JOURNAL OF BACTERIOLOGY, Feb. 1997, p. 569–575 Vol. 179, No. 3
0021-9193/97/$04.00+0

Expression and Characterization of DrrA and DrrB Proteins of *Streptomyces peucetius* in *Escherichia coli*: DrrA Is an ATP Binding Protein

PARJIT KAUR*

Department of Biology, Georgia State University, Atlanta, Georgia 30303

Received 10 June 1996/Accepted 13 November 1996

***Streptomyces peucetius*, a microorganism that produces the anticancer drugs doxorubicin and daunorubicin, is itself resistant to the action of these drugs. The genes conferring resistance to doxorubicin and daunorubicin in *S. peucetius* have been sequenced (P. G. Guilfoile and R. Hutchinson, Proc. Natl. Acad. Sci. USA 88:8553–8557, 1991). Two open reading frames, *drrA* and *drrB*, were proposed to encode for an ABC (ATP-binding cassette) type of permease that carries out export of the antibiotics in an ATP-dependent manner. This article reports subcloning of the *drrA* and *drrB* genes into *Escherichia coli* expression vectors and characterization of their gene products. Upon induction from the *lac* promoter, a 36-kDa DrrA protein could be identified on Coomassie blue-stained gels. The DrrB protein was identified by use of a polyclonal antiserum generated against a synthetic peptide corresponding to a portion of the DrrB protein. Together, the DrrA and DrrB proteins conferred resistance to doxorubicin in *E. coli*. The DrrB protein was localized to the cell membrane. The DrrA protein bound ATP or GTP in a Mg^{2+}-dependent fashion. ATP binding was enhanced on addition of doxorubicin or daunorubicin.**

Doxorubicin (Dox) and daunorubicin (Dnr), two structurally related antibiotics of the anthracycline family, are produced by *Streptomyces peucetius*. Due to their potent cytotoxic effects, these antibiotics are widely used for the treatment of human cancers. However, human cell lines become resistant to a variety of chemotherapeutic drugs, including Dox and Dnr, limiting the effectiveness of the drugs. This phenomenon of multidrug resistance (MDR) is conferred by the action of P-glycoprotein in the cell membrane (4). P-glycoprotein is a large (170-kDa) multidomain protein. It consists of two similar halves, each containing an ABC (ATP-binding cassette [3])-type nucleotide binding domain followed by a transmembrane domain of six α helices (4). P-glycoprotein has been shown to contain a drug-stimulated ATPase activity (18). Upon reconstitution of purified protein into liposomes, it demonstrates the ability to carry out transport of drugs in an ATP-dependent manner (19). However, its mode of action and the mechanism by which it can result in decreased accumulation of a variety of structurally unrelated substrates are not understood. Since *S. peucetius*, which produces Dox, is itself resistant to the inhibitory action of the drug, an understanding of its mechanism of resistance may shed light on the mechanism of resistance in human cancer cells, which is important for effective drug design.

The Dox resistance operon (*drr*) of *S. peucetius* has been sequenced (5). The operon consists of two open reading frames, *drrA* and *drrB*. Both genes were shown to be required for conferring Dox resistance in a sensitive host, *Streptomyces lividans* (5). From the deduced amino acid sequence, DrrA was found to consist of one nucleotide binding domain with homology to the ABC family of permeases, to which P-glycoprotein (4) belongs. The product of *drrB* gene is predicted to be a membrane protein consisting of six transmembrane α helices. It also shows homology to membrane components of the bacterial ABC transporters, such as NodJ, involved in oligosaccharide export in *Rhizobium leguminosarum*, and KpsM, involved in capsular polysaccharide export in *Escherichia coli* (16). It has been proposed that the DrrA and DrrB proteins might function together to carry out export of the drugs by a mechanism analogous to that employed by the mammalian MDR protein (5). However, gene products of the *drr* operon have not been identified, nor have their functions been characterized at the biochemical level. In this study, the DrrA and DrrB proteins were identified. The DrrA protein binds ATP or GTP in a Mg^{2+}-dependent fashion. ATP/GTP binding to the DrrA protein is enhanced on addition of Dox. The DrrB protein is localized to the cell membrane. Taken together, these data provide a strong indication that the DrrA and DrrB proteins form an ATP-driven transport system with DrrB the membrane carrier and DrrA the peripheral membrane protein that acts as the energy-transducing subunit.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The *E. coli* strains, phages, and plasmids used in this study are described in Table 1.

Media and growth conditions. The cells were grown in LB medium (14) at 37°C. M9 glucose medium (14) was used where indicated. Chloramphenicol was added to 20 μg/ml. Dox or Dnr was added at the indicated concentrations to test for resistance or nucleotide binding.

