Title: Overexpression and functional characterization of an ABC transporter encoded by the genes drrA and drrB of *Mycobacterium tuberculosis*

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*Running title:* The DrrAB efflux pump of *Mycobacterium tuberculosis*

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SUMMARY

The genes encoding ABC transporters occupy 2.5% of the genome of *Mycobacterium tuberculosis*. However, none of these putative ABC transporters has been characterized so far. We describe the development of expression systems for simultaneous expression of the ATP-binding protein DrrA and the membrane integral protein DrrB which together behave as a functional doxorubicin efflux pump. Doxorubicin uptake in *Escherichia coli* or *Mycobacterium smegmatis* expressing DrrAB was inhibited by reserpine, an inhibitor of ABC transporters. The localization of DrrA to the membrane depended on the simultaneous expression of DrrB. ATP binding was positively regulated by doxorubicin and daunorubicin. At the same time, DrrB appeared to be sensitive to proteolysis when expressed alone in the absence of DrrA. Simultaneous expression of the two polypeptides was essential in order to obtain a functional doxorubicin efflux pump. Expression of DrrAB in *E. coli* conferred 8-fold increased resistance to ethidium bromide, a cationic compound. 2’,7’-bis-(2-carboxyethyl)-5(-and 6)-carboxyfluorescein (BCECF), a neutral compound also behaved as a substrate of the reconstituted efflux pump. When expressed in *M. smegmatis*, DrrAB conferred resistance to a number of clinically relevant, structurally unrelated antibiotics. The resistant phenotype could be reversed by verapamil and reserpine, two potent inhibitors of ABC transporters.
Mycobacterium tuberculosis accounts for the largest number of deaths caused by a single human pathogen with predictions of an excess of 80 million new cases and 20 million deaths in the coming decade. The appearance of drug-resistant strains of M. tuberculosis and the HIV pandemic have exacerbated this situation. Effective treatment of tuberculosis infections requires the identification of new drugs, drug targets and drug resistance factors. The ABC (ATP-Binding Cassette) transporters [1], constitute a large superfamily of multi-subunit permeases that transport diverse molecules (ions, amino acids, peptides, drugs, antibiotics, lipids, polysaccharides, proteins etc.) at the expense of ATP [2, 3]. The genes encoding the ABC transporters occupy about 2.5% of the genome of M. tuberculosis. [4]. At least 37 complete and incomplete ABC transporters have been identified in M. tuberculosis based on the structural similarities of the typical subunits of ABC transporters present in all living organisms. Knowledge of these M. tuberculosis ABC transporters is necessary both for understanding their involvement in the development of multidrug resistance in M. tuberculosis as well as in the export of the unique antigenic cell surface components of this organism, such as the phthiocerol mycocerosates and lipoarabinomannan.

The prototype eukaryotic ABC transporter is the P-glycoprotein [P-gp] [5]. The 170 kDa P-glycoprotein is characterized by two membrane integral domains with six membrane-spanning helices each connected with two ATP hydrolyzing domains and the presence of the “Walker sites A and B” [6] in the primary structure of the ATP-hydrolyzing domains, which by analogy with ATP-and GTP-binding proteins, bind nucleotides [7, 8]. The doxorubicin resistance operon, first identified in Streptomyces peucetius [9] bears analogy to the eukaryotic P-glycoprotein. Two translationally coupled open reading frames drrA and drrB encode an ABC type transporter, with drrA encoding the nucleotide-binding domain and drrB encoding the membrane integral component [10]. A monomer of DrrA and DrrB form a molecule about half the size of Pgp. The likely stoichiometry of the functional transporter is DrrA₂B₂ [10]. By analogy to S. peucetius, the completed Mycobacterium tuberculosis genome [11] also contains a doxorubicin resistance operon drr. In addition, the drr operon has been identified in the genomes of M. leprae and M. avium. As powerful techniques of molecular biology have become available, the drr operon has assumed particular significance in relation to its implications in virulence of M. tuberculosis. The biosynthesis of phthiocerol dimycocerosates (DIM) involves several genes [12]. The genes pps A-E, encode a type I modular polyketide synthase responsible for the synthesis of phthiocerol and phenolphthiocerol. Another gene, mas encodes an iterative type I polyketide synthase that produces mycocerosic acid. The gene fadD28 is probably involved in the release and transfer of
mycocerosic acid from Mas onto the diols. These 7 genes are clustered on a 50 kb fragment of the chromosome containing among other genes, the ORFs of the *drr* operon and *mmpl7* encoding polypeptides similar to ABC transporters. Signature-tagged transposon mutagenesis has shown that transposon insertions in the *drr* operon or *mmpl7* lead to a strong growth defect *M. tuberculosis* in lungs of intravenously infected mice [13, 14] and in export of DIM to the cell surface [15].

