Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*

Baisakhee Saha CHOUDHURI, Sanjib BHAKTA, Rajib BARIK, Joyoti BASU, Manikuntala KUNDU and Parul CHAKRABARTI[1]
Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Calcutta 700 009, India

The genes encoding ATP-binding cassette (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 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(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.

Key words: antibiotic resistance, efflux pump, lipid transporter, mycobacteria, virulence.

INTRODUCTION

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 ATP-binding cassette (ABC) 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 P-glycoprotein [5]. The 170 kDa P-glycoprotein is characterized by two membrane integral domains with six membrane-spanning helices each connected with two ATP-hydrolysing domains and the presence of 'Walker sites A and B' [6] in the primary structure of the ATP-hydrolysing domains, which, by analogy with ATP-and GTP-binding proteins, bind nucleotides [7,8]. The doxorubicin-resistance operon, first identified in *Streptomyces peucetius* [9], shares similarity with 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 forms a molecule about half the size of P-glycoprotein. The likely stoichiometry of the functional transporter is $DrrA_2B_2$ [10]. By analogy to *S. peucetius*, the completed *M. tuberculosis* genome [11] also contains a doxorubicin-resistance operon, *drr*. In addition, the *drr* operon has been identified in the genomes of *Mycobacterium leprae* and *Mycobacterium avium*. As powerful techniques of molecular biology have become available, the *drr* operon has assumed particular significance in relation to its implications in the virulence of *M. tuberculosis*. The biosynthesis of phthiocerol dimycocerosates (DIMs) 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 on to the diols. These seven genes are clustered on a 50 kb fragment of the chromosome containing, among other genes, the open reading frames 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 of *M. tuberculosis* in the lungs of intravenously infected mice [13,14] and in export of DIM to the cell surface [15].

Abbreviations used: ABC, ATP-binding cassette; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetomethoxyl ester; DIM, phthiocerol dimycocerosate; IPTG, isopropyl β-D-thiogalactoside; MIC, minimum inhibitory concentration.

[1] To whom correspondence should be addressed (e-mail parul@bosemain.boseinst.ac.in).

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

Materials

[^{14}C]Doxorubicin and the Thermosequenase cycle sequencing kit were purchased from Amersham Bioscience (Little Chalfont, Bucks., U.K.). Restriction enzymes and antibiotics were purchased from Life Technologies (Gaithersberg, MD, U.S.A.), [α-^{32}P]ATP was purchased from NEN Life Sciences (Boston, MA, U.S.A.) and 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetomethoxyl ester (BCECF-AM) was a product of Molecular Probes (Eugene, OR, 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, MO, U.S.A.). All other reagents were of analytical grade.

Strains

Cloning was performed in *Escherichia coli* DH5α. *E. coli* LMG194 (Invitrogen) and *E. coli* BL21(DE3) (Novagen) were used for protein expression. *Mycobacterium smegmatis* mc^{2}155 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, France) using the primer pair 5′-CGGGGTACC**ATATG**CGCAACGACGACATGGC-3′ (sense) and 5′-CCCGAATTCGTCGTGATCATGGGCCGCCTAG-3′ (antisense) with asymmetric *Kpn*I and *Eco*RI sites (underlined) in the sense and antisense primers respectively. The PCR product was digested with *Kpn*I and *Eco*RI, cloned into the vector pUC19 between the *Kpn*I and *Eco*RI sites to generate the plasmid pCKB101, and sequenced on both strands. The sequenced *drrAB* gene was excised from pUC19 and cloned between the *Nde*I (indicated above in bold) and *Eco*RI sites of the expression vector pET 28a$^+$ (Novagen) to give the plasmid pCKB102.

Cloning of the *drrA* and *drrB* genes

The *drrA* gene was amplified from plasmid pCKB101 using the following primer pair: 5′-CGGGGTACC**ATATG**CGCAACGACGACATGGC-3′ (sense) and 5′-ATAGAATTCATCGCGCGGACCCCGACACCAG-3′ (antisense) with asymmetric *Kpn*I and *Eco*RI sites, and cloned into the vector pK18 [18] between the *Kpn*I and *Eco*RI sites (underlined) to generate the plasmid pCKB104. pCKB104 was sequenced and the *drrA* gene was excised and cloned between the *Nde*I (shown above in bold) and *Eco*RI sites of pET28a$^+$ to give pCKB105.

