a dedicated efflux system like DrrAB contains specificity for multiple drugs.

Our study also provides the first direct evidence for the dual role of the metalloprotease FtsH in the biogenesis of membrane proteins. We found that FtsH is not only responsible for proteolysis of unassembled DrrB protein but it also plays a much broader role in biogenesis of the DrrAB complex. DrrA and DrrB proteins expressed together in a temperature sensitive ftsH mutant strain were found to be non-functional due to their incorrect assembly. Simultaneous expression of wild-type FtsH in trans resulted in normal doxorubicin efflux. Strikingly, doxorubicin efflux could be restored in mutant cells irrespective of whether FtsH was expressed simultaneously with DrrAB or expressed after these proteins had already accumulated in an inactive conformation, thus providing crucial evidence for the ability of FtsH to refold the misassembled proteins. Complementation experiments also showed that the catalytic ATP binding domain of FtsH contains a chaperone-like activity, however both the catalytic and the proteolytic domains of FtsH are required to be present and work coordinately to participate in biogenesis of DrrAB complex in the membrane.

INDEX WORDS: Multi-drug Resistance, ABC transporter, DrrAB, FtsH, Drug efflux
CHARACTERIZATION OF DRRAB COMPLEX FROM *STREPTOMYCES PEUCETIUS* AS A MULTIDRUG TRANSPORTER

by

WEN LI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences

Georgia State University

2014
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December 2014
ACKNOWLEDGEMENTS

I owe a great many thanks to a great many people who helped me and supported me during the study of my Ph.D. I truly apologize if I have forgotten to mention someone.

First, I would like to express my deepest appreciation to my advisor Dr. Parjit Kaur, who gave me guidance, encouragement, caring and support during my Ph.D. I would never have been able to get these achievements without your help. You patiently guided me and inspired me during the hardest time. Thank you for all these special moments for my life.

I would also like to thank my committee members, Dr. Phang C. Tai and Dr. Casonya Johnson for their critical comments and suggestions for my research. I am very thankful to my fellow lab members, who listen to me, help me and support me all the time. Special thanks to Dr. Han Zhang and Sadia Jannath, who are my good friends, accompanies and excellent researchers. I would also like to express my gratitude to members in the core facility, the other professors in the biology department and staff in the biology department.

I would like to acknowledge all my friends who cares me sometimes more than myself. They make my life more colorful and meaningful.

Finally, there is never enough thanks to my family, they are part of my life and they complete me. My parents brought me up so many years; I owe a lot to them. My husband, Mr. Ning Song, is my strongest support during these years, thanks for holding my hands all the time during the good and bad times. Even though my son, Eric Song, is too small to contribute to my dissertation, his smile and love make me stronger and my life more meaningful. A special thanks to my extended family, the Song’s family, for their unconditionally support and love.
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1 GENERAL INTRODUCTION

1.1 Multidrug Resistance and ABC Transporters

In the chemotherapies of cancer and infectious diseases, multidrug resistance (MDR) is seen to develop in more than 90% of the patients. This resistance can be caused by the decreased uptake of drugs, altered cell cycle checkpoint, altered drug target or the increased efflux of drugs by drug transporter (1). In tumor cells, the major reason for multidrug resistance is the over efflux of anti-cancer drugs. The first and the most well-characterized multidrug transporter in human cells is P-glycoprotein (P-gp), which belongs to the ATP-binding cassette (ABC) superfamily. Proteins in this family share highly conserved domains and functions, including nucleotide binding domains (NBDs) and transmembrane domains (TMDs). Members of the ABC superfamily range from prokaryotes to eukaryotes. In eukaryotes, most ABC transporters only carry out drug efflux, while in prokaryotes, ABC proteins include both importers and exporters (2).

Most members of the ABC superfamily mediate the transport of highly specific substrates, following the “lock-key” hypothesis (3); however, some transporters and are categorized as multidrug transporters. Well-characterized multidrug transporters in eukaryotic or prokaryotic cells include P-gp, MsbA, LmrA and Sav1866, etc. (4-7). This study focuses on the prototype drug transporter DrrAB found in Streptomyces peucetius, a soil organism that produces anticancer drugs doxorubicin (Dox) and daunorubicin (Dnr) (8). The DrrAB system shows sequence, structural and functional similarities to the P-gp drug transporter of human cells (9,10).
1.2 Characterization of P-glycoprotein

P-gp is known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1). It was first discovered by Biedler and coworkers, who found that Chinese Hamster cells, which are resistant to actinomycin D, are also cross-resistant to other several antibiotics (11).

P-gp is encoded by a single polypeptide chain with 1280 amino acids and assembled as two homologous halves in the plasma membrane (12). These two halves are not identical, but share 43% sequence identity and 78% similarity (12). They are connected by a central linker. Each half contains one TMD (with six transmembrane helices (TMH)) and one cytoplasmic ATP binding domain or NBD (13). The two TMDs from each halve form a large drug transport channel with 12 TMHs connected by loops, while the two ATP binding domains constitute the cytosolic ATP binding pockets. The NBD contains conserved motifs shared by other proteins in the ABC transporter family, such as Walker A (P-loop), Walker B, ABC signature, Q-loop and switch region (H-loop) (14). These conserved motifs are involved in ATP binding and hydrolysis, energy transduction, and the cross-talk between NBD and TMD. The NBDs of most ABC transporters share high homology irrespective of their substrate specificities while the sequence and structure of TMDs varies significantly (15).

P-gp is expressed in tumor cells and normal tissue cells, its main physiological function is to protect sensitive organs, such as intestine, brain or placenta by pumping the drugs or toxic agents into bile, urine or lumen of gastrointestinal tract (16). In tumor cells, P-gp is over-expressed thus leading to the resistance of anti-cancer drugs used for chemotherapy.
1.3 P-glycoprotein is a multidrug transporter

P-gp can interact with a wide range of structurally unrelated chemical compounds, including natural products, anticancer drugs, steroids, fluorescent dyes, linear and cyclic peptides, etc. According to the type of interactions, these drugs are classified as substrates or modulators. **Substrates** that are transported by P-gp through the membrane, such as anthracyclines (doxorubicin, daunomycin), and vinca alkaloids (vincristine, vinblastine) (17). P-gp can also translocate fluorescent phospholipids such as C6-NBD-diacylglycerol (18) or lipid-based drugs. **Modulators** can block the function of the transporter and generate a drug concentration gradient; some of them can also be effluxed by the transporter, such as verapamil and cyclosporine A (19). A modulator has very significant clinical application by reversing the multidrug resistance problem during cancer chemotherapy. A combination of anti-cancer drugs and modulators will greatly improve the effect of cancer cells treatment (20).

1.4 Mechanism of drug transport

Gottesman and co-workers had proposed the hydrophobic vacuum cleaner model (4,17), which now has been extended to many other MDR proteins (21). In this model, substrate first interacts with the lipids phase. With the energy provided by ATP hydrolysis, P-gp detects the hydrophobic substrate from the lipid bilayer and extracts the substrates from inner leaflet to the extracellular medium (22-26). Strong evidences to support this hypothesis include the early studies by Victor Ling’s group, which suggested that P-glycoprotein transports Hoechst 33342 or LDS-751 from the cytoplasmic leaflet of the plasma membrane directly to the extracellular medium (22,27,28). LmrA, the homologue of Pgp, may also transport lipophilic substrate by a vacuum cleaner mechanism (29).
The complete process of drug efflux requires a series of conformational changes of the drug transporter, which can be divided into four steps (30,31).

1. Initial binding of substrate to the drug binding pocket;
2. ATP binding and dimerization of two NBDs;
3. Hydrolysis of ATP resulting in the efflux of the drugs, which causes drug binding affinity to switch from high to low;
4. Resetting of the transport back to the original state after the reaction cycle.

1.5 Drug binding sites on Pgp

During the last two decades, the studies of P-gp drug transporter have revealed specific drug binding locations, the kinetic interactions of multiple drugs and the energy coupling between ATP hydrolysis and drug efflux.

As P-gp can interact with unrelated chemical compounds, the question whether Pgp contains one single flexible and open drug binding site or multiple drug binding sites with different binding affinities is still not clear. The presence of multiple drug interaction sites has been demonstrated by a number of groups. For example, in 1997, Shapiro and Ling’s group identified two drug binding sites on P-gp. The H-site preferentially transports Hoechst 33342 and the R-site preferentially transports rhodamine 123 (32). These two sites interact in a cooperative manner. Later, they further discovered the third drug-binding site, which has a positively allosteric effect on the drug transport through H and R sites (33). In 2000, Richard Callaghan found the presence of at least four distinct drug interaction sties on P-gp using equilibrium and kinetic radioligand binding assays (34). Specific residues potentially involved in the drug binding were subsequently identified by Loo and Clark via a combination of cysteine scanning mutagenesis and thiol-modification techniques. The thiol-reactive compounds include
the drug substrate dibromobimane (dBBn) (35-37) and the drug substrate analogues methanethiosulfonate (MTS)-verapamil (38) or MTS rhodamine (39). First, single cysteine substitution mutation of every residue in the TMDs was generated; the expression level and function of these mutations were confirmed. The residues protected by drug substrates from being labeled by thiol-reactive analogs were identified as potential drug binding sites. This work showed that residues in TMHs 4-6 in TMD1 and TMHs 9-12 in TMD2 of P-gp are involved in the drug binding process.

P-gp has been proven to contain multiple drug binding sites by various techniques; however, other studies suggest that P-gp contains a large single drug binding pocket. Loo and Clark showed that the P-gp drug binding pocket can bind verapamil and TMEA simultaneously, and these two drugs may occupy different regions of the shared drug-binding pocket (40). This phenotype is also supported by low-resolution cryo-electron microscopy structure of P-gp, which present the existence of a large, 5 nm diameter, central chamber for drug binding inside of P-gp (41). A much clearer crystal structure was not obtained until 2009 by Chang’s group. The apo-P-gp structure contains an internal cavity of ~6,000 Å³ with a 30 Å separation of two NBDs (42). This large internal cavity could fit at least two substrates simultaneously and the drug binding pocket is mainly occupied by hydrophobic and aromatic residues (43-46).

1.6 DrrAB is a multidrug transporter

*Streptomyces peucetius* produces two anti-cancer drugs, Dox and Dnr, and is self-resistant to these drugs due to the efflux action of membrane transporter DrrAB (47). DrrA and DrrB proteins are coded by the *drrAB* operon present within the Dox biosynthesis gene cluster (47). DrrA is the catalytic subunit (36 kDa). It belongs to the ABC family of proteins and binds ATP in a Dox-dependent manner (48). DrrB is an integral membrane protein (30 kDa) with
eight transmembrane $\alpha$-helices and belongs to the ABC-2 subfamily (3). Two subunits each of DrrA and DrrB interact together to form a functional tetrameric complex (49). Both DrrAB and P-gp belong to the ABC (ATP-binding cassette) superfamily of proteins. The DrrAB system belongs to the Class III, DRA family, and DRR subfamily of ABC proteins (50). The NBD of DrrA shares 29% identity and 47% similarity to NBD1 and NBD2 of Pgp (48). Thus the bacterial DrrAB system could serve as a prototype model system for understanding the function and evolution of multidrug transporters in general.

Due to its dedicated nature, the DrrAB system was previously believed to form a single-drug-transporter. However, in our recent studies, it was shown that DrrAB is a multidrug transporter that can carry out efflux of at least 3 different drugs: Dox, Hoechst 33342 and ethidium bromide (8). Inhibition studies also suggest that DrrAB can interact with a number of other well-characterized MDR substrates, such as verapamil, rifampicin and colchicine. The interactions between two different drugs in DrrAB were also studied by kinetics analysis, which revealed competitive inhibition of Dox efflux by Hoechst 33342 and rifampicin but non-competitive inhibition of Dox efflux by verapamil. These findings indicate that DrrAB is a multidrug transporter with at least two drug binding sites. This work also highlights overlaps of the substrate specificity of the DrrAB system and Pgp as well as other bacterial MDR systems and points to a common mechanism, and perhaps origin, for most MDR proteins.

1.7 Quality control of MDR proteins in membranes

One of the major causes of MDR is the over-expression of the drug transporters, which results in efflux of anti-cancer drugs out of the cancer cells. Our previous studies have indicated that DrrA and DrrB proteins need to co-assemble in the membrane to maintain the normal function of the DrrAB complex (51). In the absence of DrrA, the expression of DrrB is
undetectable due to its proteolysis by FtsH on the membrane. FtsH, also defined as HflB, is an inner membrane-embedded, ATP and Zn$^{2+}$-dependent metalloprotease (52). It has been shown to play a role in quality control of soluble proteins, such as σ$^{32}$, as well as membrane proteins including SecY and YccA, etc. (53-55). FtsH protein consists of two domains: the N-terminal AAA domain which carries out hydrolysis of ATP, and the C-terminal proteolytic domain (52). The degradation process of FtsH is triggered by the hydrolysis of ATP and conformational changes of the hexameric ring structure of FtsH (52). Once the peptide tail is bound by FtsH, the peptide can be unfolded to an open structure, extracted from the membrane lipid bilayer and translocated through the central pore into the protease chamber for further digestion (56). As FtsH has relatively low unfolding activity, it preferentially degrades proteins with natural structure or low thermostabilities (57).

In our studies, we have demonstrated that FtsH plays dual roles in the quality control of the DrrAB complex; it is not only involved in proteolysis of the unassembled DrrB protein, but also facilitates assembly of the DrrAB complex (58). We showed that the DrrAB complex is improperly assembled in an FtsH-deficient cell and loses its drug efflux function. However, when FtsH is co-expressed in trans in FtsH-deficient cells, the function of the DrrAB complex is fully restored. Interestingly, the Dox efflux activity could be restored in FtsH-deficient cells irrespective of whether FtsH was expressed simultaneously with DrrAB or expressed after these proteins had already accumulated in a misfolded conformation. To facilitate this assembly function, both the AAA domain and proteolytic domain need to be present and work coordinately to refold the misfolded DrrAB into correct conformation.
2 DUAL ROLE OF THE METALLOPROTEASE FTSH IN BIOGENESIS OF THE DRRAB DRUG TRANSPORTER

2.1 Introduction

Membrane proteins play essential roles in cell physiology. They carry out import of nutrients, export of toxins, antibiotics and drugs, and play important roles in energy and signal transduction. Improper assembly of membrane proteins is known to result in various diseases. However, because of the complexity of the assembly process and the diverse array of factors involved, understanding mechanisms of membrane protein assembly poses a serious challenge. The bacterial DrrAB (doxorubicin resistance proteins A and B) system is an attractive model for studying assembly of ABC (ATP-Binding Cassette) transporters. In this system, the catalytic function (DrrA) and the transport function (DrrB) are present on separate subunits (59), which together form a tetrameric complex in the membrane (60) and carry out ATP-dependent efflux of the anti-cancer drugs doxorubicin (Dox) and daunorubicin (61). Previous studies from this lab suggested that for proper function of the DrrAB complex the DrrA and DrrB proteins may be required to co-assemble (51). It was also shown that interaction between DrrA and DrrB is essential for stable maintenance of DrrB in the membrane so that the expression of DrrB is undetectable in the absence of a simultaneous expression of DrrA (60). Co-expression of DrrA in cis or trans restores the wild-type levels of DrrB expression, therefore suggesting that DrrA protects DrrB from proteolysis by a cellular protease. The nature of the protease and whether it plays a specific role in quality control and biogenesis of the DrrAB complex has so far remained uncharacterized. In this study, we examine the role of FtsH (filament temperature-sensitive protein H) in this process.
FtsH is a zinc-dependent metalloprotease which belongs to the AAA (ATPases Associated with Diverse Cellular Activities) family of proteins. Along with other proteases, such as ClpAP, ClpXP, HsIUV, and Lon, these proteins form the large AAA superfamily of proteins, members of which share a similar AAA-ATPase domain (62). FtsH is evolutionarily conserved with more than 40% sequence identity observed between bacterial, yeast and human homologs (63). *E. coli* FtsH is the best studied of all known members, and it has been shown to be the only growth-essential protease in *E. coli*. Yeast cells lacking the three FtsH orthologs (two m-AAA and one i-AAA) were also found to be non-viable, demonstrating the essential function of this enzyme in eukaryotic cells (63,64). FtsH is unique in being embedded in the cell membrane in *E. coli*, and in the mitochondrial inner membrane in eukaryotes (65), where it forms homohexameric ring-like structures. The major role of FtsH is believed to be in the quality control of specific membrane proteins, such as degradation of the unassembled SecY and subunit ‘a’ of F₀ sector of the ATP synthase, in addition to modulating levels of some soluble regulatory proteins (65-67).