The biochemical characterization of the Drr transporter assumes obvious importance in the light of these recent observations. Its association with virulence suggests that it presents an attractive drug target, which if inactivated, will probably disable the pathogen in terms of its ability to export complex molecules such as DIM, a surface-exposed antigenic lipid present in seven pathogenic species of mycobacteria [16], to the cell surface. In addition, the likely importance of the *drr* operon in multidrug resistance deserves evaluation. The biochemical characterization of the *drr* operon was undertaken before most of these recent observations came to light, envisaging its likely importance in multidrug resistance of *M. tuberculosis*. The goal was to develop expression systems expressing a functional DrrAB transporter in order to evaluate the role of the transporter in drug resistance as well as to exploit the expression systems in further characterization of the transporter in relation to the roles of the conserved domains in transport function.

**EXPERIMENTAL PROCEDURES**

*Materials*- [*14C*]Doxorubicin and the Thermosequenase cycle sequencing kit were purchased from Amersham Pharmacia Biotech, Buckinghamshire, U.K. Restriction enzymes and antibiotics were purchased from Life Technologies, Gaithersberg, Maryland, U.S.A, [*α-32P*]ATP was purchased from NEN Life Sciences, Boston, U.S.A., and 2’,7’-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetomethoxyl ester (BCECF-AM) was a product of Molecular Probes, Eugene, Oregon, U.S.A. Doxorubicin, daunorubicin, ethidium bromide, reserpine, verapamil, chloramphenicol, tetracycline, erythromycin, ethambutol, rifampicin, norfloxacin, streptomycin and puromycin were products of Sigma Chemicals, St. Louis, Missouri, U.S.A. All other reagents were of analytical grade.

*Strains*- Cloning was performed in *E. coli* DH5α. *E. coli* LMG194 [Invitrogen] and *E. coli* BL21(DE3) [Novagen] were used for protein expression. *M. smegmatis* mc2155 has been described by Snapper *et al.* [17].

*Amplification and cloning of drrAB from cosmid MTCY19H9*- The *drrAB* operon was amplified from the cosmid MTCY19H9 (a kind gift from Stewart Cole, Institut Pasteur, Paris) using the primer pair: sense: 5’CGG GGT ACC ATA TGC GCA ACG ACG ACA TGCC 3’ and...
antisense 5’CCC GAA TTC GTC GTG ATC ATG GGCC GCC TAG 3’ with asymmetric KpnI and EcoRI sites (underlined) in the sense and antisense primers respectively. The PCR product was digested with KpnI and EcoRI, cloned in the vector pUC19 between the KpnI and EcoRI sites to generate the plasmid pCKB101, and sequenced on both strands. The sequenced drrAB gene was excised from pUC19 and cloned between the NdeI (indicated in bold) and EcoRI sites of the expression vector pET 28a+ (Novagen) to give the plasmid pCKB102.

Cloning of the drrA gene- The drrA gene was amplified from plasmid pCKB101 using the following primer pair: 5’CGG GGT ACC ATA TGC GCA ACG ACG ACA TGGC3’ (sense) and 5’ATA GAA TTC ATC GCG CGG ACC CCG ACA CCA G3’ (antisense) with asymmetric BglII and EcoRI sites, and cloned in the vector pK18 [18] between the BglII and EcoRI sites to generate the plasmid pCKB104. pCKB104 was sequenced and the drrA gene was excised and cloned between the NdeI and EcoRI sites of pET28a+ to give pCKB105.

Cloning of the drrB gene - The drrB gene was amplified from plasmid pCKB101 using the following primer pair: 5’ ATA GGT ACC ATA TGA GCG GCC CGG CCA TAG ATG CG 3’ (sense) and 5’CCC GAA TTC GTC GTG ATC ATG GGCC GCC TAG 3’ (antisense) with asymmetric BglII and EcoRI sites, and cloned in the vector pK18 to give pCKB107 and sequenced. The drrB gene was excised and cloned between the NdeI and EcoRI sites of pET28a+ to give pCKB108, as well as between the BglII and EcoRI sites of pBAD-HisA to give pCKB109.