The *drrB* gene was amplified from plasmid pCKB101 using the following primer pair: 5′-GAAGATCTCATATGAGCGGCCCG-3′ (sense) and 5′-CCCGAATTCGTCGTGATCATGGGCCGCCTAG-3′ (antisense) with asymmetric *Bgl*II and *Eco*RI sites, cloned in the vector pK18 to give pCKB107 and sequenced. The *drrB* gene was excised and cloned between the *Nde*I and *Eco*RI sites of pET28a$^+$ to give pCKB108, as well as between the *Bgl*II and *Eco*RI sites of pBAD-HisA to give pCKB109.

Construction of an artificial operon

pCKB108 was digested with *Nco*I and *Nde*I to eliminate a 58 bp fragment. This was replaced by oligonucleotide 5′-CATGGCTGGTACCGGGGTCAAGGAGATAACA-3′ and its reverse complement strand, with overhangs of CATG at the 5′-end and AT at the 3′ end to complement *Nco*I and *Nde*I respectively. The resulting plasmid, termed pCKB110, contained a *Kpn*I site (underlined) and a Shine–Dalgarno sequence (AGGA).

The *drrA* gene was amplified from pCKB105 using the primers 5′-GAAGATCTCATATGCGCAACGACGACATGGC-3′ (sense) and 5′-GGAATTC**GGTACC**AGATGGGTCAGAGACTCGGT-3′ (antisense) with asymmetric *Nde*I and *Eco*RI sites and cloned between the *Nde*I and *Eco*RI sites of pET28a$^+$ to give pCKB111. Plasmid pCKB110 was digested with *Kpn*I and *Eco*RI and the fragment containing the *drrB* gene with the upstream Shine–Dalgarno sequence was cloned between the *Kpn*I (shown above in bold) and *Eco*RI 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 to introduce the Myc epitope at the C-terminal end of DrrB. Using pCKB112 as a template, the *drrA* and *drrB* genes were amplified using the primer pair 5′-GAAGATCTCATATGCGCAACGACGACATGGC-3′ (sense; primer DrrA-His) and 5′-AAAAAGCTTTGGCCGCCTAGCCAAAACGTTTTGGCTAGGCGGCCA-3′ (antisense; *Bgl*II and *Hin*dIII sites in the sense and antisense primers are underlined) and cloned between the *Bgl*II and *Hin*dIII 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′-ATGGAATTCTCAGTCGACGGCGCTATTCAGATC-3′ (*Eco*RI site underlined) for amplification using pCKB113 as the template, and the product was cloned between the *Bam*HI and *Eco*RI 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 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 to mid-logarithmic phase ($D_{600} = 0.6$) in Luria broth and induction was carried out at different temperatures with different concentrations of isopropyl β-D-thiogalactoside (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 *M. 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 a D_{600} value of 0.8, followed by heat shock.

Fractionation of cells and localization of proteins

After induction *E. coli* cells were suspended in 10 mM Tris/HCl (pH 7.4), 1 mM $MgCl_2$, 1 mM PMSF, 20 μg/ml leupeptin, 10 μg/ml pepstatin and 10 μg/ml aprotinin, and sonicated thrice at 200 W for 15 s. The unbroken cells were removed by centrifugation at 500 ***g*** for 5 min. Inclusion bodies were obtained by centrifugation at 5000 ***g*** for 5 min. Membranes were obtained by centrifugation at 100000 ***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-^{32}P]ATP (Easytides; NEN) at varying concentrations and a specific activity of 0.25 mCi/mmol in 10 mM Tris/HCl, pH 7.4, containing 10 mM dithiothreitol. Where indicated, doxorubicin was added to the reaction mixture to a final concentration of 50 or 100 μM and $MgCl_2$ was added to a final concentration of 5 mM. The reaction was carried out in a volume of 25 μl in a 96-well microtitre plate kept on a block of ice directly under UV illumination (254 nm) for 30 min. Samples were analysed by SDS/PAGE and autoradiography.