FtsH contains two transmembrane helices at the N terminus, followed by a cytoplasmic domain containing the catalytic AAA domain in the middle and the proteolytic domain at the C terminus. The AAA domain (residues 144-398) consists of the conserved Walker A, Walker B and SRH (second region of homology) motifs, which are essential for ATP binding and hydrolysis (65). The proteolytic domain contains the conserved Zn^{2+} binding motif H^{417}EXXH^{421}, the third Zn^{2+}-ligand residue E^{479}, and the coiled-coil leucine-zipper sequence (65). FtsH carries out proteolysis of polypeptides in an ATP and Zn^{2+}-dependent manner, while other AAA+ proteases, such as Lon and ClpA/P, are serine peptidases (68). To initiate proteolysis of a membrane protein, the putative polypeptide binding site in the catalytic AAA
domain of FtsH is believed to capture the cytoplasmic tail (either at the N or the C terminus) of the membrane substrate, followed by dislocation and processive unfolding of the protein to an open structure (65). Therefore, both the catalytic AAA domain and the proteolytic domain are required for proteolysis.

Although the major function of the AAA⁺ proteases is in proteolysis, they also exhibit chaperone-like activities, which allow them to monitor the folding status of a protein, promote disassembly or unfolding (69), and specifically degrade non-native proteins (70). For a long time, it has been speculated that the AAA⁺ proteases may also have the ability to refold their substrate proteins (71), which remains an open question till date (72). FtsH was originally identified by Ito and co-workers in a screen to isolate factors which may assist in membrane protein assembly (73). They used SecY-PhoA fusion to screen for stop-transfer defect (Std) mutations, and found that such a mutation lies in the ftsH gene. Depletion of FtsH also resulted in significant export defects of β-lactamase and OmpA in E. coli in addition to causing a strong Std phenotype. Together, these studies indicated that FtsH may be involved in protein assembly into and through the membrane and may play a role in determining orientation of membrane proteins (73). In another study, FtsH orthologs Yta10 and Yta12 in yeast mitochondria were shown to be required for the formation of a 48 kDa assembly intermediate of the F₀ subunit 9 (74). Finally, in vitro studies showed that the purified AAA domain of Yme1, a yeast mitochondrial homolog of FtsH, suppresses aggregation of a model polypeptide (75). Despite these observations, however, no direct evidence for the role of FtsH or its homologs in functional assembly of membrane proteins has been obtained so far.

In this study, we provide the first direct evidence that FtsH is a dual function enzyme containing both the protease and assembly functions. We show that not only is FtsH responsible
for removal of the unassembled DrrB but that it is actually able to refold previously misassembled DrrAB proteins and restore Dox efflux function of the complex. Our results also show that while the AAA domain of FtsH provides recognition and specificity for binding of the substrate, both ATP hydrolysis and the proteolytic functions of FtsH are used concurrently for refolding of DrrAB and restoration of function. Our studies, therefore, not only shed light on the mechanism of assembly of the DrrAB complex but also further elucidate the function of the FtsH protease.

2.2 Materials and Methods

Strains and plasmids – The E. coli strains and plasmids are described in Table 1.

Media and growth conditions – E. coli cells were normally grown in LB medium at 30°C or 37°C, unless indicated otherwise. Chloramphenicol, kanamycin, or ampicillin was added to a final concentration of 20, 30 or 100 µg/ml, respectively, where indicated. E. coli cells used for the Dox efflux assay were grown in TEA medium (61).

Site-directed mutagenesis of ftsH – Site-directed mutagenesis of the ftsH gene was carried out by a Stratagene QuickChange Multisite-directed mutagenesis kit (La Jolla, CA). Using pUC18/ftsH plasmid as the template, Lys<sup>198</sup>, located in the conserved Walker A motif of the AAA domain, was changed to asparagine. The resulting plasmid was named pUCftsH(K198N). Another mutant, named ftsH(HEH), was obtained by substituting His<sup>417</sup>Glu<sup>418</sup>His<sup>421</sup> in the conserved HEAGH motif present in the proteolytic domain to Ala<sup>417</sup>Gln<sup>418</sup>Ala<sup>421</sup>. The primers for both substitutions are shown in Table S1.

Subcloning of the AAA domain of ftsH into pUC18 vector – To completely remove the proteolytic domain, a fragment of ftsH corresponding to the first 1194 base pairs was PCR-amplified and ligated into the pUC18 vector using the EcoRI and HindIII restriction enzymes.
resulting in pUC18(AAA). This construct is referred to as ftsH(AAA) in this article. The primers are shown in Table S1.

**Subcloning of ftsH and the groESL genes** – Wild-type ftsH, ftsH(K198N), ftsH(HEH) and ftsH(AAA) genes were subcloned into pET28a vector using SnaBI and HindIII restriction enzymes. The resulting plasmids were named pETftsH, pETftsH(K198N), pETftsH(HEH) and pETftsH(AAA), respectively. The groES/L genes were subcloned from pKY326 (76) into pUC18 vector using EcoRI and SmaI restriction enzymes resulting in pUCgroESL.

**Cloning of ftsH into the pBAD vector** – Using pBAD/HisA (Invitrogen) as a template, a 3.9kb fragment between NcoI and HindIII sites was amplified. The NcoI site was substituted with the XhoI site in the primers used for amplification (Table S1). This resulted in deletion of the multiple cloning sites and the polyhistidine region. The ftsH gene was amplified from pUCftsH using primers containing XhoI and HindIII sites and ligated to the above fragment.

**Growth and Protein expression** – *E. coli* TG1, AR796, AR797, *E. coli* SG1110, and *E. coli* SG1126 cells containing the indicated plasmids were grown at 30 °C or 37 °C to mid-log phase (A_{600nm}=0.6). The proteins were induced by addition of 0.1 or 0.25 mM IPTG, and the incubation was continued at 30 °C, 37 °C, or 42 °C for 3 h. The cells were spun down, resuspended in 5 ml lysis buffer (2 mM Tris-Cl, pH 7.5, 20% glycerol, 2mM EDTA, 1 mM DTT) and lysed by a single passage through a French pressure cell at 20,000 p.s.i. After centrifugation at 10,000 X g for 15 min, the pellet represented the inclusion body fraction. The supernatant was centrifuged at 100,000 X g for 1 h to separate the supernatant (cytosolic fraction) and the pellet (membrane fraction). The membrane, cytosol and the inclusion body fractions were analyzed by 12% SDS-PAGE, followed by Western-blot analysis using anti-DrrA or anti-DrrB antibodies (60).
**Purification of the FtsH protein** – FtsH protein was purified according to published protocols (66) with modifications. *E. coli* HMS174(DE3) cells containing the pETftsH plasmid or its variants were inoculated in 1 liter LB medium supplemented with 30 µg/ml kanamycin at 37 °C. The cells were grown to mid-log phase and induced with 0.25 mM IPTG at 20 °C overnight. The membrane fraction was prepared as described earlier (60). 5 mg of the membrane fraction was solubilized with 5 ml solubilization buffer (50 mM Tris-Cl, pH7.5, 500 mM KCl, 0.5 % (w/v) Nonidet P-40, 15% (w/v) Glycerol, 2.9 mM 2-mercaptoethanol). The solubilized protein was purified using Ni\(^{2+}\)-NTA-agarose column and eluted with a gradient of 50 to 500 mM imidazole. Fractions containing FtsH were collected and dialyzed against 2 liters of the dialysis buffer (10 mM Tris-Cl, pH7.5, 15% (w/v) glycerol, 50 mM KCl, 0.5% (w/v) Nonidet P-40, 5 mM MgCl\(_2\), 1 mM dithiothreitol) for 12 hours. The protein was stored at -80 °C until used.

**ATPase activity assay** – The ATPase activity of FtsH was detected by the malachite green-ammonium molybdate colorimetric assay (66).

**In vivo FtsH Proteolytic assay** - Membrane-bound DrrAB proteins were used as a substrate to determine the proteolytic activity of FtsH. pBAD vector or the pBADftsH plasmid was transformed into AR797 cells containing pDX101 (pSU2718drrAB). The cells were grown in LB medium to mid-log phase and the DrrAB proteins were induced with 0.25 mM IPTG at 42 °C for 1 h. To terminate the synthesis of DrrAB, 1000 µg/ml chloramphenicol was added to the cell culture and incubated at 42 °C for 30 min. The cells were washed extensively to remove chloramphenicol and IPTG. After resuspending the cells in fresh medium, synthesis of FtsH from the pBADftsH plasmid was induced by addition of 0.2% arabinose at 42 °C. An aliquot of cell culture was taken out at 0, 30, 60, 90, 120 and 180 min. Membrane fractions were prepared
as described above, and 20 µg total membrane protein was loaded onto 12% SDS-PAGE, followed by Western-blot analysis using anti-DrrA, anti-DrrB or anti-FtsH antibodies.

**In vitro FtsH Proteolytic assay** - α-Casein was used as a substrate in *vitro* to demonstrate the proteolytic activity of purified FtsH and the FtsH(HEH) protein (77). 40 µg of α-casein (Sigma Aldrich) was mixed with 40 µg purified FtsH or FtsH (HEH) in 150 µl protease buffer (50 mM Tris-Cl, pH8.0, 20 mM KCl, 5 mM MgCl₂, 12.5 µM Zn(OAc)₂, 0.5 % (w/v) NP-40, 10 % (w/v) glycerol, 1 mM dithiothreitol) at 42 °C. The reaction was initiated by adding 8 mM ATP, and an aliquot (20 µl) of the sample was taken out at the indicated time points. The reaction was terminated by adding 7 µl of 4×SDS sample buffer. The samples were analyzed by 12% SDS-PAGE, followed by Coomassie Brilliant blue staining.

**Whole cell Dox efflux assay** – The whole cell Dox efflux assay was carried out according to the protocol published previously (61). The fluorescence spectra were recorded on an Alphascan-2 spectrofluorometer (Photon Technology International, London, Ontario, Canada). The slope of the Dox efflux curve of the positive control (the first sample) in each panel was designated as 1.0. The efficiency of Dox efflux of each sample within one panel was calculated by dividing the slope of the efflux curve by the slope of sample 1. The average data obtained from three independent experiments were plotted in the histograms.

### 2.3 Results

**FtsH is responsible for the proteolysis of unassembled DrrB** – Previous studies from this laboratory showed that DrrB is undetectable in wild-type *E. coli* membranes in the absence of simultaneous expression of DrrA ((60), Fig 1A, lane 2), however stable expression of DrrB is seen when both DrrA and DrrB are expressed together (lane 1). These results suggest that DrrB is completely degraded when not complexed with DrrA. Review of recent literature suggested
that proteins of the AAA\(^+\) family, especially FtsH, may be involved in the quality control of membrane proteins (70). To determine if this is true for DrrB, three proteases, including Lon, ClpA/ClpP and FtsH, were investigated. If any of these proteases is responsible for degradation of unassembled DrrB, stable expression of DrrB will be observed in cells deficient in that protease as compared to the wild-type cells. The FtsH-deficient *E. coli* AR797 strain (Table 1) used in this study contains a temperature sensitive mutation in the *ftsH* gene, therefore it was grown at 30 °C but the temperature was switched to 42 °C to inactivate FtsH. The Lon\(^-\) (SG1110) and the ClpA\(^-\) (SG1126) cells were grown normally at 30 °C or 37 °C. The isogenic *E. coli* AR796 parent strain was used as a control. Of the 3 protease-deficient strains tested, only the FtsH-deficient *E. coli* cells showed stable expression of DrrB in the absence of DrrA (Fig. 1B, lanes 1-3) (Fig. 1C, lane 2), while the Lon\(^-\) and the ClpA\(^-\) cells showed no effect on the stability of DrrB (Fig. 1C, lanes 3-4). Since DrrB was not seen in AR796 (wild-type) cells at either 30 °C or 42 °C (Fig. 1B, lanes 1 and 2), but it was stably expressed in the 797 (FtsH\(^ts\)) cells at 42 °C (lane 3), these results show that FtsH degrades unassembled DrrB. In contrast to the expression of DrrB alone, when both DrrA and DrrB were expressed together in wild-type 796 cells at 30 °C or 42 °C, stable expression of DrrB was seen (Fig. 1B, lanes 4 and 5), confirming that DrrA protects DrrB against FtsH proteolysis. Note that the amount of DrrA and DrrB in the wild-type cells was less at 42 °C as compared to at 30 °C (Fig. 1B, lanes 4-5), suggesting that the DrrAB complex acquires a more open conformation at higher temperature resulting in partial proteolysis by endogenous FtsH. Protection of DrrB from FtsH proteolysis by DrrA was also seen in the ClpA\(^-\) and Lon\(^-\) backgrounds (Fig. 1C, lanes 5-6). Together, the data in Fig. 1 show that FtsH plays an important role in quality control of the DrrB protein when DrrA is absent. To rule out the possibility that DrrA or DrrB may aggregate when their
expression is induced at 42 °C, the membrane, cytosol, and inclusion body fractions were prepared from both wild type and FtsH<sup>ts</sup> cells and analyzed by Western blotting using anti-DrrA and anti-DrrB antibodies. The data in Fig. 2 show that although some DrrB protein is present in the inclusion body fraction in both wild type (lower panel, lane 3) and the FtsH<sup>ts</sup> cells (lane 9) at 30 °C, the induction of either strain at 42 °C did not result in any increase in the amount of inclusion body formation (lower panel, lanes 6 and 12). Moreover, no aggregated DrrAB proteins were seen in the stacking region of the gel in any of the fractions of wild type or FtsH<sup>ts</sup> cells, indicating absence of any significant aggregation under the conditions used in these experiments. Note that the anti-DrrB antibody is an anti-peptide antibody, therefore it shows some cross-reactivity with epitopes in some other <i>E. coli</i> proteins, as explained in a previous publication (1).