Construction of an artificial operon- pCKB108 was digested with NcoI and NdeI to eliminate a 58 bp fragment. This was replaced by oligonucleotides 5’CATGGCTGGTACCGGGGT CAA GGA GAT AAC A 3’ and its reverse complement strand, with overhangs of CATG at the 5’end and AT at the 3’ end to complement NcoI and NdeI respectively. The resulting plasmid termed pCKB110, contained a KpnI site (underlined) and a Shine-Delgarno (SD) sequence (AGGA).

The drrA gene was amplified from pCKB105 using the primers: sense 5’GAA GAT CTC ATA TGC GCA ACG ACG ACA TGG C 3’ and antisense 5’GAATT CGG TAC CAG ATG GGT CAG AGA CTC GGT 3’ with asymmetric NdeI and EcoRI sites and cloned between the NdeI and EcoRI sites of pET28a+ to give pCKB111. Plasmid pCKB110 was digested with KpnI and EcoRI and the fragment containing the drrB gene with the upstream SD sequence was cloned between the KpnI and EcoRI sites of pCKB111 to give pCKB112. pCKB112 therefore contains the translationally coupled drrAB genes, with the artificial ribosome binding site AGGA upstream of the drrB gene, under the control of the T7 promoter. The following cloning steps were performed in order to introduce the myc epitope at the C-terminal end of DrrB. Using pCKB112 as template, the drrA and drrB genes were amplified using the primer pair 5’ GAAGATCTCAT ATG CGC AAC GAC GAC ATG GC3’ (sense) [primer DrrA-His] and 5’AAAAAAGCTTT GGG
CCG CCT AGC CAA AAC GTT TTG GCT AGG CGG CCA3' (antisense) [BglII and HindIII sites in the sense and antisense primers underlined] and cloned between the BglII and HindIII sites of pBAD myc HisA to give plasmid pCKB113 carrying a translational fusion of the myc epitope to the C-terminal end of DrrB. Primer DrrA-His was used as the sense primer paired with the antisense primer: 5'ATG GAATTC TCA GTC GAC GGC GCT ATT CAG ATC3' [EcoRI site underlined] for amplification using pCKB113 as the template, and the product was cloned between the BamHI and EcoRI sites of pET28a+ to give pCKB114. pCKB114 carried the drrA gene fused to an upstream sequence encoding a hexahistidine tag, and the drrB gene fused to a downstream sequence encoding a myc epitope. Further cloning was performed in order to place the his-drrA-drrB-myc –encoding sequence under the control of the mycobacterial hsp60 promoter in a shuttle vector with E. coli and mycobacterial origins of replication (derived from pYUB12) [17] and a kanamycin resistance marker generating the plasmid pCKB115.

Expression of proteins in E. coli - Recombinant plasmids derived from pET28a+ were transformed in E. coli BL21 (DE3). Cells were grown upto mid log phase (OD₆₀₀=0.6) in Luria broth and induction was carried out at different temperatures with different concentrations of IPTG. Recombinant plasmids derived from pBad-HisA were transformed into E. coli LMG194 cells (Invitrogen pBAD Manual) and induced with varying concentrations of arabinose at different temperatures.

Expression of proteins in Mycobacterium smegmatis mc²155 - Electroporation of M. smegmatis mc²155 with the plasmid pCKB115 was performed as described by Larsen [19] Transformants were grown in Luria broth supplemented with 25 µg/ml kanamycin up to an A₆₀₀ of 0.8 followed by heat shock.

Fractionation of cells and localization of proteins - E. coli cells after induction were suspended in 10 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 1mM phenylmethylsulphonylfluoride (PMSF), 20µg/ml leupeptin, 10 µg/ml pepstatin and 10 µg/ml aprotinin, and sonicated thrice at 200 W for 15 sec. The unbroken cells were removed by centrifugation at 500 x g for 5 min. Inclusion bodies were obtained by centrifugation at 5000 x g for 5'. Membranes were obtained by centrifugation at 100,000 x g for 60 min, and the supernatant constituted the cytosolic extract. Membranes from M. smegmatis transformants were prepared as described by Basu et al.[20].