DNA manipulations. The conditions for plasmid isolation, DNA endonuclease restriction analysis, ligation, and sequencing have been described elsewhere (17).

Mutagenesis of the 5′ end of the *drrA* gene. The *drrA* and *drrB* genes were subcloned into M13mp18 vector to facilitate mutagenesis. The cosmid pWHM612 (5), carrying the *drrA* and *drrB* genes, was digested with *Kpn*I. The 2.3-kb fragment containing the genes of interest was purified and ligated to the *Kpn*I-digested replicative form of M13mp18 vector DNA, resulting in clone Dx106. The 5′ end of the *drrA* gene was mutagenized by using an Amersham Life Sciences Corp. in vitro mutagenesis kit to change the start codon from GTG to ATG and to introduce an *Nde*I site at the start codon. The sequence of the oligonucleotide used for mutagenesis was 5′TCGGCTGCGTGTTCATATGAC GCCCCCAGTAGTC3′. The mutagenized clone was designated Dx107.

Subcloning of the *drrA* and *drrB* genes into *E. coli* expression vectors. The 1.8-kb *Nde*I-*Hin*dIII fragment from the double-stranded replicative form of Dx107 was subcloned behind the *lac* promoter in pSU2718 vector by substitution of the *arsA* gene in pSU2718*arsA* (7). The *drrA* and *drrB* genes were also subcloned separately into pSU2718 at the *Nde*I-*Hin*dIII sites after PCR amplification of the genes. The primers used for amplification contained the sequences

* Mailing address: Department of Biology, 24 Peachtree Center Ave., Georgia State University, Atlanta, GA 30303. Phone: (404) 651-3864. Fax: (404) 651-2509. E-mail: bioppk@gsusgi2.gsu.edu.

TABLE 1. Strains, plasmids, and phages used

Strain, plasmids, or phage	Genotype or description	Reference or source
Strains		
TG1	K-12 Δ(*lac-pro*) *supE thi hsdD5*/F′ *tra36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*	17
N43	*ara-14* Δ*lac-85 acrA1 supE44*? *galK2 rpsL197 malA1* (λ^r) *xyl-5 mtl-1*	15
CC118	F$^-$ *araD139* Δ(*ara-leu*)*7697* Δ*lacX74 phoA*Δ*20 galE15 galK16 thi rpsE rpoB argE*(Am) *recA1*	13
Plasmids		
pSU2718*arsA*	*arsA*	7
pWHM612	*drrAB*	5
pJHW101	*arsDRABC*	21
pDx101	*drrAB*	This study
pDx102	*drrA*	This study
pDx103	*drrB*	This study
pDx104	Fusion of *arsD* gene with *drrA*	This study
pDx105	Fusion of *arsD* gene with *drrB*	This study
Phages		
Dx106	M13mp18 derivative containing *drrA* and *drrB* genes	This study
Dx107	Dx106 with start codon for DrrA changed to ATG and an *Nde*I site introduced at the ATG	This study
λTn*phoA*	Tn*5* IS*50*$_L$::*phoA* (Km^r)	13

for appropriate restriction sites. The primers for the amplification of *drrA* and *drrB* were as follows: for *drrA*, I (5′CCGCCGCCGCATATGAACACGCAGC CGACA3′) and II (5′CCCCCCAAGCTTTCGTCATGCCACCTTCTC3′); for *drrB*, I (5′CCGCCGCCGCATATGACGACGTCCCCCGGC3′) and II (5′GCC AGTGCCAAGCTTGCATGCCTGCAG3′).

Gene fusions of *drrA* and *drrB* with *arsD*. Fusion of the *drrA* or *drrB* gene to the first 24 codons of the *arsD* gene was created. The replicative form of Dx107 was digested with *Nde*I. The *Nde*I end was filled in with Klenow enzyme and ligated to a phosphorylated *Bgl*II linker. After digestion with *Kpn*I and *Bgl*II, a 1.8-kb fragment was purified and ligated to plasmid pJHW101 digested with *Bcl*I and *Kpn*I. This construct, designated pDx104, resulted in an in-frame fusion of the *drrA* gene to the first 24 codons of the *arsD* gene under the control of the *ars* promoter. An *arsD-drrB* fusion was created by digesting the replicative form of Dx107 with *Pvu*II and ligating it to a *Bgl*II linker. After digestion with *Bgl*II and *Kpn*I, a 0.7-kb fragment was purified and ligated to pJHW101 digested with *Bcl*I and *Kpn*I. This construct, designated pDx105, resulted in fusion of the first 24 codons of the *arsD* gene to codon 56 in *drrB*.