**Expression of DrrB alone or DrrAB together in FtsH-deficient cells results in growth inhibition**- The growth analysis of the wild type and FtsH<sup>ts</sup> cells expressing DrrB or DrrAB at 30 °C or 42 °C was carried out (Figs. 3A and 3B). The relative growth of various strains at the four hour time point was plotted in a histogram (Fig. 3C). The data in Fig. 3A show that the growth of mutant FtsH<sup>ts</sup> cells is compromised at 42 °C (Fig. 3A; 797, open diamonds; Fig. 3C, Column 4) as compared to the growth of wild-type cells under similar conditions (Figs. 3A, 796, lines; Fig. 3C, Column 2). This result is not surprising due to the essential nature of <i>E. coli</i> FtsH. Interestingly, the expression of DrrB alone in FtsH<sup>ts</sup> cells at 42 °C further inhibited the growth of these cells (Fig. 3A, compare 797, open diamonds with 797/B, filled triangles; Fig. 3C, Column 7). However, growth inhibition was not seen when DrrB was expressed in wild-type cells at 42 °C (compare 796, lines with 796/B, open rectangles; Fig. 3C, column 6). Since DrrB accumulates in FtsH<sup>ts</sup> cells but is proteolysed in wild-type cells (Fig. 1B), it allows us to
conclude that the growth defect seen in mutant cells is caused by the accumulation of unassembled DrrB protein. Surprisingly, however, growth inhibition was also seen in the FtsH\textsuperscript{ts} cells expressing DrrA and DrrB together at 42 °C (Fig. 3B, 797/AB, filled triangles; Fig. 3C, Column 7). This effect was unexpected because DrrA and DrrB can be expressed together in the wild-type cells at 42 °C without any negative effect on their growth (Fig. 3B, 796/AB, open rectangles; Fig. 3C, Column 6). These data indicate that the DrrAB proteins expressed in FtsH\textsuperscript{ts} cells at 42 °C may be misfolded, and the accumulation of misfolded membrane proteins results in growth inhibition. It was also observed that if the FtsH\textsuperscript{ts} cells initially induced at 42 °C were shifted down to 30 °C, the cell growth resumed, albeit slowly. After 43 hours of temperature shift-down, the final growth was about half as compared to the cells induced and maintained at 30 °C (data not shown), indicating that the growth inhibition of these cells is quite severe. In summary, the data in Fig. 3 suggest that FtsH is not only responsible for removing unassembled DrrB (in the absence of DrrA) but it may also be critical for proper assembly of the DrrAB complex in the membrane.

The growth defect of the FtsH\textsuperscript{ts} cells expressing DrrB or DrrAB at 42 °C could be rescued by overexpression of FtsH in trans (Figs. 3A-B, open triangles; Fig. 3C, Column 8), indicating that the absence of functional FtsH was solely responsible for this defect. The growth defect in each case was also suppressed by overexpression of the chaperone GroESL (Figs. 3A-B, filled circles; Fig. 3C, Column 9) but not to the same extent as seen with FtsH. Western blot analysis of the membrane fractions (prepared from the four hour cultures of samples # 6-9 in Figs. 3A-B) showed that while FtsH overexpression resulted in significant proteolysis of DrrAB (Fig. 3D, lanes 3 and 7), overexpression of GroESL did not (lanes 4 and 8). This might imply
that FtsH restores growth by simply removing misfolded DrrAB proteins, while GroESL is able to alter their conformation, thus alleviating growth inhibition.

**FtsH preferentially proteolyzes misfolded DrrAB** - To determine if FtsH discriminates between properly assembled and misfolded DrrAB, the effect of overexpression of FtsH proteolysis was compared in wild type or FtsH<sup>ts</sup> cells at 30 °C or 42 °C. Interestingly, overexpression of FtsH in either wild type (Figs. 4A-B, compare lanes 1 and 3) or FtsH<sup>ts</sup> cells (lanes 2 and 4) produced no significant proteolysis of DrrA and DrrB expressed at 30 °C, showing that FtsH does not proteolyze properly assembled DrrAB. However, when the DrrAB proteins were expressed in wild-type cells at 42 °C, simultaneous overexpression of FtsH resulted in significant proteolysis (Figs. 4A-B, compare lanes 3 and 7). These data suggest that the DrrAB proteins must acquire a partially unfolded conformation at a higher temperature (as also seen in Fig. 1B), thus making them more susceptible to proteolysis by over-expressed FtsH. As expected, overexpression of FtsH in FtsH<sup>ts</sup> cells also showed significant proteolysis of DrrAB expressed at 42 °C (Figs. 4A-B, lanes 4 and 8). This is consistent with the data in Fig. 3, which suggested that the DrrAB proteins expressed at 42 °C in FtsH<sup>ts</sup> cells are misfolded. (Please note that the conformation of the DrrAB proteins in wild-type cells at 42 °C is completely different from the DrrAB expressed in mutant FtsH<sup>ts</sup> cells at 42 °C even though they are both sensitive to overexpressed FtsH. In a later experiment in Fig. 8, it is shown that the DrrAB proteins expressed in wild type cells at 42 °C retain normal function, while the DrrAB expressed in FtsH<sup>ts</sup> cells are inactive due to their misfolding)

The rate of proteolysis of misfolded DrrAB by FtsH was analyzed in a separate time-course experiment. The misfolded DrrAB proteins were first allowed to accumulate in the membranes of FtsH<sup>ts</sup> cells by induction with IPTG for 1 hour at 42 °C. Chloramphenicol was
added to stop further synthesis, as described under Methods. The synthesis of FtsH from pBAD/ftsH was induced by addition of arabinose, and the proteolysis of DrrA and DrrB by FtsH was determined by Western blot analysis. The data in Figs. 4C and 4D (filled circles) show that synthesis of FtsH (Fig. 4E, lower panel) resulted in increasing proteolysis of misfolded DrrA and DrrB from the membrane. At 120 minutes after addition of arabinose, about 75-80% of DrrA and DrrB were removed from the membrane. These observations are in agreement with the dislocation model proposed previously for the activity of FtsH (65). No significant proteolysis of DrrAB was seen in the absence of FtsH synthesis (Figs. 4C and 4D, filled rectangles).

The AAA domain of FtsH contains a chaperone-like activity, but it is not sufficient by itself to restore the Dox efflux function - The growth experiment in Fig. 3B suggested that FtsH may be critical for the assembly of the DrrAB complex. Further support for this idea was obtained by comparing the rate of assembly of the DrrAB complex in wild type and FtsH<sup>ts</sup> cells. The data in Fig. 5 show that the assembly of DrrA and DrrB in the membrane of FtsH<sup>ts</sup> cells is significantly compromised already at 30 °C as compared to in the wild type cells. A significant difference in the amounts of DrrAB in the membrane of wild type and mutant cells was seen at all time points tested (Fig. 5, compare lanes 1-3 with 7-9). However, this difference is most evident at the early time points, which suggests that the rate of assembly of DrrAB is affected by FtsH. This is most likely due to the partial defect of FtsH function in FtsH<sup>ts</sup> cells already at 30 °C, leading to the low efficiency of the DrrAB complex formation.

To determine if the ability to promote assembly of DrrAB resides in the AAA domain of FtsH, variants containing mutations in the Walker A motif of the AAA domain (K198N mutation) or the conserved amino acids in the proteolytic domain (the HEH mutation and the AAA subclone are described under Methods) were created. The K198N allele contained a
defective AAA domain, while the HEH mutant and the AAA subclone contained an intact AAA domain. As expected, the K198N mutation resulted in a significantly reduced ATPase activity, however the HEH mutant and the AAA subclone were unaffected (Table 2). The *in vitro* proteolytic activity assay showed that while the wild-type FtsH completely proteolyzed α-casein in one hour, no significant reduction in the α-casein level was seen with the HEH mutant even after two hours of incubation (Fig. 6). These analyses confirmed that the AAA and the proteolytic domain mutants behave as expected. Therefore, they were used in two different complementation experiments (described below) to determine if the AAA domain by itself is sufficient for promoting assembly of the DrrAB complex.

The expression of the DrrAB proteins in FtsH<sup>ts</sup> cells was previously shown to result in severe growth inhibition (Fig. 3B, filled triangles). This inhibition was reversed by simultaneous expression of FtsH (Fig. 3B, open triangles). In the next experiment, we asked whether co-expression of the HEH allele or the AAA subclone can rescue FtsH<sup>ts</sup> cells from the growth inhibition resulting from DrrAB expression. The data in Fig. 7 show that the simultaneous expression of either the HEH mutant (Fig. 7A, open circles) or the AAA subclone (Fig. 7A, filled triangles) with DrrAB can complement the growth defect of FtsH<sup>ts</sup> cells, indicating that the AAA domain of FtsH indeed contains a chaperone-like activity. Interestingly, the HEH mutant showed much better complementation of the growth defect as compared to the AAA subclone, perhaps due to a more native conformation of the full-length HEH protein as compared to the AAA subclone. The K198N mutation, on the other hand, showed no growth complementation effect (Fig. 7A, open triangles), showing that the ATPase activity associated with the AAA domain is important for the chaperone function of FtsH. Western blot analysis showed that the DrrAB complex is membrane-associated in the FtsH(HEH), FtsH(AAA), and FtsH (K198N)-
containing strains (Figs. 7B and 7C, lanes 5-7), and the amounts of DrrA and DrrB in these cells were comparable to the levels in their absence (lane 3). Therefore the restoration of growth by the HEH and AAA clones must result from a change in conformation of the DrrAB proteins brought about by the functional AAA domain present in these two clones. Whether the HEH and AAA variants of FtsH can also restore function of the DrrAB complex is addressed in the next experiment.

We previously showed that the wild-type DrrA and DrrB proteins together carry out ATP-dependent efflux of the anticancer drug doxorubicin (61). Here, we investigated whether co-expression of the AAA subclone or the HEH allele can restore the Dox efflux function of the misassembled DrrAB proteins expressed in FtsH\textsuperscript{ts} cells at 42 °C (Fig. 8). The data in Fig 8A indicate that the rate of DrrAB-mediated Dox efflux at 30 °C in the wild-type 796 and FtsH\textsuperscript{ts} 797 cells is comparable (Columns 1 and 3). Induction of wild-type cells at 42 °C showed only a slight reduction in Dox efflux (Column 2), which is likely due to the destabilization effect produced by high temperature on DrrAB, as seen earlier in Figs. 1 and 4. By contrast, the FtsH\textsuperscript{ts} cells induced at 42 °C showed very little or no DrrAB-mediated Dox efflux (Fig. 8A, column 4), which was comparable to the efflux seen with control cells containing empty vector (Fig. 8A, Columns 5 and 6). These data confirm that the DrrAB proteins are misfolded in the absence of functional FtsH. Simultaneous overexpression of the FtsH variants, K198N, HEH or the AAA subclone, in FtsH\textsuperscript{ts} cells did not restore function of misassembled DrrAB (Fig. 8B, columns 4-6), even though restoration of growth by HEH and the AAA subclone was earlier seen in Fig. 7A. Similarly, overexpression of GroESL also did not complement the DrrAB-mediated Dox efflux in FtsH\textsuperscript{ts} cells (Fig. 8B, column 7). One possible explanation for these data could be that even though the AAA domain of FtsH (or GroESL) is able to alter the conformation of the DrrAB
proteins and relieve growth inhibition, it is not sufficient by itself to restore proper conformation required for full function of the complex.

**Wild-type FtsH can refold previously misassembled DrrAB and restore function** - Surprisingly, co-expression of wild-type FtsH restored the Dox efflux function of the misassembled proteins expressed in FtsH<sup>ts</sup> cells at 42 °C resulting in a significant recovery (about 45%) of Dox efflux by the DrrAB complex (Fig. 8B; compare columns 2 and 3). Since this could only have resulted if FtsH expressed in *trans* facilitated assembly of the complex, these results imply that the AAA and the proteolytic domains of FtsH must work hand-in-hand to bring about functional assembly of the DrrAB complex. In this experiment, however, FtsH and the DrrAB proteins were expressed simultaneously by IPTG induction, therefore it was not possible to determine if FtsH assists only the newly synthesized DrrAB to achieve proper conformation, or if it can also bring about refolding of the DrrAB proteins that have already been misfolded.

Therefore, in the next experiment, DrrAB and wild-type FtsH were expressed in FtsH<sup>ts</sup> cells in a sequential manner. The *ftsH* gene subcloned under the control of the *araBAD* promoter was induced by arabinose, while the *drrAB* genes remained under the control of the *lac* promoter induced by IPTG. The FtsH<sup>ts</sup> cells containing both the plasmids (797/AB/pBADftsH) were grown at 30 °C to mid-log phase. The DrrAB proteins were first induced with IPTG at 42 °C for 1 hour (this condition inactivates FtsH and renders DrrAB inactive as seen in Figs. 8A and 8B). The cells were then washed several times to remove extracellular IPTG and stop further synthesis of DrrAB. The expression of FtsH was induced by arabinose for 1 hour at 42 °C, and the cells were subjected to the Dox efflux assay. To maintain the chromosomally-encoded FtsH in an inactive conformation, the temperature was maintained at 42 °C throughout the duration of
the experiment. As previously seen in Fig. 8B, simultaneous expression of DrrAB and FtsH resulted in restoration of Dox efflux (Fig. 8C; 797/AB/pUCftsH, column 3). More interestingly, however, even greater restoration of the DrrAB-mediated Dox efflux was seen when FtsH was induced after DrrAB proteins had been pre-synthesized in these cells (Fig. 8C, 797/AB/pBADftsH+ara, column 5). In the absence of arabinose induction of FtsH, much lower restoration of Dox efflux was seen (Fig. 8C, 797/AB/pBADftsH – ara, column 4). These data, therefore, show that the sequential expression of DrrAB and FtsH can still restore the function of the previously misfolded DrrAB proteins to the same (or even higher) extent as seen with simultaneous expression.

Finally, the effect of over-expression of FtsH on Dox efflux function of DrrAB expressed at different temperatures was investigated. Irrespective of whether the DrrAB proteins were induced at 30 °C, 37 °C, or 42 °C in FtsH<sup>ts</sup> cells, simultaneous expression of FtsH resulted in a very similar final Dox efflux efficiency (Fig. 8D). At 42 °C, co-expression of FtsH enhanced Dox efflux of misfolded DrrAB 5-fold (compare columns 3 and 6), yielding about 45% Dox efflux efficiency. Interestingly, over-expression of FtsH at 30 °C or 37 °C reduced the efficiency by about half (compare columns 1-2 with 4-5), once again yielding final Dox efflux efficiency of about 45%. These results imply that FtsH produces an optimal level of functional complexes in the membrane perhaps by exerting both proteolytic and refolding effects concurrently.

2.4 Discussion

Non-native proteins, especially unassembled membrane proteins, interfere with cellular processes and are known to become toxic to the cells. Therefore, quality control systems, consisting of chaperones and proteases, play essential roles by monitoring their folding and either refolding or degrading misfolded proteins (78-80). Hsp60 (GroEL/ES) and Hsp70 (DnaK)
proteins provide classical examples of ATP-dependent chaperones which prevent aggregation of newly translated proteins and promote their refolding (79). A special class of chaperones (e.g. ClpB in bacteria and its homologs Hsp78 and Hsp104 in eukaryotes) is known to resolubilize protein aggregates and, in cooperation with the Hsp70 chaperones (specifically DnaKJE), can result in regaining function of the affected protein (72). On the other end of the spectrum are proteins classically defined as proteases, for example Lon, ClpA/P, ClpX/P and FtsH, whose major function is considered to be removal of irreversibly damaged proteins from the cell (63,71,81). Despite their differences, however, both classical chaperones and proteases share common features. For example, both have the ability to recognize and bind non-native polypeptides and both bring about unfolding of their substrates, which are subsequently refolded (by a chaperone) or degraded (by a protease)(69,79,82). Because of the ATP-dependent unfolding function of AAA+ proteases, it has been speculated that they may also have the ability to refold substrate proteins and may participate in protein biogenesis. However, very little direct evidence is available for the role of FtsH or other AAA proteases in biogenesis, especially of membrane protein complexes. In this study, we provide clear evidence that the *E. coli* FtsH is able to both degrade and refold misassembled DrrAB proteins, resulting in regaining the Dox efflux function of the membrane complex.

We show that in the absence of the DrrA protein, DrrB acquires an FtsH-sensitive conformation and is completely proteolyzed. However, in the absence of functional FtsH, the DrrB protein accumulates even in the absence of DrrA confirming that FtsH monitors the folding status of DrrB and removes it if it is improperly assembled. The molecular details of proteolysis of DrrB by FtsH are currently unknown, however based on the prevalent model for its action (65,83) we assume that FtsH could initiate proteolysis of DrrB either at the N- or the C-terminal
end (both of which are found in the cytoplasm, (84)). Cross-linking studies previously showed that the N terminus of DrrB is the major site of interaction with DrrA (61,84); therefore we propose that proteolysis of DrrB initiates at its N-terminal tail, and binding of DrrA to this region of DrrB protects it from proteolysis by FtsH.