ATP binding experiment -Photolabelling of membranes was carried out with [α-³²P]ATP (NEN, Easytides) at varying concentrations and a specific activity of 0.25 mCi/mmol in 10 mM Tris HCl, pH 7.4 containing 10 mM DTT. Where indicated, doxorubicin was added to the reaction mixture to a final concentration of 50µM and 100 µM and MgCl₂ was added to a final concentration of 5 mM. The reaction was carried out in a volume of 25µl in a 96-well
Microtitre plate kept on a block of ice directly under UV (254 nm) illumination for 30 min. Samples were analyzed by SDS-PAGE and autoradiography.

**Minimum inhibitory concentrations (MIC)** - *E. coli* BL21(DE3)/pCKB114 or *M. smegmatis*/pCKB115 were induced as described above in order to express DrrA and DrrB. MICs were determined by the broth microdilution method according to National Committee for Clinical Laboratory Standards guidelines [21]. Cells were added at a CFU of $10^6$ per ml in microtitre wells (in duplicate) containing serial dilutions of different drugs. Controls without drug were also run. The plate was incubated at 37°C overnight (for *E. coli*) or 48 h (for *M. smegmatis*). MIC was defined as the lowest concentration of drug with complete absence of growth (Absorbance at 600 nm >0.05 i.e., a value equal to visible growth). Each MIC was determined at least thrice using three different transformants.

**Uptake of $[^{14}C]$-doxorubicin** - Cells expressing DrrA and DrrB were grown up to mid-log phase, induced, harvested and suspended to an OD$$_{600}$ of 20 in 50 mM K-phosphate, pH 7.4 containing 5 mM MgSO$$$_4$$. Uptake was initiated (in the absence or in the presence of inhibitors) by the addition of doxorubicin (5 µM; 10-50 µCi/mmol). Cells were diluted into ice-cold 10 mM K-phosphate, pH 7.4 containing 0.1 M LiCl, filtered on glass fibre (Whatman GF/C) filters, dried and counted in a liquid scintillation counter.

**Uptake of 2',7'-bis-(2-carboxyethyl)-5(-and 6)-carboxyflurescein acetomethoxyl ester (BCECF-AM)** - This was done as described by Bolhuis et al. 1996 [22]. The cells were harvested and washed thrice with 50 mM HEPES buffer, pH 7.3 containing 25 mM K$_2$SO$_4$ and 5 mM MgSO$_4$. Subsequently, the cells were resuspended in the buffer to a final OD$_{600}$ of 20. BCECF-AM (2mM stock solution in dimethyl sulphoxide) was added to the cell suspension to a final concentration of 1 µM. BCECF fluorescence was monitored continuously at excitation and emission wavelengths of 502 and 525 nm respectively, in a HITACHI (F-4500) spectrofluorimeter, equipped with a thermostatically-controlled (37°C), magnetically-stirred cuvette holder.

**RESULTS**

**Expression and localization of DrrA in *E. coli*** - DrrA was expressed in *E. coli* BL21(DE3)/pCKB105 at 30°C at an IPTG concentration of 100 µM for 4h (Fig. 1, Panel A). The protein localized primarily in the inclusion bodies (data not shown).

**Expression of DrrB in *E. coli*** - Attempts to express DrrB (cloned in the vector pE28a+) in *E. coli* BL21(DE3) were abortive. The expressed protein appeared to be extremely sensitive to proteolysis. DrrB was also cloned in the vector pBAD-HisA (Invitrogen) under the control of the
araBAD promoter and expressed in *E. coli* LMG-194. This was done in view of the fact that the araBAD promoter (pBAD) of *E. coli* allows regulated expression facilitating optimum expression of protein in its properly folded form. Briefly, transformed cells were grown up to an OD<sub>600</sub> of 0.6 in RM-glucose medium with 50µg/ml ampicillin as described in the Invitrogen pBAD Manual. Cells were induced with 0.002 % arabinose at 30°C for 4 h. Induced cells were pelleted down, subjected to three cycles of freezing and thawing, and run on SDS-polyacrylamide (12.5%) gels. Expressed DrrB migrated as a polypeptide of apparent molecular mass 31 kDa (Fig. 1, panel B). DrrB localized exclusively to the membranes. However, it still remained sensitive to proteolytic degradation. Attempts to extract it from membranes using various detergents, were abortive.