Expression of the *drrA* and *drrB* gene products and fusion proteins. The expression of proteins was analyzed by induction of mid-log-phase *E. coli* cells containing the appropriate plasmid with 0.25 mM isopropylthiogalactopyranoside (IPTG) or 10 μM sodium arsenite (8). Samples were withdrawn at the indicated times after induction. Where indicated, the growth of cultures was monitored by recording the optical density at 600 nm (OD_{600}). One-milliliter cell suspensions were centrifuged, and the cell pellets were resuspended in 100 μl of 4× Laemmli sample buffer (9). The cells were solubilized by heating at 55°C for 10 min unless otherwise indicated. The proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 12% gels as described elsewhere (9).

Generation of antisera to the DrrA and DrrB proteins. Slices containing the ArsD-DrrA fusion protein were excised from a 12% polyacrylamide gel. Antiserum was raised at a commercial facility (Great Lakes Biomedical and Environmental Research, Romeo, Mich.) by injecting homogenized gel slices into New Zealand White rabbits. Anti-DrrB serum was generated against a 24-residue peptide (amino acid residues 260 to 283 in DrrB) synthesized in the Core Facility of the Department of Biology at Georgia State University. The 24-residue peptide was conjugated to bovine serum albumin (6) before being injected into the rabbits. Specificity of the antiserum against the DrrA or DrrB protein was tested by probing Western blots prepared from induced *E. coli* cells containing plasmid pSU2718 with or without the *drrA* and *drrB* genes.

Expression of Dox resistance in *E. coli*. Sensitivity to Dox was tested in liquid medium by making serial dilutions of the drug or by the disk diffusion method on agar plates. The strain to be tested was grown to late log phase in liquid medium from a fresh plate. A lawn of the cells was prepared on nutrient agar plates. Sterile Whatman paper disks (6-mm diameter) containing various concentrations of the drug (0 to 10 μg/disk) were placed on the plates. The plates were incubated at 37°C for 16 h, and the sizes of the zones of inhibition were recorded. When liquid medium was used for determination of the MIC, serial dilutions ranging from 0 to 10 μg of Dox per ml were prepared in M9 medium containing 0.25% Casamino Acids. The medium was inoculated with 1:100 dilution of a late-log-phase culture, and the tubes were incubated with aeration in the dark at 37°C for 16 h. Growth was measured by recording the OD_{600} of the cultures.

Nucleotide binding to DrrA. Photolabeling of the DrrA protein with [α-^{32}P]ATP was studied by a modification (7) of a procedure described earlier (22). Photolabeling was performed in cell extracts prepared from cells expressing DrrA. *E. coli* cells bearing plasmid pSU2718 or pDx101 were grown in LB medium and induced with 0.25 mM IPTG. Growth was continued for an additional 4 h at 37°C. The cells were spun down and lysed by a single passage through a French pressure cell at 20,000 lb/in^2. The cell lysates were centrifuged at 10,000 × *g* for 10 min to remove the unbroken cells. The supernatant fluids were used directly in photolabeling experiments. To determine the specificity of nucleotide binding of DrrA, an excess of unlabeled ATP, ADP, AMP, or GTP was added to the reaction mixture as indicated. Dox or Dnr was added to the reactions at various concentrations. The samples were analyzed by SDS-PAGE and analyzed by autoradiography.

Localization of DrrA and DrrB. (i) Fractionation of cells into cytosolic and total membrane fractions. Induced *E. coli* cells containing pDx101 were lysed by passage through a French pressure cell at 20,000 lb/in^2. After centrifugation at 10,000 × *g* for 10 min to remove unbroken cells, the cell lysates were fractionated into cytosolic and total membrane fractions by ultracentrifugation at 100,000 × *g* for 1 h. The fractions were analyzed for the presence of the DrrA or DrrB protein. Cross-contamination of the fractions was checked by testing for the presence of SecY, chloramphenicol acetyltransferase (CAT), and elongation factor G (EFG). Quantitation of protein bands on the Western blots was carried out by using the PDI densitometric package.

(ii) AP fusions. Random fusions of the promotorless *phoA* gene that lacked the leader sequence into the *drrA* and *drrB* genes were isolated. CC118 cells containing pDx101 were infected with λTn*phoA* phage as described previously (13). The infected cells were screened for blue color on medium containing 5-bromo-4-chloro-3-indolylphosphate (XP; 30 μg/ml) and kanamycin (75 μg/ml). Two blue and two white colonies were used for determination of the fusion junction by nucleotide sequence analysis. Fusion proteins were identified by probing Western blots with antibodies to alkaline phosphatase (AP) (5′-3′ Inc., Boulder, Colo.). Relative amounts of the fusion proteins were determined by densitometric analysis using the PDI densitometric scanning package. AP activity was determined in French pressure cell lysates by a published procedure (2).