Interestingly, we found that the function of FtsH is not limited to proteolysis of unassembled DrrB, but it also plays an essential role in folding and assembly of the DrrAB complex. This conclusion is supported by several lines of evidence presented in this paper. First, the expression of DrrA and DrrB together in FtsH<sup>ts</sup> cells at 42 °C (which results in inactivation of FtsH) was found to be growth inhibitory (Fig. 3B) suggesting that the complex is improperly assembled in the absence of a functional FtsH. Second, the rate of assembly of the DrrAB complex in the FtsH<sup>ts</sup> cells at 30 °C was found to be significantly reduced as compared to the wild-type cells (Fig. 5). Third, functional analysis showed complete absence of the DrrAB-mediated Dox efflux in FtsH<sup>ts</sup> cells under conditions of FtsH inactivation, suggesting that most or all of the DrrAB proteins expressed in these cells at 42 °C are misassembled. By contrast, the DrrAB proteins expressed in wild-type cells at 42 °C retained on average 85-90% of the Dox efflux activity (Fig. 8A). Finally, co-expression of FtsH in trans in FtsH<sup>ts</sup> cells restored the ability of DrrAB to carry out Dox efflux, confirming that FtsH facilitates assembly of the DrrAB complex (Fig. 8B). Nevertheless, this result was surprising because FtsH contains a functional proteolytic domain. It’s overexpression in FtsH<sup>ts</sup> cells results in proteolysis of the misfolded DrrAB proteins (as seen in Fig. 4), however the data in Fig. 8B show that FtsH also facilitated some folding resulting in about 45% recovery of the Dox efflux activity. Either the AAA domain by itself or the GroESL chaperone was unable to complement the Dox efflux function of DrrAB in FtsH<sup>ts</sup> cells, even though each was able to alleviate the growth defect. Therefore,
together these data suggest that both the AAA and the protease domains of FtsH are essential for promoting functional assembly of DrrAB.

The most crucial evidence for the refolding function of FtsH, however, came from the sequential expression studies. Irrespective of whether FtsH was expressed simultaneously with DrrAB or expressed after the non-functional DrrA and DrrB proteins had already accumulated in FtsH<sup>ts</sup> cells, it was able to restore the function of the complex (Fig. 8C), thus showing conclusively that FtsH not only facilitates assembly of the DrrAB complex but it is also actively involved in refolding previously misassembled DrrAB proteins. Interestingly, we also found that the sequential expression of DrrAB and FtsH resulted in a significantly higher recovery of the Dox efflux function of DrrAB as compared to simultaneous expression (Fig. 8C). This finding suggests that FtsH treats its substrate differently during its synthesis as compared to after it has already been synthesized.

In summary, our studies confirm that the AAA domain of FtsH can recognize and bind substrates and change their conformation, which is in agreement with the previous studies (75). However, we also show that the two activities (ATPase and proteolytic) of FtsH must be present simultaneously and occur in a coordinated manner to facilitate assembly and refolding of DrrAB. Much more extensive analysis will be required in the future to understand the nature of the molecular processes involved in refolding of DrrA and DrrB and to determine if other factors also play a role in the assembly of the DrrAB complex. Further studies will also provide clues about how degradation and assembly of multi-subunit complexes are regulated, and whether other AAA+ proteases may also contain chaperone activity. This study raises intriguing questions about the distinction between classical chaperones like GroESL (that can prevent aggregation of many proteins) and the classical proteases like FtsH that not only carry out
proteolysis but also actively participate in refolding of their specific substrates, as shown in this study. Bukau and Mogk previously (79) coined four terms to describe the various activities of chaperones and proteases: Holders (small heat shock proteins, Hsps), Folders (GroESL and DnaK), Unfolders (ClpA, ClpX, and ClpB), and Proteases (Lon, ClpP, and FtsH). In light of the findings reported in this article, we propose a new term ‘Specific Refolder’ to describe the function of FtsH and possibly other AAA+ proteases that may be shown in the future to contain such an activity.
### Table 2.1 Bacterial strains, plasmids and anti-sera

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<td>This study</td>
</tr>
<tr>
<td>pETFtsH</td>
<td>ftsH in pET28a with C-terminal histag, Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pETFtsH(K1)</td>
<td>ftsH(K198N) in pET28a with C-terminal histag, Kan'</td>
<td>This study</td>
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<tr>
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<tr>
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<tr>
<td>pBADftsH</td>
<td>ftsH in pBAD/HisA, Amp'</td>
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### Table 2.2 The ATPase activity of purified FtsH and its variants

<table>
<thead>
<tr>
<th>FtsH Variant</th>
<th>ATPase activity (nmol Pi/min/mg)</th>
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<tr>
<td>Wild-type FtsH</td>
<td>139.5</td>
</tr>
<tr>
<td>FtsH(K198N)</td>
<td>18.1</td>
</tr>
<tr>
<td>FtsH(HEH)</td>
<td>147.8</td>
</tr>
<tr>
<td>FtsH(AAA)</td>
<td>155.6</td>
</tr>
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FIGURE 2.1 Role of DrrA and FtsH in stable maintenance of DrrB. (A) DrrA is required for stable expression of DrrB. Wild-type E. coli (TG1) cells expressing DrrAB (pDX101) or DrrB alone (pDX103) were grown at 37°C to mid-log phase ($A_{600nm}=0.6$) and induced with 0.25 mM IPTG for 3 h. 30 µg membrane proteins were loaded onto 12% SDS-PAGE gels, followed by Western blotting against anti-DrrA (upper panel) and anti-DrrB (lower panel) antibodies. (B) FtsH is responsible for proteolysis of unassembled DrrB. Wild-type E. coli (796) or the E. coli 797 (ftsH') cells expressing DrrAB (pDX101) or DrrB alone (pDX103) were grown at 30°C to mid-log phase and induced with 0.25 mM IPTG at either 30 °C or 42 °C for 3 h. Western blot analysis of the membrane fraction was carried out as in Fig. 1A. (C) ClpA/P or the Lon
protease is not involved in quality control of DrrB. Wild-type *E. coli* (796), *E. coli* 797 (*ftsH*)*, E. coli* SG1110 (Lon)*, and *E. coli* SG1126 (CtpA*)* cells expressing DrrAB (pDX101) or DrrB alone (pDX103) were grown at 30 °C or 37 °C to mid-log phase and induced with 0.25 mM IPTG at either 42 °C or 37 °C for 3 h, as indicated. Analysis was carried as described in (B) above.

Figure 2.2 Western blot analysis of the cytosol, membrane, and inclusion body fractions of *E. coli* 796 or 797 cells expressing DrrAB at 30 °C or 42 °C.
FIGURE 2.2 Western blot analysis of the cytosol, membrane, and inclusion body fractions of *E. coli* 796 or 797 cells expressing DrrAB at 30 °C or 42 °C. Wild-type *E. coli* 796 or the *E. coli* 797 (ftsH<sup>ts</sup>) cells expressing DrrAB (pDX101) were grown at 30 °C to mid-log phase and induced with 0.25 mM IPTG at either 30 °C or 42 °C for 3 h. Cell fractions were prepared, as described under Methods. 20 µg of each fraction was loaded onto 12% SDS-PAGE gels, followed by Western blotting against anti-DrrA (upper panel) and anti-DrrB (lower panel) antibodies. (m, membrane; c, cytosol; IB, inclusion body). Note that the anti-DrrB antibody is an anti-peptide antibody, therefore it shows some cross-reactivity with epitopes in other *E. coli* proteins, as explained in a previous publication (59).

Figure 2.3 Growth inhibition resulting from expression of DrrB alone or DrrAB together in *E. coli* 797 cells can be relieved by the overexpression of FtsH or GroESL.

These data were contributed by Wen Li and Divya K. Rao
FIGURE 2.3 Growth inhibition resulting from expression of DrrB alone or DrrAB together in *E. coli* 797 cells can be relieved by the overexpression of FtsH or GroESL. (A) Effect of overexpression of FtsH or GroESL on growth of 797 cells expressing DrrB. 797 cells containing pDX103 (DrrB) and pUCftsH or pUCgroESL were grown at 30 °C and induced at 42 °C, as in Fig. 1B. The growth was monitored at O.D. 600 nm for four hours after induction.  (B) Effect of overexpression of FtsH or GroESL on growth of 797 cells expressing DrrAB. 797 cells containing pDX101 (DrrAB) and pUCftsH or pUCgroESL were analyzed, as described in (A) above.  (C) Quantitation of the growth of 796 or 797 cells expressing DrrB or DrrAB at 4 hours after induction. The growth of the sample #1 at 4 hours in Fig. 3A or 3B was designated as 1.0. The relative growth of each culture at 4 hours was calculated. The histogram represents an average of 3 experiments. Columns 1-9 correspond to the cultures 1-9 in Figs 3A (filled) and 3B (open). (D) Western blot analysis. The membrane fractions were prepared from sample #s 6-9 collected at the 4 hour time point in Fig. 3A and 3B. Western blot analysis was carried out as in Fig. 1(A).
**Figure 2.4 FtsH preferentially degrades misfolded DrrAB.**

**FIGURE 2.4 FtsH preferentially degrades misfolded DrrAB.** (A) *E. coli* 796 or 797 cells containing pDX101(DrrAB) and pUCftsH were grown and analyzed by Western blotting, as in Fig. 1A. (B) **Quantitative analysis of the amounts of DrrA and DrrB.** The intensity of the bands on the nitrocellulose membrane in Fig. 4A was determined by densitometric scanning. The intensity of DrrA and DrrB in lane 1 was designated as 1. The data represent an average of 3 experiments. Columns 1-8 correspond to the lanes 1-8 in Fig. 4A. (C), (D) and (E) **In vivo FtsH proteolytic assay.** The assay was carried out as described under Methods. The membrane fractions were prepared from the cells collected at the indicated time points after addition of arabinose. 20 µg total membrane protein was loaded onto 12% SDS-PAGE, followed by Western blot with anti-DrrA, anti-DrrB, or anti-FtsH antibodies. The intensity of the bands on the nitrocellulose membrane was determined by densitometric scanning. The intensity of DrrA and DrrB on the membrane at 0 min was designated as 1.0. (C) Quantitation of DrrA in the
membrane at the indicated time points after addition of arabinose. The data shown represent an average of 3 experiments. (D) Quantitation of DrrB in the membrane. (E) Western blot analysis showing amount of FtsH present in the membrane at various times after addition of arabinose. Top panel: cells containing pBAD vector only. Bottom panel: cells containing the plasmid pBADftsH.

Figure 2.5 FtsH promotes assembly of the DrrAB complex
FIGURE 2.5 FtsH promotes assembly of the DrrAB complex. (A) *E. coli* 796 or 797 cells expressing DrrAB were grown at 30 °C to mid-log phase and the culture was divided into two halves. One half was induced with 0.25 mM IPTG at 30 °C (lanes 1-3 for 796 and lanes 7-9 for 797) and the other half was induced at 42 °C (lanes 4-6 for 796 and lanes 10-12 for 797). Aliquots of each sample were collected at 30 min intervals. Hours of induction are shown at the top of the gel. 0 hour represents $A_{600 \text{nm}} = 0.6$ at which time IPTG was added. Western blot analysis was carried out as in Fig. 1A. (B) The quantitative analysis of DrrA and DrrB. The intensity of bands on the nitrocellulose membrane was determined by densitometric scanning. The intensity of DrrA and DrrB in lane 1 was designated as 1. The data represent an average of 3 experiments.

![Figure 2.5](image)

**Figure 2.5 In vitro digestion of α-casein by purified FtsH or FtsH(HEH)**

FIGURE 2.6 In vitro digestion of α-casein by purified FtsH or FtsH(HEH). (A) α-casein was mixed with purified wild-type FtsH or FtsH (HEH) protein in the protease buffer as described in the Methods. The samples were analyzed by 12% SDS-PAGE, followed by Coomassie Brilliant blue staining. (B) Quantitative analysis of the amounts of α-casein. The intensity of the bands on the nitrocellulose membrane was determined by densitometric scanning. The intensity of α-casein in lane 1 was designated as 1. The intensities represent an average of 3 experiments.

![Figure 2.6](image)
Figure 2.7 The AAA domain of FtsH is sufficient to complement the growth defect resulting from the expression of DrrAB in 797 cells. These data were contributed by Wen Li and Divya K. Rao
FIGURE 2.7 The AAA domain of FtsH is sufficient to complement the growth defect resulting from the expression of DrrAB in 797 cells. *E. coli* 797 cells containing the *drrAB* genes (pDX101) and the wild-type or a mutated version (K198N, HEH, or AAA subclone) of the *ftsH* gene on a compatible plasmid were grown at 30 °C and induced at 42 °C, as in Fig. 1B. (A) **Growth analysis at 600 nm.** (B) **Western blot analysis.** Membranes prepared from the cells collected at the 3 hour time point were analyzed, as in Fig. 1A. (C) **Quantitative analysis of DrrA and DrrB.** The intensity of the bands on the nitrocellulose membrane in Fig. 7B was determined by densitometric scanning. The intensity of DrrA and DrrB in lane 1 was designated as 1.0. The data shown represent an average of 3 experiments. The columns 1-8 correspond to samples 1-8 in Fig. 7B.
FIGURE 2.8 DrrAB-mediated Dox efflux. (A) Dox efflux in *E. coli* 796 or 797 cells. The cells containing pDX101 (DrrAB) or the empty vector were grown at 30 °C to mid-log phase and induced with 0.1 mM IPTG at either 30 °C or 42 °C for 1 h. The washed cells were loaded with Dox, and efflux was initiated by addition of glucose and detected fluorometrically. The slope of the efflux curve of sample 1 in each panel was designated as 1.0. The efficiency of Dox efflux was then calculated by dividing the slope of each efflux curve by the slope of sample 1. The average data obtained from three independent experiments are shown in the histograms. (B) Complementation of the DrrAB-mediated Dox efflux in 797 cells by simultaneous overexpression of wild-type FtsH or its variants. *E. coli* 797 cells containing pDX101(DrrAB) and pUCftsH (wild-type FtsH), pUCftsH(K198N), pUCftsH(HEH), pUCftsH(AAA) or pUCgroESL, were analyzed as described under (A) above. (C) Effect of sequential expression of DrrAB and FtsH on complementation of Dox efflux. *E. coli* 797 cells containing pDX101 (DrrAB) and pBADftsH were grown at 30 °C to mid-log phase and induced with 0.1mM IPTG for 1 h at 42 °C. The cells were washed extensively to remove IPTG and divided into two halves. One half was kept at 42 °C for 1 h without any induction (-ara, 42 °C) and the other half was induced by 1% arabinose at 42 °C (+ara, 42 °C) for 1 h. As controls, 796/AB, 797/AB and 797/AB/pUCftsH were grown at 30 °C to mid-log phase and induced with 0.1 mM IPTG at 42 °C for 2 h. The cells were loaded with Dox, and efflux was measured. The data were analyzed as described under (A) above. (D) Effect of simultaneous over-expression of FtsH on Dox efflux by DrrAB proteins expressed at different temperatures. 797 cells containing pDX101 (DrrAB) and pUCftsH were grown at 30 °C to O.D. 0.6, induced with 0.1 mM IPTG at 30 °C, 37 °C or 42 °C for 1 h, and analyzed as in (A) above.
3 THE DrrAB SYSTEM OF STREPTOMYCES PEUCETIUS IS A MULTI-DRUG TRANSPORTER OF A BROAD SUBSTRATE SPECIFICITY

2.5 Introduction

Multidrug resistance (MDR) has emerged as a major clinical problem in recent years both for the treatment of infectious diseases and for chemotherapy of cancer. Although many different mechanisms for drug resistance are known, a common strategy consists of active efflux of drugs from the cells (87). Drug transporters are categorized into either single-drug efflux systems (which are specific for a drug or a group of drugs) or multi-drug efflux systems that exhibit a broad specificity and can transport structurally and functionally unrelated compounds. These proteins function as either primary active (belonging to the ATP Binding Cassette superfamily) or secondary active transporters (88). The phenomenon of multidrug resistance was first characterized in mammalian cancer cells, where exposure to anticancer drugs was seen to result in over-expression of ABC-type efflux pumps, such as P-glycoprotein and MRP1 (88). These proteins have since been shown to transport hundreds of structurally unrelated compounds, including amphipathic anti-cancer drugs, peptides, and fluorescent dyes, etc., thus conferring MDR in cancer cells. MDR is also widespread among bacteria - the best known ABC family members include the bacterial proteins LmrA and LmrCD in L. lactis, Sav1866 in S. aureus, and MsbA in E. coli.