**Tandem expression of DrrA and DrrB**- When *E. coli* BL21(DE3)/pCKB102 harbouring the drrAB genes was induced with 50µM IPTG, the expression of DrrA was visible on polyacrylamide gels, but the expression of DrrB could not be visualized. An artificial operon was therefore constructed to allow the simultaneous expression of DrrA and DrrB. An artificial ribosome-binding site was introduced upstream of the drrB gene (Fig. 1, panel D), and the drrA and drrB genes were fused downstream of a hexahistidine encoding sequence and upstream of a myc epitope encoding sequence respectively in the vector pET28a+ to give the plasmid pCKB114. Induction of DrrA and DrrB was found to be optimal when carried out using 50µM IPTG at 30°C for 4 h. Membranes, cytosolic extracts and inclusion bodies were prepared as described above. In crude cell extracts, bands corresponding to both DrrA and DrrB were visible on SDS-polyacrylamide gels. Both DrrA and DrrB were present exclusively in the membranes of these cells (Fig. 1, panel C, lanes 1 and 2) as confirmed by Western blotting with anti-His and anti-myc antibodies (Fig. 1, panel C, lanes 3 and 4). *E. coli* transformants could be stored as glycerol stocks at −70°C for several weeks without loss of expression of DrrAB.

**Expression of DrrAB in *M. smegmatis*** – In order to evaluate the functional characteristics of the DrrAB efflux pump in mycobacteria, conditions were optimized for expression of both DrrA and DrrB under the control of the heat shock promoter, hsp60, in *M. smegmatis*. Heat shock was given at temperatures ranging from 37 to 45°C for varying periods of time (30 min to 2 h). Optimum expression was observed following heat shock at 42°C for 45 min. Both components of the pump localized to the membranes of induced cells as judged by western blotting using anti-His and anti-myc antibodies (Fig. 1, panel E). *M. smegmatis* transformants could be stored as glycerol stocks at −70°C for two weeks after which transformants showed very slow growth when cultured in liquid medium.
[α-32P] ATP binding - UV-catalyzed binding of [α-32P]ATP to DrrA was observed when *E. coli* membranes expressing DrrA and B simultaneously were used for binding studies. The binding was enhanced in the presence of both doxorubicin (Dox) and daunorubicin (Dnr). It is possible that both these drugs induce a conformational change in DrrA favouring interaction with ATP.

Uptake of [14C]-doxorubicin - [14C]-doxorubicin uptake was studied in *E. coli* cells harbouring plasmid pCKB114 designed for tandem expression of DrrA and DrrB. Doxorubicin was used at a concentration of 5µM (which did not affect cell viability). Doxorubicin uptake was significantly lower in cells induced to express DrrAB compared to uninduced cells suggesting that the tandemly expressed DrrA and B proteins were associating to form a functional doxorubicin efflux pump (Fig. 3, panel A). This was further confirmed by determining doxorubicin accumulation after addition of reserpine (at sublethal concentrations), an inhibitor of ATP-dependent efflux pumps. On addition of reserpine, doxorubicin accumulation in cells expressing both DrrA and B increased to levels similar to that observed in uninduced cells. DrrAB therefore appeared to associate to form a functional doxorubicin efflux pump. Accumulation levels in cells expressing DrrA or DrrB alone were similar to uninduced cells (data not shown). The uptake of doxorubicin in *M. smegmatis* was again found to be inhibitable by reserpine (Fig. 3, panel B), suggesting that the pump is functional in *M. smegmatis*. In *E. coli* and in *M. smegmatis*, the difference in accumulation in the induced and uninduced states while being statistically significant, ranged between 2.5 and 3-fold.

BCECF-AM transport - BCECF-AM is a non-fluorescent, neutral compound which diffuses across the cytoplasmic membrane. Once inside the cell, BCECF-AM is rapidly hydrolyzed by non-specific esterases, trapping the non-permeant hydrophilic free acid BCECF within the cell. BCECF accumulated after the addition of BCECF-AM to *E. coli* BL21(DE3)/pET28a+ or to *E. coli* BL21(DE3)/pCKB114. Intracellular accumulation was strongly reduced in the latter case compared to cells containing vector alone. The expression of DrrAB therefore appeared to play a role in decreased accumulation of BCECF, suggesting that neutral compounds may also serve as substrates of the Drr pump. Addition of reserpine was able to partially restore the level of accumulation of BCECF to that of cells expressing vector alone (Fig. 4). The likely presence of reserpine-insensitive pump(s) in *E. coli* BL21(DE3) probably accounts for the inability of reserpine to fully restore BCECF accumulation. Cells expressing only DrrA or DrrB behaved in a manner similar to cells harbouring the vector pET28a+ or the vector pBAD-HisA alone (data not shown). The presence of constitutive extracellular mycobacterial esterases made it impossible to study BCECF accumulation in *M. smegmatis*. 