RESULTS

Cloning of the *drrA* and *drrB* genes into *E. coli* expression vectors. To clone the *drrA* and *drrB* genes into an *E. coli* expression vector, an *Nde*I site was introduced into the 5′ end of the *drrA* gene. At the same time, the start codon of the *drrA* gene was changed from GTG to ATG. After mutagenesis, the 1.8-kb *Nde*I-*Hin*dIII fragment was subcloned into pSU2718*arsA* by substitution of the *arsA* gene.

Expression of the DrrA and DrrB proteins was examined by induction of the *lac* promoter in the pDx101 construct. In this construct, the *drrA* gene utilizes the *lac* promoter from the vector and the ribosomal binding site of the *arsA* gene. The

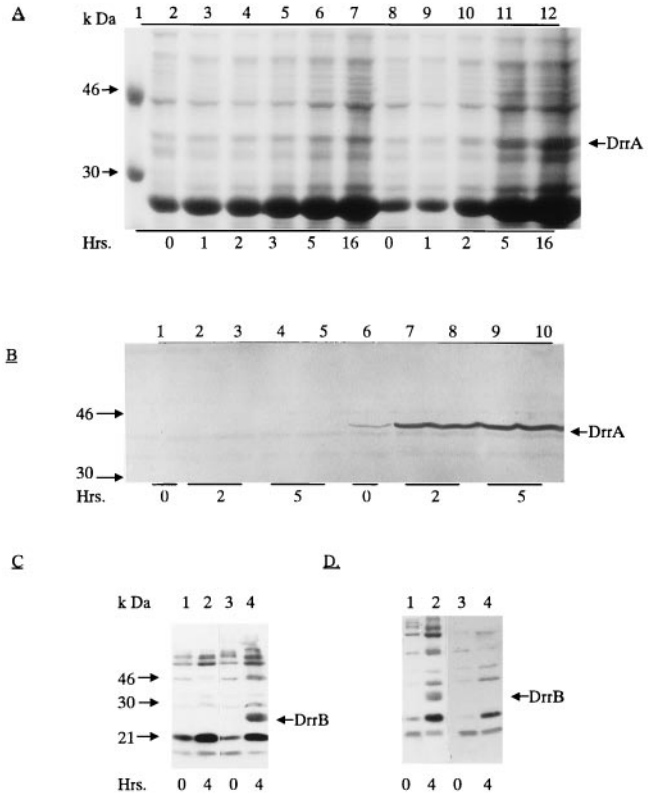

FIG. 1. Expression of the DrrA and DrrB proteins. Cultures of cells were grown and induced with 0.25 mM IPTG as described under Materials and Methods. Cells were suspended in Laemmli sample buffer and analyzed by SDS-PAGE on 12% polyacrylamide gels. (A) Coomassie blue-stained gel. Lane 1, molecular weight standards; lanes 2 to 7, with vector; lanes 8 to 12, with plasmid pDx101. At any particular time point, amounts of total cell protein loaded in the control and test samples were the same. (B) Immunoblot analysis using anti-DrrA serum. Lanes 1 to 5, with vector; lanes 6 to 10, with plasmid pDx101. Horseradish peroxidase-conjugated secondary antibody was used to give a color reaction on the blot. (C) Immunoblot analysis using anti-DrrB serum. Lanes 1 and 2, with vector; lanes 3 and 4, with plasmid pDx101. (D) Quenching of anti-DrrB serum with the DrrB peptide. Lanes 1 and 2, with plasmid pDx101, unquenched serum; lanes 3 and 4, with plasmid pDX101, quenched serum. A Bio-Rad chemiluminescence detection kit was used for detection of bands reactive to the anti-DrrB antibody in panels C and D. The time of induction is indicated below each panel. Migration of the standard proteins is shown on the left in panels B and C.