While most of the ABC proteins mentioned above have served as useful models to characterize and understand the basis of MDR (7,88), the most extensive biochemical analysis of the nature of multidrug specificity has been carried out with Pgp (89,90). Together the analyses carried out by many different groups suggested that Pgp contains a large drug binding chamber which can accommodate several drugs simultaneously. It was also suggested that the drug
binding chamber in Pgp is lined by several trans-membrane (TM) helices, including TMs 4-6 in TMD1 and TMs 9-12 in TMD2 (89). Most recently, the crystal structure of Pgp confirmed many findings of the biochemical analysis and showed that Pgp indeed contains a large and flexible drug binding cavity made of mostly hydrophobic and aromatic residues (42). Different drugs were seen to interact with residues in different parts of the flexible cavity, mostly through hydrophobic interactions, thus providing an explanation for the poly-specific nature of Pgp. The crystal structure of Pgp also revealed that some drugs were bound to a single site, while some others bound to two different locations within the cavity. The drug binding cavity of Pgp was found to reside within the cell membrane, and it showed the presence of two portals formed by TMs 4 and 6 and TMs 10 and 12 which allow direct entry of hydrophobic molecules from the membrane (42). This observation supports previous models, which proposed that Pgp can extract drugs directly from the lipid bilayer and remove them by a hydrophobic vacuum cleaner mechanism (17). The crystal structure of another MDR protein, AcrB, a secondary-active transporter, is also available. This structure also showed the presence of a very large central drug binding cavity, which could accommodate several ligand molecules simultaneously (91).

However, the drug binding cavity, though present in the membrane, was found to be accessible to the periplasmic domain. Thus, in contrast to Pgp (where the drug portals are located in the membrane), the periplasmic region of AcrB seems to play a major role in determining substrate specificity, suggesting differences between the mechanisms of different MDR proteins.

The bacterial ABC drug transporters that have also been studied in significant detail include the E. coli ABC transporter MsbA (responsible for export of Lipid A, the core moiety of LPS), L. lactis homologue LmrA and S. aureus Sav1866 (5,7,92). All of these proteins have been shown to transport multiple drugs. Interestingly, MsbA was found to contain overlapping
substrate specificity with LmrA and Sav1866 (93). Some newly identified members, such as VcaM from Non-O1 Vibrio cholerae and the YccC (BmrA) from Bacillus subtilis were also shown to transport multiple drugs (94,95). Therefore, a significant progress has indeed been made in understanding the phenomenon of multidrug resistance. However, the available information is based on the analysis of only a handful of drug transporters described above. Most other annotated drug transporters, especially those found in the antibiotic/drug producer organisms, have not been analyzed for their ability to confer MDR. Moreover, not much is known about why and how the ability to confer multidrug resistance evolves.

In this study, we analyzed the ABC transporter DrrAB, which confers self-resistance to two related anticancer antibiotics doxorubicin (Dox) and daunorubicin (Dnr) in the producer soil organism Streptomyces peucetius. The genes for this system are present in an operon located within the gene cluster for biosynthesis of Dox and Dnr, therefore they code for a dedicated transporter for these antibiotics. The DrrAB system represents the simplest form of an ABC drug transporter, which is assembled from two molecules each of DrrA (the catalytic subunit) and DrrB (integral membrane subunit) (49). In the mammalian Pgp, the two catalytic and two integral membrane domains are naturally fused into a single large polypeptide, possibly the result of an evolutionary gene fusion event (4). Both DrrAB and Pgp confer resistance to the anticancer agents Dox and Dnr: DrrAB in the producer organism and Pgp in cancer cells. Therefore, the overall structure and function of the DrrAB transporter bears significant similarity to Pgp even though these two transporters belong to different classes of ABC proteins (50). Subcloning of the drrAB locus in E. coli was previously shown to confer doxorubicin resistance in this host (48). It was also previously shown that the DrrAB system confers resistance to Dox by an energy-dependent efflux mechanism (58). However, it is not known if this system is
specific for Dox and Dnr, or if, like Pgp, it can also recognize and transport multiple drugs. Since this is a prototype drug resistance mechanism found in the producer organism, analysis of this system could shed light on the nature of substrate specificity and elucidate how the ability to confer multidrug resistance evolves in proteins.

In this paper, we provide in-depth characterization of drug transport by the DrrAB system and show that, contrary to the generally held assumption, this system forms a multidrug transporter. Using both *E. coli* whole cells and inside-out membrane vesicles (IOVs), it is shown that the DrrAB system can efficiently transport not only Dox, but also Hoechst 33342 and ethidium bromide, two substrates most commonly used to establish the MDR phenotype (5,7,27). We also found that the DrrAB-mediated Dox efflux is inhibited by a number of other well-characterized MDR substrates, such as verapamil, rifampicin, vinblastine and colchicine, suggesting that these drugs are also substrates of the DrrAB pump. Interestingly, DrrAB-mediated efflux could be coupled to the energy of either ATP or GTP hydrolysis, and, as expected, the function of this transporter was found to be completely independent of the proton motive force (pmf). Since multiple drugs were found to inhibit Dox efflux by the DrrAB system, kinetics analysis was carried out to understand the mechanism of inhibition and interaction of drugs with DrrAB. Our studies revealed that inhibition of Dox efflux by Hoechst 33342 and rifampicin occurs by a competitive mechanism, whereas verapamil inhibits Dox transport by a non-competitive mechanism, suggesting that the DrrAB transporter may contain at least two drug binding sites. The findings of this paper demonstrate for the first time that the dedicated Dox transport system, DrrAB, can recognize and transport multiple drugs. This study highlights overlaps between the substrate specificity of the DrrAB system and Pgp and points to a common mechanism, and perhaps origin, for most MDR proteins.
2.6 Materials and Methods

Materials used – verapamil, vinblastine, rifampicin, doxorubicin hydrochloride, ethidium bromide, quinine, quinidine, colchicine, succinate, sodium fluoride, NADH (β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate), ATP, GTP, and sodium 2-vanadate were purchased from Sigma Aldrich. Hoechst 33342, rhodamine 123, rhodamine 6G, rhodamine B, and TMRM (Tetramethylrhodamine) were obtained from Life Technology. Creatine kinase and creatine phosphate were purchased from Roche Diagnostics.

In vivo Dox efflux in cells – *E. coli* LE392ΔuncIC cells (Table 1) containing either vector pSU2718 or pDX101(pSU2718/ΔrrAB) were grown in 200 ml TEA medium [50 mM triethanolamine HCl, pH 6.9, 15 mM KCl, 10mM (NH4)2SO4, 1mM MgSO4] supplemented with 0.5% (w/v) glycerol, 2.5 μg/ml thiamine, 0.5% (w/v) peptone and 0.15% (w/v) succinate to mid-log phase and induced with 0.1 mM IPTG for 1 hr. The harvested cells were washed twice and resuspended in 100 μl TEA buffer. 10 μl of the cell suspension from above was incubated in 3 ml of TEA medium containing 10 μM doxorubicin and 5 mM 2,4-dinitrophenol for 11 h at 37 °C. The loaded cells were washed twice with 0.1 mM MOPS buffer, pH 7.0 and resuspended in 3 ml of MOPS buffer containing 2 mM MgSO4. The fluorescence spectra were recorded on an Alphascan-2 spectrofluorometer (Photon Technology International, London, Ontario, Canada). The excitation wavelength for doxorubicin was 480 nm, and emission was monitored at 590 nm. The excitation and emission slit widths were both set at 1.00 mm, and a time-based script was run. After an initial recording of fluorescence for 100 s at 37 °C, energy was provided in the form of either 20 mM glucose or 20 mM succinate, and recording was continued for an additional 400 s. The rate of Dox efflux was determined from the slope in the steady-state range.
(300 s to 500 s). Where indicated, 10 mM sodium fluoride was added as inhibitor of ATP synthesis.

**In vivo ethidium bromide (EtBr) efflux in cells** – *E. coli* LE392ΔuncIC cells containing the indicated plasmid were grown, induced, and loaded with various concentrations (1 µM to 100 µM) of EtBr, as described above, except that the loading time used was 1 hr at 37 °C. The loaded cells were washed twice with 0.1 mM MOPS, pH 7.0 and resuspended in 3 ml MOPS buffer containing 2 mM MgSO₄. EtBr efflux from loaded cells was measured fluorometrically (exi, 500 nm; emi, 580 nm) on an Alphascan-2 spectrofluorometer (Photon Technology International). After 100 s, energy was provided in the form of 20 mM glucose. The recording was continued for additional 400 s. The rate of EtBr efflux was determined from the slope in the steady-state range (300 s to 500 s).

**Preparation of inside-out membrane vesicles (IOVs)** – *E. coli* LE392ΔuncIC cells containing indicated plasmids were grown in 1 L LB medium at 37 °C until mid-log phase and induced with 0.25 mM IPTG at 37 °C for 3 hr. The cells pellet was re-suspended in 20 ml 1xPBS buffer, pH 7.4 and lysed with French Press at 16,000 p.s.i. twice. The membrane fraction was prepared according to the previously published protocol (49), except that the membrane vesicles were washed twice with 20 ml 1xPBS buffer.

**In vitro Dox efflux in IOVs and kinetic analysis** – 250 µg IOVs were resuspended in 3 ml 1x PBS buffer, pH 7.4 supplemented with 0.1 mg/ml creatine kinase and 5 mM creatine phosphate. Dox was added to a final concentration of 1.0 µM, or as indicated. The fluorescence spectra were recorded on an Alphascan-2-spectrofluorometer with excitation wavelength of 480 nm and emission wavelength of 590 nm. The excitation and emission slit width were set to 1.00 mm and data were collected at 0.1-s intervals. After 100 s, the detection was paused, 1 mM Mg²⁺ and 1
mM ATP, pH7.5 were added into the reaction and the detection was continued for additional 400 s. Where indicated, ATP was substituted with 1 mM GTP or 5 mM NADH. The rate of Dox transport was determined from the slope of the initial linear range between 100 s and 200 s. To determine the kinetics of Dox transport, efflux was measured at a wide range of Dox concentrations (0.1 µM to 6 µM). The data were fitted by the Michaelis–Menten equation ($V = \frac{V_{\text{max}}[S]}{(K_m) + [S]}$) by Sigma Plot Kinetics software in Single-Substrate Format.

**In vitro Hoechst 33342 Efflux in IOVs** – The DrrAB-mediated efflux of Hoechst 33342 was studied in IOVs, as described above for Dox Efflux with some modifications. Briefly, 250 µg IOVs were resuspended in 3 ml 1x PBS buffer, pH 7.4 supplemented with 0.1 mg/ml creatine kinase and 5 mM creatine phosphate and various concentrations of Hoechst 33342 (0.1 µM to 2.5 µM). The excitation and emission wavelengths of Hoechst 33342 were 355 nm and 457 nm, respectively. The rate of Hoechst 33342 transport was determined from the initial slope of the linear range between 100 s to 200 s.

**Vanadate inhibition of Dox efflux in IOVs** – 250 µg IOVs were resuspended in 3 ml 1x PBS buffer, pH 7.4 supplemented with 0.1 mg/ml creatine kinase, 5 mM creatine phosphate, 1 µM Dox, and various concentrations of sodium o-vanadate (0 µM – 100 µM). The measurement of Dox efflux was performed, as described above. The rate of Dox transport was determined from the slope of the linear range between 100 s and 200 s.

**Determination of IC50** – 250 µg IOVs were resuspended in 3ml 1xPBS buffer, pH 7.4 supplemented with 0.1 mg/ml creatine kinase and 5 mM creatine phosphate, 1 µM Dox, and various concentrations of the inhibitory drug. The measurement of Dox efflux was performed, as described above. The rate of Dox transport was determined from the slope of the initial linear range between 100 s and 200 s. Designating the efflux rate of the sample without inhibitor as
1.0, the relative rate of each sample was calculated. The average data of three independent experiments were plotted by ‘Scatter Plot with simple error bars’ in Sigma Plot 11.0 software and fitted by the dynamic curve fit (equation: \( y = ae^{-bx} \), \( x \): concentration of inhibitor; \( y \): relative rate). The IC\(_{50}\) value was determined based on the concentration of the drug that brings about 50% inhibition of the DrrAB-mediated Dox efflux at a Dox concentration of 1 µM.

**Kinetics of Dox efflux inhibition by known MDR substrates** – To study the kinetic inhibition of Dox efflux by Hoechst 33342, four different concentrations of Dox (0.25, 0.5, 0.75 and 1.0 µM) were individually mixed with a fixed concentration of Hoechst 33342 in 1x PBS buffer containing 250 µg IOVs, 0.1 mg/ml creatine kinase and 5 mM creatine phosphate. In total, four different concentrations of Hoechst 33342 (0, 0.2, 0.6 or 0.8 µM) were studied. Similar assays were set up to study kinetics of Dox inhibition by different drugs. Initial rate of Dox transport was determined, as described above. The rate of Dox transport obtained with 1 µM Dox and 0 µM Hoechst 33342 (or another drug) was designated as 1.0. The relative rates were then calculated for each efflux curve, and the data were plotted by Lineweaver-Burk plot using SigmaPlot - Kinetics software in Single-Substrate/Single-Inhibitor Kinetics Format. The error bars represent three independent experiments. The type of inhibition was determined based on the AICc value (Akaike’s Information Criterion corrected); lower AICc values correspond to better fits to the data.

**Point Mutations in DrrA** – Site-directed mutagenesis of the *drrA* gene was carried out by a QuikChange Multisite-directed mutagenesis kit (Stratagene, La Jolla, CA). Using pDX101 (pSU2718/drrAB) plasmid as the template, Gln197, located in the Switch motif of DrrA, was changed to Histidine. The resulting plasmid was named pDX101(Q197H). Another plasmid, generated by substituting Tyr198 to Arginine, was named pDX101(Y198R). Double mutations
of Gln197Tyr198 to His197Arg198 and His197His198 were also created, and these plasmids were named pDX101(Q197H/Y198R; HR) and pDX101(Q197H/Y198H; HH). Other mutations used in this study were described previously (Table 1).

ATPase activity in IOVs - 7.5 µg IOVs expressing either wild type DrrAB or DrrAB with mutations in the Switch motif were incubated in 1 ml reaction mixture containing 50 mM MOPS, pH 7.5, 1mM dithiothreitol, 10µl PK/LDH enzyme (Sigma Aldrich), 5 mM ATP, 0.25 mM NADH (β-Nicotinamide adenine dinucleotide) and 1.25 mM PEP[phosphor(enol)pyruvic acid] at 37 ºC for 10 min, as described previously (30). The reaction was started by addition of 2.5 mM MgCl₂. The optical density at 340 nm was monitored for 10 min using Shumadzu UV1601 spectrophotometer and the UV probe 2.20 Kinetics software. The slope of the linear portion of each curve (between 200 s and 400 s) was used to calculate ATPase activity. The activity of the control IOVs (without DrrAB) was subtracted from the activity of each test sample to obtain DrrAB-specific activity. Relative activity was then calculated by dividing the activity of each sample by the activity of the wild type sample.