Minimum inhibitory concentrations (MICs)

E. coli BL21(DE3)/pCKB114 or *M. smegmatis*/pCKB115 were induced as described above 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 10^6 colony-forming units/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 ($A_{600} > 0.05$, 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-logarithmic phase, induced, harvested and suspended to a D_{600} value of 20 in 50 mM potassium phosphate, pH 7.4, containing 5 mM $MgSO_4$. Uptake was initiated (in the absence or presence of inhibitors) by the addition of doxorubicin (5 μM; 10–50 μCi/mmol). Cells were diluted into ice-cold 10 mM potassium 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 BCECF-AM

This was done as described by Bolhuis et al. [22]. The cells were harvested and washed thrice with 50 mM Hepes buffer, pH 7.3, containing 25 mM K_2SO_4 and 5 mM $MgSO_4$. Subsequently, the cells were resuspended in the buffer to a final D_{600} value of 20. BCECF-AM (2 mM stock solution in DMSO) 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 4 h (Figure 1A). The protein localized primarily in the inclusion bodies (results 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 *ara*BAD promoter and expressed in *E. coli* LMG-194. This was done in view of the fact that the *ara*BAD 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 a D_{600} of 0.6 in RM-glucose medium with 50 μg/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 (Figure 1B). 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 50 μM 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 (Figure 1D), and the *drr A* 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 50 μM 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 (Figure 1C, lanes 1 and 2), as confirmed by Western blotting with anti-His and anti-Myc antibodies (Figure 1C, 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-

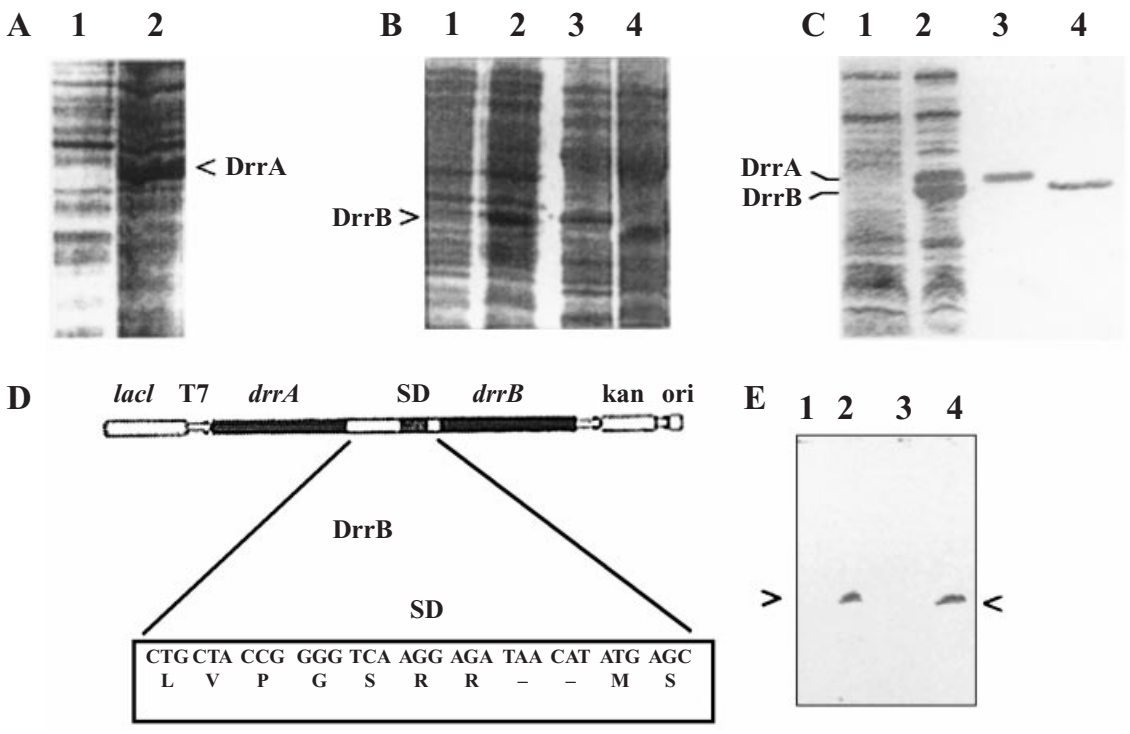

Figure 1 Expression of the DrrA and DrrB proteins

Cells were grown and induced as described in the Experimental section. 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 1–4 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. SD, Shine–Dalgarno sequence. (**E**) Western blots of *M. smegmatis*/pCKB115 membranes from uninduced (lanes 1 and 3) and induced (lanes 2 and 4) cells, probed with anti-His (lanes 1 and 2) or anti-Myc (lanes 3 and 4) antibodies.