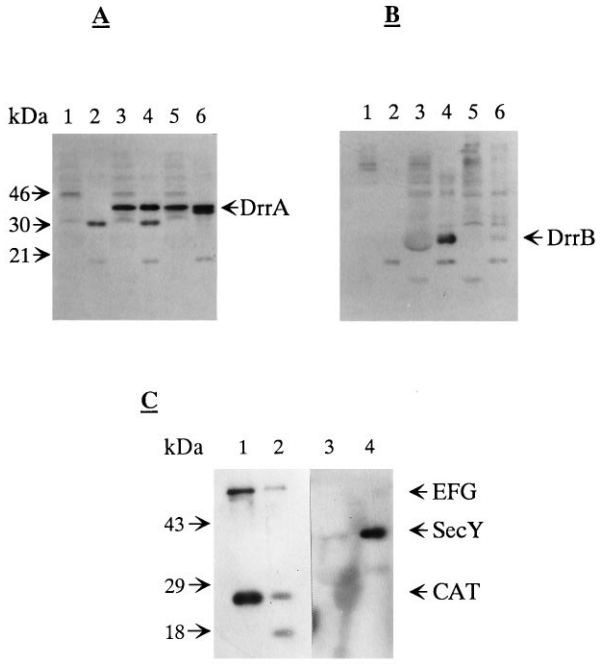

FIG. 2. Localization of DrrA and DrrB in cell fractions. *E. coli* cells were induced with IPTG, French pressed, and fractionated into the cytosolic and total membrane fractions by centrifugation at 100,000 × *g* for 1 h. The fractions were analyzed by SDS-PAGE followed by Western blot analysis using anti-DrrA or anti-DrrB serum. Equal volumes of the cytosolic and membrane fractions were loaded on the gel. A Bio-Rad chemiluminescence detection kit was used for detection of bands. (A) Immunoblotting using anti-DrrA; (B) immunoblotting using anti-DrrB. Lanes 1 and 2, with vector; lanes 3 and 4, with plasmid pDX101; lanes 5 and 6, pDX101 heated at 90°C for 10 min; lanes 1, 3, and 5, cytosol; lanes 2, 4, and 6, total membrane. (C) Analysis of fractions from cells containing pDX101 for cross-contamination by use of anti-SecY, anti-CAT, or anti-EFG serum. Lanes 1 and 2, probed with anti-CAT and anti-EFG sequentially; lanes 3 and 4, probed with anti-SecY; lanes 1 and 3, cytosol; lanes 2 and 4, membrane.

nucleotide sequences of the *drrA* and *drrB* genes indicated that the molecular masses of the DrrA and DrrB proteins are 35.5 and 30.6 kDa, respectively. Samples were collected after induction of *E. coli* cells with IPTG. Cell lysates prepared from *E. coli* cells were analyzed by Coomassie blue staining of the SDS-polyacrylamide gels (Fig. 1A). A protein band corresponding to 36 kDa could be visualized upon induction of cells containing the pDx101 construct (lanes 8 to 12). Control and test samples contained roughly equal amounts of total cell protein at any particular time point. A band corresponding to the 30-kDa DrrB protein was not detected for reasons discussed below.

Anti-ArsD-DrrA serum was found to be specific for the DrrA protein, as seen from Western blots (Fig. 1B) probed with the serum. A 36-kDa band corresponding to DrrA was seen in lysates from *E. coli* cells containing pDx101 upon induction (lanes 6 to 10) and not in cells containing vector alone (lanes 1 to 5) when equal amounts of total cell protein were compared. Anti-DrrB serum also showed a specific band corresponding to the DrrB protein (Fig. 1C) in lysates prepared from cells carrying pDx101 (lane 4). This band was absent from pSU2718-containing cells (lanes 1 and 2). The band corresponding to DrrB on the Western blot was seen to migrate faster than the predicted molecular mass of 30.6 kDa (Fig. 1C). Since DrrB protein is predicted to be a hydrophobic protein, faster migration on SDS-gels is expected. The nonspecific bands seen with anti-DrrB antiserum in both kinds of cells (with or without the *drrA* and *drrB* genes) are likely due to cross-reactivity of the antipeptide antibody with epitopes in different proteins. The band corresponding to the DrrB protein could be quenched by preincubating anti-DrrB serum with the 24-residue peptide (Fig. 1D, lane 4), indicating that it is specific for the DrrB protein.

E. coli TG1 cells expressing the DrrA and DrrB proteins were fractionated into cytosolic and total membrane fractions. The fractions were analyzed by Western blotting using anti-DrrA or anti-DrrB serum, and the intensities of bands were determined by densitometric analysis. Western blot analysis using anti-DrrA antiserum (Fig. 2A) suggests that a significant proportion of the DrrA protein is associated with the membrane fraction (Fig. 2A, lane 4). Results averaged from two experiments suggest that roughly 60% DrrA is membrane associated. The band corresponding to DrrA is not present in the membrane or cytosolic fraction of the control cells containing the vector alone (lanes 1 and 2). Analysis of a similar gel with anti-DrrB serum shows that DrrB migrates with the same mobility as the *cat* gene product (Fig. 2B). CAT is very highly