2.7 Results

Characterization of the DrrAB-mediated Dox efflux under in vivo and in vitro conditions – An in vivo assay for studying DrrAB-mediated Dox efflux was reported previously (58,61) and is shown in Fig. 1A. In this study, we established conditions to study DrrAB-mediated Dox efflux under in vitro conditions using E. coli inside out membrane vesicles (IOVs) (Fig. 1B). Both in vivo and in vitro assays utilize the fluorescent nature of Dox to measure efflux in E. coli LE392ΔuncIC cells (or IOVs). This strain of E. coli contains a deletion in the unc genes; as a result it is unable to carry out synthesis of ATP using proton gradients or establish a proton gradient by hydrolysis of ATP (96). Therefore, it is possible to establish conditions where only
the proton motive force (pmf) or ATP is available as a source of energy (described below). Dox is fluorescent in solution, however its accumulation inside the cells results in quenching of its fluorescence and its efflux results in an increase in fluorescence intensity (97).

**In vivo Dox efflux** – The basic strategy for studying efflux under *in vivo* conditions consists of loading of the de-energized cells with Dox (58), followed by addition of an energy source, which is expected to result in efflux of Dox and an increase in its fluorescence (Fig. 1A). Under the conditions used in our experimental system, use of succinate as energy will generate only proton motive force while glucose will generate both pmf and ATP, therefore allowing us to discriminate between the energy sources used by the DrrAB system. The data in Fig. 1A compare the rate of Dox efflux in DrrAB-containing cells in the presence of glucose or succinate. *E. coli* LE392ΔuncIC cells (containing empty vector) were used as negative control in these experiments. Two conclusions can be made from the data shown in Fig. 1A. First, the rate of Dox efflux by the DrrAB-containing cells in the presence of glucose is about 5-fold higher as compared to the rate in control cells (Fig. 1A.2, compare columns 1 and 4). A small increase in Dox efflux efficiency seen in control cells on addition of glucose is likely due to the action of one of the several MDR pumps known to be present in *E. coli* (87). Secondly, in contrast to glucose, use of succinate as energy showed no increase in fluorescence intensity in DrrAB-containing cells (Fig. 1A.1, curve 2; Fig. 1A.2, column 2), indicating that pmf does not support Dox efflux by the DrrAB proteins. Confirmation of these results was obtained by addition of sodium fluoride, a specific inhibitor of ATP synthesis by substrate-level phosphorylation, to DrrAB-containing cells in the presence of glucose. The data show a drastic reduction in Dox efflux by the DrrAB-containing cells (Fig. 1A.1, curve 3; Fig. 1A.2, column 3), resulting in the same background levels of efflux as seen with succinate (column 2). These studies show that
doxorubicin efflux by the DrrAB pump is solely ATP-dependent, and pmf is not required for this process, thus highlighting similarities between DrrAB and other MDR proteins of the ABC superfamily (6,27,98).

**In vitro Dox efflux** – To understand the kinetics of Dox transport, an *in vitro* Dox efflux assay was optimized using inside-out membrane vesicles, as described under Methods. In this assay, the vesicles were mixed with Dox, and efflux was initiated by addition of ATP and Mg$^{2+}$. Because of the inverted nature of the IOVs, DrrAB-mediated efflux results in accumulation of Dox inside the vesicles which is seen as quenching of its fluorescence (Fig. 1B.1). The data in Fig. 1B.1 show that addition of ATP/Mg$^{2+}$ first results in a rapid non-specific decrease in Dox fluorescence due to interaction between Dox and ATP (97). This quick phase is then followed by a slower rate of quenching, which corresponds to the DrrAB-dependent efflux in the vesicles. The initial rate of Dox efflux was therefore determined from the linear slope of the fluorescence spectra between 100 s and 200 s. As seen in Fig.1B.1 and 1B.2, Dox transport activity in vesicles containing DrrAB was found to be almost 12-fold higher as compared to the control vesicles prepared from cells containing vector alone (Fig. 1B.2, compare columns 1 and 4). Absence of either Mg$^{2+}$ (column 2) or ATP (column 3) resulted in the failure of these vesicles to transport Dox. Use of NADH as an energy source also did not support Dox efflux (column 6), once again confirming that proton motive force is not used by the DrrAB proteins as a source of energy. Surprisingly, when GTP was used as an energy source instead of ATP, even higher transport activity was observed (column 5). Dox-dependent ATP and GTP binding to DrrA was shown previously (48). Together these data indicate that either ATP or GTP (and Mg$^{2+}$) can serve as a source of energy for the Dox transport function of DrrAB.
Kinetic Analysis of Dox efflux in vitro – To understand the kinetics of Dox efflux by the DrrAB system, efflux was analyzed (as shown in Fig. 1B.1) at a wide range of Dox concentrations. The initial rate (slope between 100 s-200 s) of each efflux curve was determined. Efficiency of Dox efflux was then calculated, as described under Methods and Figure Legends. The data were fitted to a hyperbola using the Michaelis-Menten equation (with an $R^2$ of 0.89), yielding an apparent $K_m$ of 0.38 µM and $V_{max}$ of 1003 (arbitrary units, a.u.) (Fig. 1C). These data showed a linear increase in the rate of Dox transport at concentrations ranging between 0.1 µM and 1 µM, which became saturated after 3µM. The Dox transport data could also be fitted equally well by the Hill equation; the implication of this finding is discussed later.

Inhibition of Dox transport activity by sodium o-vanadate – Vanadate (Vi) is a known inhibitor of the ATPase activity of ABC proteins (99-102). Since it functions as an analog of Pi, the ADP·Vi·Mg$^{2+}$ complex is trapped in the ATP binding pocket after a single catalytic turnover, thus blocking further hydrolysis of ATP as well as drug transport. To determine if the DrrAB system is inhibited by vanadate, Dox transport was measured in IOVs in the absence or presence of increasing amounts of vanadate (5 µM to 100 µM). The data in Fig. 1D show that vanadate is a potent inhibitor of the Dox transport of DrrAB, with an $IC_{50} = 11$ µM. A complete inhibition of the Dox transport activity was seen at 100 µM vanadate. These data are consistent with the previously reported studies on the inhibitory effect of vanadate on Pgp and MsbA (101,103).

Point mutations in the nucleotide binding domain of DrrA compromise Dox transport activity – The N-terminal nucleotide binding domain of DrrA contains a 200 amino acids long ABC cassette consisting of all the conserved motifs (Walker A, Walker B, Signature motif/C-loop, Q-loop, and the Switch motif/H-loop) involved in ATP binding and hydrolysis (Fig. 2A.1). We previously reported that mutations in the conserved residues of DrrA confer Dox sensitivity
Mutations in Walker A, as expected, also compromised ATP binding (104). Here we evaluated the effect of several mutations on DrrAB-mediated Dox efflux in IOVs. The data in Fig. 2B show that, as expected, single point mutations in Walker A (such as G44A, G44S or K47R), signature (S141R), or the Walker B (E165Q) motif of DrrA result in a drastic effect on Dox transport activity. Since the sequence of the Switch motif of DrrA is different from most other ABC proteins (Fig. 2A), this region was analyzed in greater detail, as described below.

Most ABC proteins normally contain a highly conserved histidine residue in their Switch motif, followed by an arginine, histidine, or a lysine (Fig. 2A.1). The conserved histidine of the Switch motif and a conserved glutamate immediately following the Walker B motif (both are shown as highlighted areas in Fig. 2) are together believed to be critical for formation of the active sites in ABC proteins. Zaitseva et al recently proposed that these two residues together form a catalytic dyad which functions in substrate-assisted catalysis (105). However, despite the high conservation of these two residues in ABC proteins, deviations in the sequence of these motifs are sometimes seen. Most commonly, a glutamine replaces the histidine in the Switch motif and an aspartate replaces the glutamate in the Walker B region. When present (for example in TAP1 and LmrC), these deviations are seen to result in asymmetrical ATP binding pockets with one site being catalytically non-functional (106,107). Interestingly, however, despite the presence of the non-canonical glutamine residue (Q197 followed by Y198, resulting in QY sequence) in the Switch motif of DrrA (as well as its close prokaryotic homologs, Fig. 2A.2), it is able to form a functional drug transporter with DrrB, as seen in Fig. 1. Note that the close eukaryotic homologs of DrrA most often contain an HH sequence in the Switch region (Fig. 2A.3), and both prokaryotic and eukaryotic groups contain the conserved glutamate in the Walker B region (specifically E165 in DrrA) (Figs. 2A.2 and 2A.3)
Since histidine is conserved in the Switch of most ABC proteins, we wondered if the DrrAB transporter will become more efficient if Q197 is substituted with a histidine or if the QY sequence is changed to the commonly occurring sequence HR or HH. Surprisingly, we found that the Q197H mutation in DrrA produces a drastic effect on Dox efflux, however mutation of Y198 to Y198R retains about 35% Dox efflux function (Fig. 2C). Interestingly, a double mutation HR (Q197H/Y198R) also resulted in Dox transport activity of about 38% indicating that the second mutation partially masked the harmful effect of the Q197H single mutation (Fig. 2C). By contrast, the double mutation HH (Q197H/Y198H) exhibited extremely low Dox efflux (Fig. 2C). To understand the role of Q197 and Y198 in catalysis, the effect of the above-mentioned mutations on the ATPase activity of the DrrAB complex was determined. We found that while Q197H produces a drastic effect on ATPase activity, both HR and HH double mutations show significant ATPase activity (43% and 61%, respectively) (Figs. 2D and 2E), indicating that the second mutation in each case partially compensates for the negative effect of the Q197H mutation on catalysis. Overall the findings in Fig. 2 indicate that residues Q197 and Y198 in the Switch motif of DrrA function together and that the QY sequence works much better than the HH or HR sequence for the overall Dox efflux function of the DrrAB complex.

Interestingly, the HH double mutant still exhibits 60% ATP hydrolysis activity, which suggests that either the QY or the HH sequence could participate in the formation of functional catalytic sites in DrrA. However, the HH allele seems to be defective in specific communication between DrrA and DrrB resulting in significantly reduced Dox efflux (less than 5%). Therefore the context in which the Switch motif functions in different ABC proteins may determine the nature of this motif. The conserved glutamate E165 present near the Walker B region served as a control in these experiments. Analysis of the E165Q mutation showed a drastic effect on both
hydrolysis of ATP and Dox efflux (Figs. 2B-D), which is consistent with the critical role of this residue previously reported in literature (108). How a glutamine residue participates in the formation of functional ATP binding pockets in DrrA is still an open question. However, we can draw from the analogous situations present in other ATP/GTP-binding proteins (including RecA and H-ras p21) which contain a catalytic glutamine residue (Q194 in RecA and Q61 in H-ras p21) in their switch II domain in the position corresponding to Q197 in DrrA (1,109,110). The 3-d structures of RecA and H-ras indicate that these glutamines show similar interactions with the catalytic glutamate and γ phosphate of ATP to those seen with histidine in the ABC proteins, therefore suggesting that the glutamine functions in a manner similar to histidine in producing the overall conformation of the active site. This is consistent with our observation that either QY or HH sequence can indeed participate in formation of the catalytic sites in DrrA although the HH sequence is deficient in energy transduction. In summary, the analyses in Fig. 2 confirm that a functional nucleotide binding domain of DrrA is essential for Dox efflux by the DrrAB system. The analysis of the Switch mutations, in particular, also indicates that the in vitro efflux assay described here can provide a sensitive and a valuable approach for elucidating the role of critical residues in the catalytic and drug transport function of the DrrAB complex.

**DrrAB-mediated Dox efflux is inhibited by multiple MDR substrates** – To determine if the DrrAB transporter can recognize and bind other known MDR substrates, inhibition of Dox efflux by different drugs was investigated in IOVs. These assays were carried out at a wide range of the inhibitor concentrations while maintaining a constant concentration of Dox, as described under Methods (determination of IC\textsubscript{50} values). The data in Fig. 3 show that many known MDR drugs, including H 33342 (H 33342) (Fig. 3A), verapamil (Fig. 3B), and rifampicin (Fig. 3C), inhibit DrrAB-mediated Dox efflux with high efficiency. The data in Fig 3, panel D, summarize
the inhibitory effects of many different MDR substrates and indicate that the IC_{50} values vary dramatically for different substrates. For example, H 33342, vinblastine, verapamil and rifampicin show relatively low IC_{50} values, while much higher concentrations of EtBr, quinidine, and colchicine were required for the same level of inhibition. These data suggest that the DrrAB system has multiple substrates, which bind with varying affinities. However, whether these drugs bind to the same site, or if there are multiple drug binding sites, can’t be ascertained from these data. To understand the nature of drug binding, kinetics of DrrAB-mediated Dox efflux was studied at multiple Dox concentrations in the presence of several concentrations of each inhibitory drug, which is described below.

**Kinetics of Dox Inhibition by MDR drugs** – The data in Fig. 1C showed that the rate of Dox efflux by DrrAB-containing IOVs increases linearly between 0.1 µM and 1 µM Dox concentration, therefore four evenly distributed concentrations of Dox within this range (0.25, 0.50, 0.75, and 1.0 µM) were used in the kinetics analysis. At each concentration of Dox, efflux was measured in the presence of our different concentrations of H 33342, verapamil, or rifampicin. These drugs were chosen based on their low IC_{50} values seen in inhibition studies (Fig.3D). (Since Dox and ethidium together were incompatible in the fluorescence-based assays due to their overlapping excitation/emission spectra, this combination could not be used in this study). The initial slope (100-200 seconds) for each Dox efflux curve was determined. The slope of the Dox efflux curve at 1.0 µM Dox and 0 concentration of the inhibitor was designated as 1, which was then used to calculate the relative slope of each curve obtained in the presence of the inhibitor, as described under Methods. The relative slopes were plotted by the Lineweaver-Burk plots using the Sigma Plot kinetics software (Fig. 4). The data in Fig. 4A indicate that the inhibition of Dox efflux by H 33342 is characteristic of competitive inhibition; the K_m of DrrAB-
mediated Dox transport increased at increasing concentration of H 33342, whereas the $V_{\text{max}}$ remained unchanged. These data suggest that Dox and H33342 may bind to the same site in DrrAB. The apparent inhibition constant ($K_i$ for H33342) was found to be 0.64 µM, which corresponds well to the IC$_{50}$ value of H 33342 (Fig 3, panel D). When studying the inhibition of Dox transport by rifampicin, a similar pattern was observed, which indicated a competitive inhibition between Dox and rifampicin (Fig. 4C). However, the kinetics of inhibition of Dox transport by verapamil showed a different pattern (Fig. 4B). The $V_{\text{max}}$ decreased at increasing concentration of verapamil, while $K_m$ remained unaltered, indicating a non-competitive inhibition between Dox and verapamil. These data suggest that verapamil may bind to both the unliganded DrrAB and the binary DrrAB-Dox complex. The $K_i$ value for verapamil inhibition was found to be 9.4 µM, which also matches well to the IC$_{50}$ of verapamil (Fig. 3, panel D). The observed competitive and non-competitive inhibitions of DrrAB-mediated Dox transport in IOVs imply that DrrAB must contain at least two drug binding sites.

The DrrAB system is a multidrug transporter – To determine whether the DrrAB system can actually transport other drugs in addition to Dox, two substrates (H 33342 and EtBr) commonly used to establish the MDR phenotype (5,27), were tested in IOVs, as described under Methods. In addition, five other fluorescent dyes or drugs (including Hoechst 34580, Hoechst 33258, quinine, TMRM, and Rhodmine 123) were also tested. Of these substrates, H 33342 and EtBr were successfully transported by the DrrAB system, as described below.