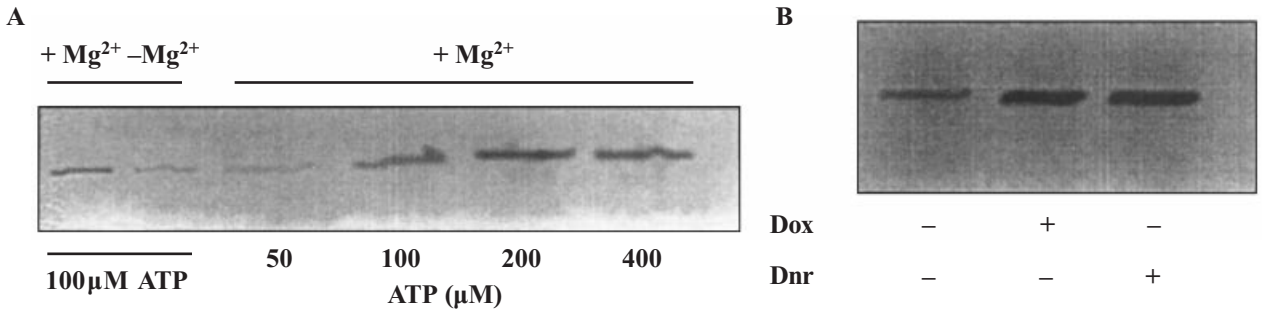

Figure 2 [α-^{32}P]ATP binding to DrrA

UV-catalysed adduct formation between DrrA and [α-^{32}P]ATP was performed in membrane fractions of cells expressing DrrA and DrrB. Adduct formation was catalysed as described in the Experimental section. 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 (Dox; 40 μM) and daunorubicin (Dnr; 40 μM) on adduct formation carried out in the presence of 5 mM Mg^{2+} and 200 μM ATP.

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 (Figure 1E). *M. smegmatis* transformants could be stored as glycerol stocks at −70 °C for 2 weeks, after which transformants showed very slow growth when cultured in liquid medium.

[α-^{32}P]ATP binding

UV-catalysed binding of [α-^{32}P]ATP to DrrA was observed when *E. coli* membranes expressing DrrA and DrrB were used sim-

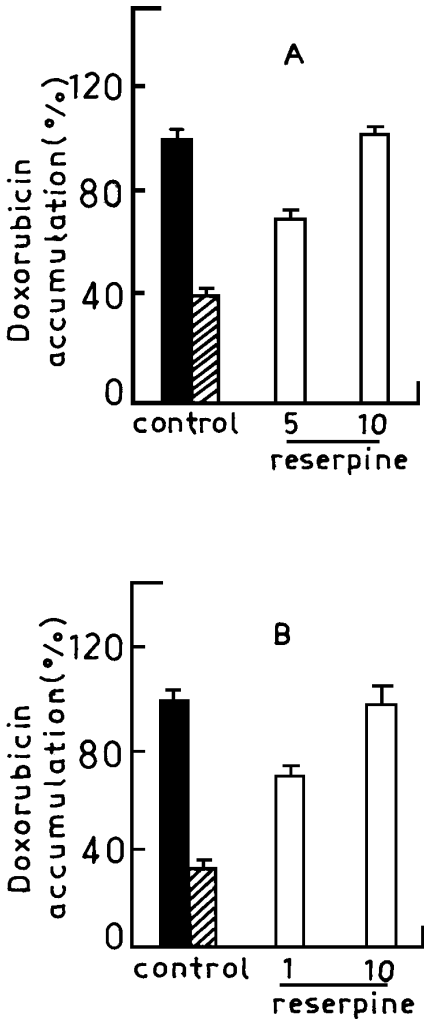

Figure 3 [^{14}C]Doxorubicin accumulation in *E. coli* (A) and *M. smegmatis* (B) expressing DrrA and DrrB

Steady-state accumulation levels of doxorubicin in uninduced (solid bars) cells were taken to be 100%. Accumulation in uninduced and induced (hatched bars) cells in the absence of inhibitors was termed controls. Where indicated, induced cells were incubated with 5 μM doxorubicin and increasing concentrations (μg/ml) of reserpine. Data expressed represent the means $\pm$ S.D. of three separate determinations using three different batches of transformants.

ultaneously for binding studies (Figure 2). The binding was enhanced in the presence of both doxorubicin and daunorubicin (Figure 2). It is possible that both these drugs induce a conformational change in DrrA, favouring interaction with ATP.