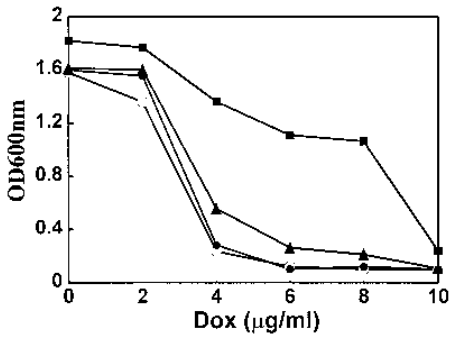

FIG. 3. Dox resistance in *E. coli. E. coli* N43 bearing the indicated plasmids was tested for resistance in M9 medium containing various concentrations of Dox. ○, vector; ■, pDx101; ●, pDx102; ▲, pDx103.

expressed in these cells, and it masks DrrB on the Coomassie blue-stained gel (Fig. 1A). Furthermore, DrrB is localized primarily in the membrane fraction of pDx101-containing cells (Fig. 2B, lane 4). Densitometric analysis suggests that about 92% of DrrB is membrane bound. The cytosol and cell membrane fractions were analyzed for cross-contamination by using antibodies to SecY, an integral membrane protein, and to CAT and EFG, two cytosolic proteins. Figure 2C shows that only trace amounts of SecY are present in the cytosolic fraction, indicating that the cross-contamination is minimal. Densitometric analysis of the bands in Fig. 2C showed around 95% SecY, 9% EFG, and 11% CAT in the membrane fraction. Taking the cross-contamination into account, distribution of DrrA in the cytosolic and membrane fractions is about 1:1. Interestingly, heating of the DrrB-containing samples to 90°C for 10 min results in a significant reduction in signal due to DrrB (Fig. 2B, lanes 5 and 6), indicating a very hydrophobic nature and a membrane location for the DrrB protein. This signal is completely absent in the heated cytosolic fraction (lane 5) and is reduced to less than 5% of the original level in the membrane fraction (lane 6).

Resistance to Dox in *E. coli*. To determine if the *drrA* and *drrB* genes conferred resistance to Dox in *E. coli* cells carrying pDx101, a host sensitive to the drug was needed. Wild-type *E. coli* K-12 was found to be intrinsically resistant to Dox. Mutants of *E. coli* defective in the barrier function were tested for Dox sensitivity. *E. coli* strains bearing mutations in (strain N43 [15]) or deletions of (strain HN818 [12]) the *acr* locus were found to be sensitive to Dox, though to different levels. Plasmid pDx101 was transformed into strain N43 to determine if expression of the *drrA* and *drrB* genes would now make this strain resistant to Dox. The data in Fig. 3 suggest that indeed the expression of the *drrA* and *drrB* genes confers resistance to Dox. pDx102 or pDx103 bearing the *drrA* or *drrB* gene alone did not confer significant Dox resistance in *E. coli*. A difference in Dox sensitivity of strains carrying a plasmid with or without the *drrA* and *drrB* genes could also be observed in strain HN818 by disk diffusion assay (data not shown). Due to the susceptibility of Dox to self-polymerization, variability in results of the Dox resistance assay was sometimes observed. It was crucial to work with freshly transformed cells and to protect Dox from light.

ATP or GTP binding to the DrrA protein. Nucleotide binding properties of the DrrA protein were analyzed in cell lysates prepared from *E. coli* cells carrying either the vector or pDx101. [α-^{32}P]ATP binding in a UV-catalyzed cross-linking experiment shows (Fig. 4) that the 36-kDa DrrA protein can bind ATP. Photoadduct formation with ATP was greatly enhanced upon addition of Mg^{2+} (lanes 1, 3, 5, and 7). Interestingly, the photoadduct formation with Mg^{2+}-ATP was enhanced when Dox was added to the reaction mixture before exposure to UV light (lanes 3, 4, 7, and 8). The ATP cross-linked 36-kDa band was not seen in control cell lysates (lanes 9 to 12) prepared from pSU2718-containing cells in the presence or absence of Mg^{2+} or Dox. The identity of the 25-kDa protein labeled in the absence of Mg^{2+} (lanes 2, 4, 6, 8, 10, and 12) is unknown. However, it is unrelated to DrrA, as this band was seen in cells with or without the *drrA* and *drrB* genes. To determine if ATP binding was specific for the nucleotide binding site of DrrA, the UV cross-linking experiment was carried out in the presence of excess nonradioactive ATP, ADP, or AMP (Fig. 5). Tenfold excess nonradioactive ATP or ADP prevented labeling of DrrA with [α-^{32}P]ATP almost completely (lanes 2 and 4), whereas AMP at 10-fold excess had less effect (lane 6). Interestingly, GTP at a concentration equal to that of ATP (10 μM) significantly affected ATP binding (lane 7). At a 10-fold-higher concentration, it was found to completely abolish photoadduct formation with ATP (lane 8).