Hoechst is a cell-permeable dye, which is fluorescent only when bound to the cell membrane and loses its fluorescence in aqueous environment. Thus a decrease in fluorescence is expected when Hoechst is transported by the DrrAB system from the membrane to the inner aqueous environment in the IOVs. Ethidium bromide, on the other hand, becomes fluorescent
when bound to DNA inside the cells. Therefore, DrrAB-mediated efflux of EtBr was studied in preloaded cells, as described under Methods. EtBr efflux under this condition is expected to result in quenching of fluorescence. The data in Fig. 5 show that both H 33342 (Fig 5A.1) and EtBr (Fig 5B.1) are transported efficiently by the DrrAB system. In the case of H 33342, the rate of transport was seen to decrease after an initial linear phase of transport. This is likely due to depletion of H 33342 in the membrane and due to passive rebinding of H 33342 to the membrane from the aqueous phase, as shown earlier in the case of Pgp (27). To determine the kinetic parameters for the transport of H 33342 and EtBr, efflux of each substrate was analyzed at a wide range of concentrations. The initial rate of H 33342 transport in DrrAB-containing IOVs was measured between 0.1 µM and 2.5 µM (Fig 5A.2). The efficiency of H 33342 transport was calculated, as described earlier for Dox (Fig. 1C). The data were plotted using the Michaelis-Menten equation by the Sigma Plot kinetics software and could be fitted to a hyperbola with an $R^2$ of 0.90, yielding an apparent $K_m$ of 0.78 µM and $V_{max}$ of 16000 (a.u.). To study kinetics of EtBr transport, energy-depleted cells were loaded with different concentrations of EtBr (ranging from 1 µM to 100 µM) for 1 hour, and efflux was initiated by addition of 20 mM glucose (Fig 5B.2). The data fitted to the hyperbola (Michaelis-Menten equation) with an $R^2$ of 0.97, yielding an apparent $K_m$ of 21µM and $V_{max}$ of 550 (a.u.). The data in Fig 5 show that both H 33342 and EtBr are transported efficiently by the DrrAB system. A comparison of the $K_m$ and $V_{max}$ values for transport of Dox, H 33342, and EtBr is shown in Fig 5, panel C. Five other fluorescent substrates (Hoechst 34580, Hoechst 33258, quinine, tetramethylrhodamine(TMRM), and Rhodamine 123) tested in this study were not found to be exported by the DrrAB system (data not shown). In general, the transport data agreed with the inhibition data shown in Fig. 3, panel D. Interestingly, of the three Hoechst dyes tested, only H 33342 was transported. Recent studies
showed that a certain balance between the hydrophobic and hydrophilic forces in the structure of the target substrate is essential for optimum efflux by Pgp (111). Since H 33342 is the most amphiphilic of the three dyes, this may explain why DrrAB is able to preferentially transport H 33342 but not the other two dyes.

2.8 Discussion

Multidrug resistance is conferred by the action of specialized proteins in the cell membrane that have the ability to carry out energy-dependent efflux of structurally and functionally unrelated hydrophobic compounds. This phenomenon defies the concept of single enzyme-single substrate (the lock and key model), which is applicable to most other enzymes. Since the discovery of multidrug resistance in human cancer cells and bacteria (112-114), two important questions have remained at the forefront of this field: 1) what is the basis of poly-specific drug recognition and transport by MDR proteins? 2) How and why does MDR evolve? Extensive biochemical analysis of Pgp (87,89,90) as well as the availability of three-dimensional structures of the drug-bound forms of Pgp, AcrB, BmrR and QacR (42,91,115,116) has contributed significantly to understanding the molecular basis of poly-specific drug binding. These studies indicate that the MDR proteins contain an extensive (and flexible) drug binding pocket which can accommodate multiple drugs simultaneously via interactions with specific (sometimes overlapping) aromatic and polar residues present in different parts of the pocket. The 3-D structure of Pgp, in particular, also provides support for the hydrophobic vacuum cleaner model, which was proposed early on by Gottesman and colleagues (4,17) to explain the mechanism of multidrug transport. According to this model, Pgp substrates (which are primarily cationic, lipid soluble, planar molecules) partition spontaneously into the membrane and are picked up by Pgp directly from the membrane (instead of from the cytoplasm) and then removed
by its ability to flip drugs from the inner to the outer leaflet. This model, which has now been extended to many other MDR proteins (21), makes a clear distinction between the classical solute transporters that remove molecules from an aqueous compartment and the MDR proteins that are proposed to function as flippases (117). A clear relationship between the ability of drugs to partition into the membrane and their binding and transport by Pgp has since been documented (24,118,119). Additional evidence in support of this model comes from 1) the ability of most MDR proteins studied to date to extrude the lipophilic dye H 33342 directly from the membrane (7,27,32,120), thus making it an ideal molecule to establish the MDR phenotype and 2) the ability of many MDR proteins to flip-flop lipids (121-124). Together, these observations led to the idea that hydrophobic drugs and lipids may use a common pathway or flipping mechanism for extrusion.

Despite significant progress in understanding the basis of poly-specific drug-binding, the origin of multidrug resistance still remains an enigma. It is generally accepted that most of the antibiotic and drug resistance genes found in clinical settings have their origins in the environmental bacteria (87). Indeed many antibiotic producing organisms contain dedicated export systems to protect themselves against their own antibiotics (125,126). Understanding these systems could hold the key to unraveling the origin and evolution of multidrug specificity. It is generally assumed that the dedicated systems found in producer organisms belong to the category of single-drug transporters as opposed to multi-drug transporters (88), however their substrate specificity has never been investigated in any significant detail thus providing the impetus for this study.

We show for the first time that the DrrAB system functions as a typical multidrug transporter that can carry out efflux of structurally unrelated substrates, including Dox, ethidium
bromide and H 33342. Inhibition studies further demonstrate that the substrate range of the DrrAB system includes many additional substrates, such as verapamil, vinblastine, and rifamicin, among others. Thus the substrate specificity of DrrAB overlaps with most other known MDR proteins, including Pgp (127-129). We found, however, that unlike Pgp, neither quinine nor rhodamine 123 serve as substrates for export by the DrrAB system nor did they show inhibition of Dox transport. Therefore, in spite of the broad substrate range of DrrAB, it appears that the range of substrates recognized by Pgp may still be larger (130), implying that the ability to bind some of these substrates may have evolved later in Pgp. Interestingly, the IC₅₀ values varied significantly among different drugs, indicating the different binding affinities of each substrate. The highest IC₅₀ was observed for colchicine, which is consistent with the data previously reported for Pgp (118,131,132). This may be related with low hydrophobicity of colchicines (133), therefore indicating that the ability to partition into the membrane may be an important factor for transport by both Pgp and DrrAB.

Kinetic characterization of Dox, H 33342, and EtBr transport by the DrrAB system revealed single-site transport kinetics. Further, the Kₘ values of 0.38 µM and 0.78 µM for transport of Dox and H33342, respectively, reflect high affinity of these substrates for the DrrAB system and correspond well with the Kₘ values seen earlier for Dox transport by Pgp (134) and Hoechst transport by MsbA and LmrA (5). Interestingly, however, we found that the kinetic data for Dox as well as H 33342 could also be fitted by the Hill equation (shown in Fig. 5C) providing the n_Hill value of 1.7 for Dox (R² = 0.93) and 2.0 for H 33342 (R² = 0.95), which suggests co-operative interaction between two or more drug binding sites in DrrAB. It is not clear from these data, however, whether both H 33342 and Dox bind to the same sites in the DrrAB system. Since ethidium transport by DrrAB exhibited only single-site transport kinetics
(n_{Hill}=1.1; \ R^2 = 0.98) \ (\text{Fig. 5C}), \ it\ possibly\ interacts\ with\ only\ one\ of\ the\ Dox/Hoechst\ binding
sites, \ or\ it\ may\ bind\ to\ a\ completely\ different\ site\ in\ DrrAB. \ The\ kinetic\ analyses\ shown\ in\ Fig.
4\ provide\ evidence\ for\ competitive\ inhibition\ of\ Dox\ transport\ by\ H\ 33342\ and\ rifampicin\ and
non-competitive\ inhibition\ by\ verapamil. \ Therefore,\ we\ conclude\ that\ both\ H33342\ and
rifampicin\ bind\ to\ the\ same\ site(s)\ as\ Dox,\ whereas\ verapamil\ may\ bind\ to\ a\ different\ site\ in
DrrAB.\ Evidence\ for\ two\ or\ more\ non-identical\ drug\ binding\ sites\ has\ also\ been\ obtained
previously\ with\ MsbA\ (5,122),\ LmrA\ (6)\ and\ Pgp\ (5,34,135). \ In\ Pgp,\ the\ two\ sites\ were\ defined
as\ the\ H\ and\ R\ sites\ based\ on\ their\ preferential\ binding\ to\ Hoechst\ and\ Rhodamine,\ respectively
(136). \ Together,\ these\ data\ point\ towards\ the\ presence\ of\ two\ or\ more\ drug\ binding\ sites\ in\ the
DrrAB\ system. \ Gleaning\ from\ the\ knowledge\ previously\ gained\ from\ studies\ with\ Pgp\ and
AcrB,\ these\ different\ drug\ binding\ sites\ are\ likely\ part\ of\ the\ same\ binding\ pocket\ in\ DrrB.
However,\ in\ the\ absence\ of\ a\ crystal\ structure\ of\ the\ DrrAB\ transporter,\ this\ conclusion\ is\ only
tentative.

Even\ though\ the\ present\ study\ does\ not\ provide\ an\ answer\ to\ the\ origin\ of\ poly-specific
drug\ recognition,\ it\ does\ indeed\ show\ that\ a\ simple\ system\ like\ DrrAB\ (which\ may\ be\ closely
related\ to\ the\ ancestral\ system)\ already\ contains\ the\ ability\ to\ recognize\ and\ transport\ multiple
substrates. \ This\ leads\ us\ to\ conjecture\ that\ the\ ability\ to\ transport\ multiple\ drugs\ is\ an\ inherent
property\ of\ a\ certain\ class\ of\ proteins\ that\ followed\ a\ different\ evolutionary\ path\ than\ the
classical\ transporters. \ This\ work\ also\ opens\ new\ questions\ regarding\ the\ mechanism\ by\ which
the\ DrrAB\ system\ extrudes\ multiple\ hydrophobic\ substrates. \ The\ most\ important\ of\ these
questions\ is\ whether\ this\ system\ relies\ on\ a\ hydrophobic\ vacuum\ cleaner-like\ mechanism\ and\ if
it\ is\ capable\ of\ flippase\ action? \ Doxorubicin\ is\ produced\ inside\ S.\ peucetius\ cells,\ therefore
presumably\ the\ function\ of\ the\ DrrAB\ system\ is\ to\ remove\ it\ from\ the\ cytoplasmic\ compartment
to the outside. Being amphipathic, however, Dox could conceivably partition into the cell membrane and then be picked up by the DrrB protein directly from the membrane, as suggested in an early model proposed for AcrB function (91). This strategy for its removal could also provide protection to the cell from the toxic effects of free Dox inside the cell. The ability to transport Hoechst no doubt indicates that the DrrB protein can indeed extract this fluorescent dye directly from the membrane by a mechanism similar to the one used by Pgp and other proteins (5,27,129). Future studies will therefore focus on an in-depth analysis of the mechanism of drug extrusion by the DrrAB system.

This and previous studies on MDR proteins raise many other intriguing questions; for example, is there evolutionary relatedness between MDR proteins and lipid flippases? What is the real difference between the single-drug and multi-drug transporters? What makes certain proteins specific for a substrate and some others multi-specific? What is the contribution of high aromatic amino acid content of the TM helices of MDR proteins in conferring poly-specificity? Since the transmembrane helices of Pgp and other MDR proteins, such as the bacterial BMR, are highly enriched in aromatic amino acids (18% and 15.4% for Pgp and BMR, respectively) as compared to the single-drug transporter TetA (9.4%), Pawagi et al. proposed that a greater number of aromatic amino acids may correlate with a decrease in substrate specificity of a transporter by providing additional binding sites for drugs containing aromatic rings in their structure (137). Moreover, it was shown that mutagenesis of a single residue Ser941 to Phe in TM11 of Pgp significantly altered its drug efflux profile (138). The recent 3-D structure confirmed that the drug-binding pocket of Pgp is made up of mostly hydrophobic and aromatic residues (42). Interestingly, we found that the aromatic amino acid content of predicted TM helices of DrrB (84) is also relatively high (15%) which is close to the aromatic content of Pgp
(18%) and BMR (15.4%). Whether this plays a role in conferring multi-specificity in DrrB will be determined in future studies by mutagenesis of specific aromatic and other residues in DrrB.

Table 3.1 Bacterial strains and plasmids

<table>
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<th>Description</th>
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<td>supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1 Δ uncIC</td>
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<td>Cloning vector, pACYC184 derivative, Cm^r</td>
<td>(59)</td>
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<td>drrAB in pSU2718, Cm^r</td>
<td>(59)</td>
</tr>
<tr>
<td>pDX102</td>
<td>drrA in pSU2718, Cm^r</td>
<td>(59)</td>
</tr>
<tr>
<td>pDX103</td>
<td>drrB in pSU2718, Cm^r</td>
<td>(59)</td>
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<td>drrAB in pSU2718 with mutation of Gly^{44} to Ala^{44} in the Walker A domain of drrA</td>
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<td>(104)</td>
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<td>(104)</td>
</tr>
<tr>
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<td>(104)</td>
</tr>
<tr>
<td>pDX101(E165Q)</td>
<td>drrAB in pSU2718 with mutation of Glu^{165} to Gln^{165} in the switch motif of drrA</td>
<td>(104)</td>
</tr>
<tr>
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<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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This study
Figure 3.1 Characterization of the DrrAB-mediated Dox efflux under in vivo and in vitro conditions. These data were contributed by Wen Li and Madhu Sharma