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Effect of DrrAB expression on drug susceptibility - E. coli BL21(DE3)/pCKB114 expressing DrrA and DrrB simultaneously showed increased resistance to ethidium bromide, doxorubicin, daunorubicin, chloramphenicol and puromycin. (Table 1). Cells expressing DrrA or DrrB alone behaved like cells harbouring the vector pET28a+ or the vector pBAD-HisA alone (data not shown).

The DrrAB pump is likely involved in the transport of phthiocerol dimycocerosate to the cell surface in M. tuberculosis. In addition, another physiological role of such a pump could be to pump out toxic lipophilic metabolites or hydrophobic compounds which are encountered in the extracellular environment. To address the role of DrrAB in antibiotic resistance in mycobacteria, the MICs of M. smegmatis expressing DrrAB towards a range of clinically relevant antibiotics were determined. DrrAB conferred resistance to a broad range of clinically relevant antibiotics, including tetracycline, erythromycin, ethambutol, norfloxacin, streptomycin and chloramphenicol. This suggested a possible role that it might play in antibiotic resistance of M. tuberculosis.

The differences in the –fold enhancement of MICs (in the case of some drugs) due to the expression of DrrAB observed between E. coli and M. smegmatis may be attributed to: (a) different roles of the permeability barrier, (b) roles of other efflux pumps, (c) differences in target sensitivities and (d) different drug-inactivating mechanisms in the two organisms.

DISCUSSION

The principal physiological role of the Drr proteins of M. tuberculosis appears to be in the export of complex lipids to the cell exterior. The role of ABC transporters in lipid transport is now being acknowledged widely [23]. The role of the drr operon in transport of DIM to the cell surface in M. tuberculosis has also been documented [15 ]. The results of drug susceptibility profiling in the E. coli and M. smegmatis expression systems for DrrAB developed by us suggest a role of this pump in resistance against hydrophobic drugs. The attempts to express the two proteins individually in E. coli, suggest that neither of the two proteins retains its integrity in the absence of the other protein. DrrB appeared to be exquisitely sensitive to proteolysis in the absence of DrrA. The use of the artificial operon to achieve simultaneous expression of DrrA and DrrB, allows the demonstration of the interdependence of the two proteins. The targeting of DrrA to the membranes is facilitated by the coexpression of DrrB, while the stability of DrrB in the membranes is facilitated by the simultaneous presence of DrrA in the membranes. In E. coli, both cationic hydrophobic compounds such as doxorubicin and neutral compounds such as BCECF appeared to be substrates of the functional DrrAB efflux pump. The expression of the pump in E.
coli imparted 8-fold increased resistance to ethidium bromide, a cationic substrate of ABC transporters. Increased adduct formation with $[\alpha-^{32}P]ATP$ in the presence of doxorubicin or daunorubicin raised the possibility that substrate binding to the pump induces a conformational alteration favouring ATP binding to DrrA. This is similar to the observation that Dox stimulates ATP binding to DrrA of \textit{S. peucetius} [24]. Whether it does so by binding to a site in the DrrA protein or exerts an indirect effect after binding to DrrB is open to question. Drug-stimulated ATPase activity has been reported in the case of the human P-glycoprotein [25]. The overexpression of a functional doxorubicin efflux pump from \textit{M. tuberculosis} and its inhibition by known inhibitors of ABC transporters such as reserpine, exemplifies the characterization of an ABC transporter for the first time from this globally important pathogen, and demonstrates that it imparts resistance to hydrophobic drugs. The use of doxorubicin accumulation to determine functionality of the DrrAB pump provides an assay system for biochemical characterization of this pump. The difference in accumulation levels between induced and uninduced cells range between 2.5- and 3-fold in \textit{E. coli} and \textit{M. smegmatis}. This suggests that only inhibitors that inhibit the pump almost completely are likely to be picked up without ambiguity using this assay system. Similarly, the effects of mutations of conserved amino acid residues are likely to be reflected unambiguously when such mutations inhibit the pump almost completely. Nevertheless the expression systems described here deserve further evaluation considering that development of an assay system based on the likely natural substrate DIM, appears even more technically challenging and elusive. The differences in MIC observed in \textit{M. smegmatis} expressing DrrAB suggest that this pump confers resistance towards a broad range of structurally unrelated drugs in mycobacteria. This is like the observation that LmrA, the \textit{Lactococcus lactis} counterpart of the human P-glycoprotein, shows broad substrate specificity [26]. The \textit{M. smegmatis} assay system is likely to be useful in evaluating drugs of potential against mycobacteria by virtue of their ability to inhibit DrrAB. On the other hand, the \textit{E. coli} expression system is more robust; \textit{E. coli} transformants being more stable than \textit{M. smegmatis} transformants when stored as glycerol stocks at –70°C. \textit{E. coli} also has the advantage of faster generation time than \textit{M. smegmatis}.