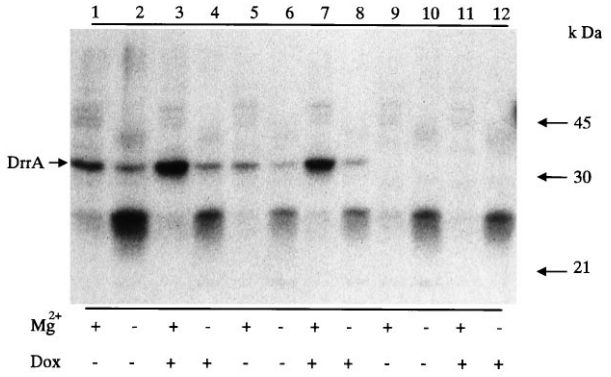

FIG. 4. [α-^{32}P]ATP binding to DrrA protein. UV-catalyzed adduct formation between DrrA protein (50 or 100 μg of cytosolic protein) and [α-^{32}P]ATP (10 μM) was performed in French-pressed cell lysates as described in Materials and Methods. The proteins were resolved by SDS-PAGE on 12% gels and electrophoretically transferred to nitrocellulose membrane. The membrane was autoradiographed. Lanes 1 to 4, 50 μg of cytosolic protein containing the DrrA protein; lanes 5 to 8, 100 μg of cytosolic protein containing the DrrA protein; lanes 9 to 12, 100 μg of cytosol without the DrrA protein. The reactions also contained 35 μM Dox and 250 μM Mg^{2+} where indicated. The migration of standard proteins is shown on the right.

To determine if GTP could substitute for ATP at the nucleotide binding site, [α-^{32}P]GTP was used in nucleotide cross-linking experiments. The data in Fig. 6 show that the DrrA

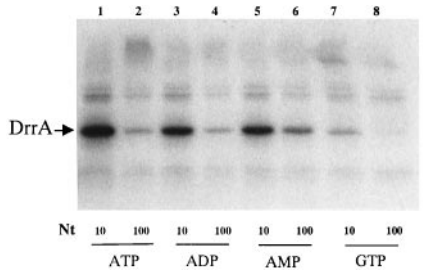

FIG. 5. Competition with excess unlabeled nucleotides. Photoadduct formation between DrrA (50 μg of cytosolic protein) and [α-^{32}P]ATP was carried out as described in the legend to Fig. 4. The reaction mixtures, in addition, contained unlabeled nucleotides (Nt). The numbers next to Nt indicate the concentration (micromolar) of the unlabeled nucleotide in the reaction. The reactions also contained 250 μM Mg^{2+} and 35 μM Dox.

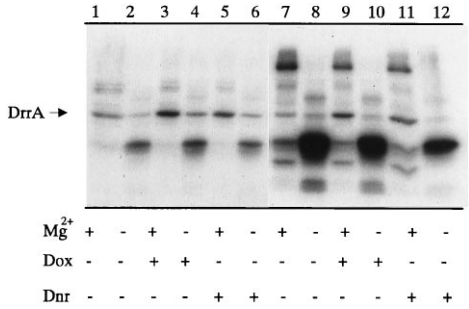

FIG. 6. [α-^{32}P]GTP binding to DrrA protein. UV-catalyzed adduct formation between DrrA protein (50 μg of cytosol protein) and [α-^{32}P]GTP or ATP was performed as described in Materials and Methods. Lanes 1 to 6, [α-^{32}P]ATP; lanes 7 to 12, [α-^{32}P]GTP. Dox (35 μM), Dnr (35 μM), or Mg^{2+} (250 μM) was added to the reactions where indicated.

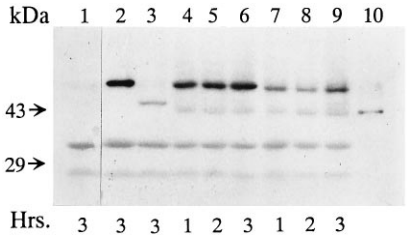

FIG. 7. Identification of the DrrA-AP or DrrB-AP fusion proteins. *E. coli* cells were grown to mid-log phase and induced with 0.25 mM IPTG. At the indicated time points, French-pressed cell lysates were analyzed by SDS-PAGE on 10% gels. Western blot analysis was carried out with anti-AP serum. Bands were detected by the chemiluminescence method. Lane 1, with vector; lane 2, with plasmid expressing A159; lane 3, A28; lanes 4 to 6, B189; lanes 7 to 9, B151; lane 10, standard (AP). Time of induction is indicated at the bottom. The migration of standard proteins is shown on the left. The top bands in lanes 2 to 9 represent the intact fusion protein; the bands below in lanes 4 to 9 represent the degradation product.

protein can bind GTP (lanes 7 to 12) as well as ATP (lanes 1 to 6) in the presence of Mg^{2+}. In addition, these data indicate that GTP binding to DrrA was enhanced by Dox (lanes 9 to 10) and Dnr (lanes 11 to 12) just like ATP binding was (lanes 3 to 6).