FIGURE 3.1 Characterization of the DrrAB-mediated Dox efflux under *in vivo* and *in vitro* conditions. (A) *In vivo* analysis of DrrAB-mediated Dox efflux using *E. coli* cells. *E. coli* LE392ΔuncIC cells containing either vector or pDX101 (DrrAB) were loaded with 10 µM Dox, and the whole cell Dox efflux assay was performed, as described under Methods and in ref (61). Dox efflux was measured fluorometrically (exi, 480 nm; emi, 590 nm) on an Alphascan-2 spectrofluorometer (Photon Technology International). Energy was provided in the form of 20
mM glucose or 20 mM succinate at 100 seconds (marked with an arrow) and fluorescence was monitored for an additional 400 seconds. 10 mM sodium fluoride (NaF) was added to the samples, where indicated. **A.1**: Curve 1, pDx101(DrrAB)/glucose; Curve 2, pDx101 (DrrAB)/succinate; Curve 3, pDx101 (DrrAB)/glucose/NaF; Curve 4, pSU2718 (vector)/glucose; Curve 5, pSU2718/succinate; Curve 6, pSU2718/glucose/NaF. **A.2**: Quantitative presentation of the Dox efflux data shown in Fig 1A.1. The slope of the linear portion of each curve shown in Fig 1A.1 was calculated. The slope of curve 1 was designated as 1.0. Relative slope of each curve was then obtained by dividing the slope of the curve by the slope of curve 1. The average data obtained from three independent experiments are shown in the histogram. **(B) In vitro analysis of DrrAB-mediated Dox efflux using inside-out membrane vesicles (IOVs).** *E. coli* LE392ΔuncIC cells containing either vector or pDX101 (DrrAB) were grown to mid-log phase and induced with 0.25 mM IPTG at 37 °C for 3 hr. The membrane fraction was prepared (49) and Dox efflux in DrrAB-containing IOVs was performed, as described under Methods. **B.1**: *In vitro* Dox efflux assay was carried out using 250 µg IOVs in the presence of 1 µM Dox, 0.1 mg/ml creatine kinase and 5 mM creatine phosphate in 3 ml PBS buffer, pH 7.5, as described under Methods. Vector, Dox efflux in IOVs prepared from cells containing empty vector; DrrAB, Dox efflux in IOVs prepared from cells containing pDX101 (DrrAB). **B.2**: Quantitative presentation of Dox efflux in DrrAB-containing IOVs under various conditions. *In vitro* Dox efflux assay was carried out in the presence or absence of 1 mM ATP, 1mM GTP, 1 mM Mg or 5 mM NADH, as described under Methods. The initial rate of Dox efflux was determined from the linear slope of the fluorescence spectra between 100 s and 200 s. The slope of the efflux curve obtained by incubation of the DrrAB-containing IOVs with ATP and Mg$^{2+}$ (column 4) was designated as 1.0. The relative slope for each curve was then
calculated by dividing the slope of the curve by the slope of sample 4. The average data obtained from three independent experiments are shown in the histogram. **(C) Kinetic analysis of DrrAB-mediated Dox efflux in IOVs.** *In vitro* Dox efflux was analyzed using 250 µg IOVs in the presence of increasing concentrations of Dox (0.1 µM to 6.0 µM) and 1 mM ATP/Mg^{2+}. The initial linear rate of Dox efflux was determined for each curve. The data obtained from three independent experiments were fitted to the Michaelis-Menten equation and plotted by SigmaPlot-Kinetics software using the equation for Single Substrate Format. **(D) Inhibition of DrrAB-mediated Dox efflux by sodium o-vanadate.** *In vitro* Dox efflux was measured using 250 µg IOVs in the presence 1 µM Dox, 1 mM ATP and 1 mM Mg^{2+} and increasing concentrations of Vi (0 – 100 µM). The initial slope of the efflux curve obtained with 0 µM Vi was designated as 1.0. The relative slope of each curve was determined, as described underpanel A.2. The average data obtained from three independent experiments are shown in the histogram.
Figure 3.2 Effect of point mutations in the nucleotide binding domain of DrrA on DrrAB-mediated Dox efflux in IOVs.
FIGURE 3.2 Effect of point mutations in the nucleotide binding domain of DrrA on DrrAB-mediated Dox efflux in IOVs. (A) ClustalW alignment of the NBD of DrrA and its prokaryotic or eukaryotic homologs. The conserved motifs present in the NBD are marked at the top. A.1, alignment of the NBD of DrrA with ABC proteins (MalK, Sav1866, HlyB, TAP1, TAP2, LmrC, LmrD and LmrA) from diverse families. A.2, alignment of the NBD of DrrA with close prokaryotic homologs (belonging to the DRA family/DRR subfamily (50) identified by NCBI BLAST. A.3, alignment of the NBD of DrrA with close eukaryotic homologs belonging to the DRA family/ABCA subfamily (50). (B) Effect of point mutations in Walker A, Walker B, or the Signature motif of DrrA on DrrAB-mediated Dox efflux in IOVs. E. coli LE392ΔuncIC cells containing either pSU2718 vector, pDX101 (drrAB in pSU2718), pDX102 (drrA only, in pSU2718), pDX103 (drrB only, in pSU2718) or pDX101 containing mutations of Walker A (G44A, G44S, K47R), Signature motif (S141R), or the Walker B (E165Q) were induced with IPTG, and the IOVs were prepared, as described under Methods. The initial rate of Dox efflux was determined for each sample, and the relative slopes were calculated, as described for Fig. 1. The average data obtained from three independent experiments are shown in the histogram. (C) Effect of point mutations in the Switch motif of DrrA on DrrAB-mediated Dox efflux in IOVs. E. coli LE392ΔuncIC cells containing either pSU2718 vector, pDX101 (drrAB in pSU2718), or pDX101 with mutations of the Walker B region (E165Q) or the Switch motif (Q197H, Y198R, Q197H/Y198H, Q197H/Y198R) were induced with IPTG, and the IOVs were prepared, as described under Methods. The initial rate of Dox efflux was determined for each sample, and the relative slopes were calculated, as described for Fig. 1. The data presented are averages of three independent experiments. Error bars represent standard deviation. (D) Effect of point mutations in the Switch motif of DrrA on DrrAB-mediated ATPase activity
IOVs. IOV samples from Fig. 2(C) were subjected to the NADH-coupled ATPase activity assay, as described under Methods. The relative ATPase activity of each sample was obtained by dividing the activity of each sample by the activity of wild type. The data presented are averages of two independent experiments. Error bars represent standard deviation. (E) Summary of the ATPase activity and Dox efflux activity of wild type DrrAB and Switch motif mutants.

Figure 3.3 Inhibition of DrrAB-mediated Dox efflux by known MDR substrates.
FIGURE 3.3 Inhibition of DrrAB-mediated Dox efflux by known MDR substrates. Dox efflux was measured using 250 µg IOVs in the presence 1 µM Dox and increasing concentrations of the inhibitory substrate in 3ml PBS, pH 7.5. The initial linear rate (100 s - 200 s) of Dox efflux was determined after addition of 1mM ATP/Mg²⁺. The slope of the efflux curve obtained at 0 concentration of inhibitor was designated as 1.0. The relative slope of each curve was then determined. The average slopes resulting from three independent repeats were plotted by Sigma Plot software using ‘scatter plot with error bars’, and IC₅₀ values were determined. (A) Kinetic analysis of the inhibitory effect of Hoechst 33342 on Dox efflux activity. The experimental conditions were the same as described above. The assay was carried out in the presence of increasing concentrations of H 33342 ranging from 0 – 1.6 µM. (B) Kinetic analysis of the inhibitory effect of verapamil. The experimental conditions were the same as for Fig. 3(A), except that the concentration of verapamil ranged from 0 µM to 100 µM. (C) Kinetic analysis of the inhibitory effect of Rifampicin. The experimental conditions were the same as for Fig. 3(A), except that the concentration of rifampicin ranged from 0 µM to 75 µM. (D) A table showing summary of the IC₅₀ values. Kinetic analysis of the inhibitory effect of various drugs on DrrAB-mediated in vitro Dox efflux was determined, as described above for panels 3A-C. The IC₅₀ values were calculated as described under Methods.
FIGURE 3.4 Kinetic characterization of the inhibition of DrrAB-mediated Dox efflux by Hoechst 33342, verapamil or rifampicin. The kinetics of DrrAB-mediated Dox efflux was determined in the presence of fixed concentrations of inhibitor, as shown in panels 4A-C. (A) Competitive inhibition by H 33342. DrrAB-mediated Dox efflux was studied at four different concentrations of Dox (0.25, 0.5, 0.75 and 1.0 µM) in the presence of a fixed concentration of H33342. In total, four different concentrations of H 33342 (0, 0.2, 0.6, 0.8 µM) were studied. The rate of Dox transport obtained with 1 µM Dox and 0 µM H 33342 was designated as 1.0. The relative rates were then calculated for each efflux curve, and the data were plotted by Lineweaver-Burk plot using Sigma Plot- Kinetics software in Single Substrate – Single Inhibitor Kinetics Format. The error bars represent three separate experiments. The type of inhibition
was determined based on the rank of both AICc and $R^2$. (B) **Non-competitive inhibition by verapamil.** The experiment was performed as described under panel (A). Four different concentrations of verapamil (0, 3.5, 7, 14 µM) were used. (C) **Competitive inhibition by rifampicin.** The experiment was performed as described under panel (A). Four different concentrations of rifampicin (0, 5, 10, 20 µM) were used. (D) **Summary of the kinetics constants obtained for inhibition of Dox efflux by H 33342, verapamil and rifampicin.**

![Graphs and tables]  

**Figure 3.5** The DrrAB system forms a multidrug transporter. (A.1) DrrAB-mediated Hoechst 33342 efflux in IOVs.
FIGURE 3.5 The DrrAB system forms a multidrug transporter. (A.1) DrrAB-mediated Hoechst 33342 efflux in IOVs. The *E. coli* inside-out membrane vesicles were prepared, as described under Methods. 250 µg IOVs were mixed with 0.5 µM 33342, 0.1 mg/ml creatine kinase and 5 mM creatine phosphate in 3ml PBS buffer, pH 7.4. The fluorescence spectra were recorded on an Alphascan-2-spectrofluorometer with excitation of 355 nm and emission of 457 nm. After 100 s, the detection was paused, and 1mM Mg$^{2+}$ and 1mM ATP, pH 7.5 were added into the reaction. The detection was continued for additional 250 s. Vector, IOVs prepared from cells containing empty vector; DrrAB, IOVs prepared from cells expressing DrrAB. (A.2) Kinetic analysis of DrrAB-mediated H 33342 efflux in IOVs. The experimental conditions were the same as described under A.1 above. However, H 33342 efflux was analyzed at concentrations ranging between 0.1 µM to 2.5 µM. The initial (between 100 s-200 s) linear rate of H 33342 efflux was determined. The data obtained from three independent experiments were fitted by the Michaelis-Menten equation using Sigma Plot – Kinetics software in Single Substrate Kinetics Format. (B.1) DrrAB-mediated ethidium bromide efflux in *E. coli* cells. *E. coli* cells containing empty vector or DrrAB were loaded with 25 µM EtBr for 1 hr at 37 °C. Energy was provided in the form of 20 mM glucose, and EtBr efflux was measured, as described under Methods. (B.2) Kinetic analysis of DrrAB-mediated EtBr Efflux in *E. coli* cells. The experimental conditions were the same as described under B.1 above. However, EtBr efflux was analyzed at concentrations ranging between 1 µM to 100 µM. The steady-state linear rate of EtBr efflux was determined. The data obtained from three independent experiments were fitted by the Michaelis-Menten equation using Sigma Plot – Kinetics software in Single Substrate Kinetics Format. (C) Summary of the kinetic parameters obtained by using Michaelis-Menten or Hill equation for DrrAB-mediated Dox, H33342, and EtBr efflux.
4 GENERAL DISCUSSION

The fast development of therapeutic agents has been compromised by the increasing problems of multidrug resistance both in infectious microorganisms as well as in cancer cells. *Streptomyces peucetius*, a soil microorganism that produces anti-cancer drugs Dox and Dnr, is self-resistant to these drugs due to the expression of the DrrAB drug efflux system on the membrane. Because of the limited knowledge of the mechanism as well as the evolution of multidrug resistance, the prototype DrrAB system found in the producer organism can serve as an excellent model to understand various aspects of the phenomenon of MDR.

In our current studies, we have demonstrated that DrrAB is a multidrug transporter. It can efficiently transport Dox, Hoechst 33342 and ethidium bromide in whole cells as well as membrane vesicles. Dox transport activity could be inhibited by verapamil, rifampicin, vinblastine and colchicine, suggesting that these drugs are also substrates of DrrAB. DrrAB belongs to the ABC family of transporters and shares high similarities in sequence, structure and function with P-gp. Our studies showed that DrrAB and P-gp share some of the substrates, although Pgp can transport a much larger range of structurally unrelated compounds. Kinetics studies of drug efflux by DrrAB indicated competitive inhibition of Dox transport by Hoechst 33342 and rifampicin but non-competitive inhibition by verapamil. These studies raise intriguing questions about whether DrrAB contains more than one drug binding site, how these drugs interact with DrrB, and how does the conformation of DrrA and DrrB change during the drug transport process? Further studies such as identification of the drug binding sites, analysis of specific drug transport mechanism in purified and reconstituted proteoliposome, and the cross-talk between NBDs and TMDs during ATP binding, hydrolysis and drug efflux are required.
The drug binding sites of P-gp have been under investigation for several decades. In early 1994, Pawagi and Deber showed that the TMHs of P-gp are highly enriched in aromatic amino acids (18%) as compared to the single-drug transporter TetA (9.4%) (137). Later, Loo and Clark used biochemical approaches to identify the drug binding sites of P-gp. Their work showed that the drug binding interfaces are located at TMHs 4, 6, 10, 11 and 12 (29,35,37-39,139). Substitution of a single serine to phenylalanine in TM11 of P-gp significantly altered its drug efflux profile, indicating the significant roles of residues in TMH11 in the recognition and transport of their specific substrates (138). Not until recently, the crystal structure of P-gp with higher resolution was presented by Chang’s group (42). In their description, P-gp with two bundles of six TMHs forms a large internal cavity open to both the inner leaflet and the cytoplasm. The drug binding pocket is made up of mostly hydrophobic and aromatic residues; only 13 of the 73 residues in the internal cavity are polar or charged. The upper half of the pocket contains mostly hydrophobic/aromatic residues while the lower half pocket mainly consists of charged/polar residues. Interestingly, based on the topological structure and modeling analysis of DrrB (84), the aromatic amino acid content of predicted TMHs of DrrB (15%) is close to the aromatic content of P-gp (18%) and the bacterial MDR protein (15.4%) (137). The hydrophobic and aromatic residues are mainly located in TMHs 1, 5, 6 and the N-terminal loop of the single monomer of DrrB. The N-terminal loop and TMH1 might be particularly important since they directly connect the TMDs of DrrB to the catalytic domain of DrrA (10). To determine whether these domains are involved in drug binding, single point mutations can be introduced in the potential drug binding domains in DrrAB. Mutations in any residues that are essential for drug binding will affect the transport of substrates (36,140,141).
Another major question of DrrAB drug transport is the cross-talk between DrrA and DrrB. The role of the conserved EAA motif found in the first intracellular loop of the TMD in communication with the NBD has been studied in bacterial importers and exporters (142). In our lab, we identified an EAA-like motif at the N-terminal cytoplasmic tail of DrrB (10). Sequence alignment analysis showed that DrrB, LmrA, MDR1, CFTR, MsbA and BtuC share a conserved EAA-like motif. Chemical cross-linking analysis indicated that DrrA cross-links with cysteines introduced in both the N-terminal and C-terminal ends of DrrB. Thus, this EAA-like motif may be involved in the interaction between DrrA and DrrB proteins. Later in our laboratory, a CREEM motif (C-terminal Regulatory Glu (E)-rich Motif), located in the extreme C terminus of DrrA, was found to interact with the EAA-like motif in the N-terminal cytoplasmic tail of DrrB (10,61). Taken together, both the EAA-like motif at the N terminus of DrrB and the CREEM motif at the C terminus of DrrA are significant for the interaction between DrrA and DrrB protein as well as the biogenesis of the DrrAB complex (61). However, how energy is transduced between NBDs to TMDs and after the drug was transported still remains to be understood (143).

In this work, I also studied the role of the FtsH protease in quality control and biogenesis of the DrrAB complex in the membrane. FtsH was first identified by Ito and co-workers, who suggested that FtsH may be involved in protein assembly into and through the membrane and in determining the orientation of membrane proteins (73). Chaperone function was also proposed for other ATP-dependent proteases, such as ClpAP/XP, YTA10/12 and Lon (144-147). However, no direct evidence for the role of FtsH or any other protease in the functional assembly of membrane proteins was obtained. In our studies, we provide the first direct evidence that FtsH can perform both proteolytic and refolding functions in the assembly of the DrrAB
complex. The most critical evidence for the refolding function of FtsH was obtained through the sequential expression studies. We show that FtsH expressed after nonfunctional DrrA and DrrB had accumulated in the membrane, was still able to restore the drug efflux function of the DrrAB complex. Our studies also allow us to conclude that although the AAA domain of FtsH provides recognition and specificity for binding of the substrate, both ATP hydrolysis and the proteolytic functions of FtsH are used concurrently for the refolding of DrrAB and restoration of its efflux function.

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