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Table 1. Drug susceptibility of cells expressing DrrAB

<table>
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<tr>
<th>Drug</th>
<th>E. coli uninduced</th>
<th>E. coli induced</th>
<th>M. smegmatis uninduced</th>
<th>M. smegmatis induced</th>
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* N.D., not determined
LEGENDS TO FIGURES

Fig. 1. Expression of the DrrA and DrrB proteins. Cells were grown and induced as described under Experimental Procedures. Cells were suspended in Laemmli sample buffer, run on SDS-polyacrylamide (12%) gels and visualized by staining with Coomassie Blue. (A) DrrA in *E. coli* BL21(DE3)/pCKB105 lanes 1 and 2 represent uninduced and induced cells respectively. (B) DrrB in *E. coli* LMG194/pCKB109, lanes 1, 2, 3 and 4 represent uninduced cells, induced cells, and membranes from induced cells and uninduced cells, respectively. (C) Membranes from uninduced (lane 1) and induced (lanes 2-4) *E. coli* BL21(DE3)/pCKB114 were run on SDS-polyacrylamide (12%) gels, transferred on to nitrocellulose and either stained with Amido Black (lanes 1 and 2) or probed with anti-His (lane 3) or anti-myc (lane 4) antibodies. (D) Schematic representation of the artificial operon constructed for translational coupling and simultaneous expression of DrrA and DrrB. (E) Western blots of *M. smegmatis* /pCKB115 membranes from uninduced (lanes 1 and 3) and induced (lanes 2 and 4), probed with anti-His (lanes 1 and 2) or anti-myc (lanes 3 and 4) antibodies.

Fig. 2. [α-^32^P]ATP binding to DrrA. UV-catalyzed adduct formation between DrrA and [α-^32^P]ATP was performed in membrane fractions of cells expressing DrrA and B. Adduct formation was catalyzed as described under Experimental procedures. Proteins were resolved on SDS-polyacrylamide (12%) gels, followed by autoradiography. (A) Effect of Mg^{2+} (5 mM) and varying concentrations of ATP on adduct formation. (B) Effect of doxorubicin (40µM) and daunorubicin (40µM) on adduct formation carried out in the presence of 5 mM Mg^{2+} and 200 µM ATP.
Fig. 3. $[^{14}C]$Doxorubicin accumulation in *E. coli* (panel A) and *M. smegmatis* (panel B) expressing DrrA and DrrB. Steady state accumulation levels of doxorubicin in uninduced (solid bars) cells were taken to be 100%. Accumulation in uninduced and induced (shaded bars) cells in the absence of inhibitors have been termed as controls. Where indicated, induced cells were incubated with 5 µM doxorubicin and increasing concentrations (µg/ml) of inhibitors. Data expressed represent the mean ± SD of three separate determinations using three different batches of transformants.

Fig. 4. Uptake of BCECF by cells expressing DrrA and DrrB. BCECF-AM (1 µM) was added to: *E. coli* BL21(DE3)/pET28a+ energized with glucose (A) or *E. coli* BL21(DE3)/pCKB112 induced with IPTG and energized with glucose (C) or *E. coli* BL21(DE3)/pCKB114 induced with IPTG, energized with glucose and incubated with reserpine (10 µg/ml) (B).
ABBREVIATIONS

BCECF-AM, 2’,7’-bis-(2-carboxyethyl)-5(-and 6)-carboxyfluorescein acetomethoxyl ester;

DIM, phthiocerol dimycocerosate; IPTG, isopropyl β-D-thiogalactoside
Fig. 2

A

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<th>+Mg^{2+}</th>
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<td>100 μM ATP</td>
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B

| Dox | + | - |
| Dnr | - | + | + | - |

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Fig. 3
Fig. 4