Uptake of [^{14}C]doxorubicin

[^{14}C]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 5 μM (which did not affect cell viability). Doxorubicin uptake was significantly lower in cells induced to express DrrAB compared with uninduced cells, suggesting that the tandemly expressed DrrA and DrrB proteins were associating to form a functional doxorubicin efflux pump (Figure 3A). 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

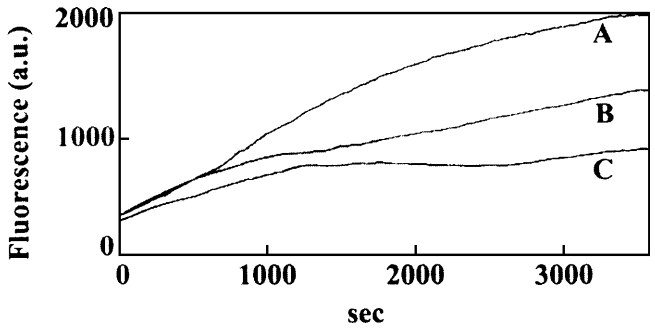

Figure 4 Uptake of BCECF by cells expressing DrrA and DrrB

BCECF-AM (1 μM) was added to: *E. coli* BL21(DE3)/pET28a$^+$ energized with glucose (**A**), *E. coli* BL21(DE3)/pCKB114 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**).

cells expressing both DrrA and DrrB 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 (results not shown). The uptake of doxorubicin in *M. smegmatis* was again found to be inhibitable by reserpine (Figure 3B), 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 hydrolysed 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 with 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 (Figure 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 (results not shown). The presence of constitutive extracellular mycobacterial esterases made it impossible to study BCECF accumulation in *M. smegmatis*.

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 (results not shown).

The DrrAB pump is likely involved in the transport of DIM to the cell surface in *M. tuberculosis*. In addition, another physio-

Table 1 Drug susceptibility of cells expressing DrrAB

Both reserpine and verapamil were administered at 10 μg/ml. N.D., not determined.

	MIC of transformants (μg/ml)					
	E. coli		*M. smegmatis*			
	Uninduced	Induced	Uninduced	Induced		
Drug				No inhibitor	+ Reserpine	+ Verapamil
Ethidium bromide	20	160	3	12	6	6
Daunorubicin	20	40	5	20	5	5
Ethambutol	N.D.	N.D.	0.5	4	1	0.5
Doxorubicin	20	60	20	60	25	20
Puromycin	20	40	N.D.	N.D.	N.D.	N.D.
Chloramphenicol	1	2	6	36	12	6
Erythromycin	N.D.	N.D.	10	40	20	10
Norfloxacin	N.D.	N.D.	2.5	10	5	2.5
Streptomycin	N.D.	N.D.	1	8	4	4
Tetracycline	N.D.	N.D.	0.25	4	1	0.25

logical 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 co-expression 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 [α-^{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 doxorubicin stimulates ATP binding to DrrA of *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 *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 ranges between 2.5- and 3-fold in *E. coli* and *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 *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 *Lactococcus lactis* counterpart of the human P-glycoprotein, shows broad substrate specificity [26]. The *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 *E. coli* expression system is more robust; with *E. coli* transformants being more stable than *M. smegmatis* transformants when stored as glycerol stocks at -70 °C. *E. coli* also has the advantage of a faster generation time than *M. smegmatis*.

This work was supported in part by grants from the Department of Biotechnology and the Department of Science and Technology, Government of India to P.C.

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Received 18 April 2002/21 May 2002; accepted 11 June 2002
Published as BJ Immediate Publication 11 June 2002, DOI 10.1042/BJ20020615