Membrane localization of the DrrB protein. To determine if DrrB is an integral membrane protein, gene fusions of a reporter gene (*phoA*) lacking the leader sequence to the *drrA* and *drrB* genes were isolated. Random insertion of λTn*phoA* phage into CC118 cells carrying pDx101 was carried out. Nucleotide sequence analysis of two transfectants that expressed active AP (as determined by visual screening for blue colonies on XP-containing medium) showed that the fusion junction was located in the *drrB* gene at nucleotides 451 and 568, respectively. AP activity in cell extracts containing these fusion proteins after 3 h of induction was found to be roughly 15- to 20-fold higher than in the control cells containing the vector alone (Table 2). Two other transfectants that showed no or very little AP activity had their fusion junctions in *drrA* at nucleotides 85 and 478, respectively (Table 2). Identification and quantitation of the fusion proteins was done by Western blot analysis using anti-AP antiserum, followed by a densitometric analysis of the intensity of the bands. Data in Fig. 7 (lanes 2 to 9) show that all four fusion proteins are synthesized and that their sizes correspond to the molecular mass predicted from the location of the fusion junction. The band corresponding to the fusion protein is absent from the control cells containing just the vector (lane 1). Fusions in DrrA were found to be completely stable during the 3-h induction time used in the experiment (lanes 2 and 3). Despite the use of proteolytic inhibitors, fusions in DrrB were subject to some degree of degradation (lanes 6 and 9). A time course of induction of B151 and B189 showed that the degradation was present even at much earlier time points (lanes 4, 5, 7, and 8). The synthesis units shown in Table 2 represent the sum of the intensities of full-length proteins and their degradation products. The synthesis units were used to calculate the relative activities of the fusion proteins shown in Table 2.

Effects of induction of ArsD-DrrA fusion protein and ArsD-DrrB protein. Fusion of the *drrA* or *drrB* gene to the *arsD* gene was created to facilitate overexpression of the DrrA and DrrB proteins. *E. coli* cells containing plasmid pDx104 or pDx105 were grown to mid-log phase and induced with 10 μM sodium arsenite. Control cells containing pJHW101 (*arsDRABC*) or pDx104 (containing the ArsD-DrrA fusion) continued to grow after induction with arsenite (Fig. 8). However, growth of cells containing pDx105 (containing ArsD-DrrB) was arrested upon induction with arsenite, indicating that expression of DrrB alone is toxic. The same inhibition was observed even if cells were induced with sodium arsenite concentrations as low as 1 μM (data not shown).

DISCUSSION

One of the drugs commonly used in cancer chemotherapy is Dox produced by *S. peucetius*. This antibiotic belongs to the family of anthracycline drugs, which also includes Dnr. *S. peucetius* is itself resistant to the action of the drug. Cloning and sequencing of the *drr* operon conferring resistance to Dox in *S. peucetius* (5) suggested that resistance might be conferred by an MDR-like efflux ATPase. The *drr* operon consists of two open reading frames; one encodes a soluble protein, DrrA, that shows homology to the ATP binding domain of ABC transporters; the other encodes a membrane protein, DrrB, that has six potential α-helical transmembrane domains. To-

TABLE 2. AP activity of CC118 cells containing *phoA* fusions in *drrA* or *drrB*[a]

Fusion protein	Fusion junction	Colony phenotype	Activity (U/mg)	Synthesis units	Relative activity[b]
A28	*drrA* nt 85 GGC CTG GAC CCT GAC	White	13.60	1.79	7.59
A159	*drrA* nt 478 ACC CCC GAC CCT GAC	White	15.30	5.59	2.73
B151	*drrB* nt 451 ACC ACG GTG GCT GAC	Blue	111	1.73	64.16
B189	*drrB* nt 568 GCC CTG GGC GCT GAC	Blue	106	2.97	35.69
Control		White	7.60		NA

[a] CC118 cells containing control plasmid pSU2718 or pDx101 containing fusions with *phoA* at different locations in the *drrA* or *drrB* gene were grown to mid-log phase and induced with 0.25 mM IPTG for 3 h. The AP activity was determined in French-pressed cell lysates as described in Materials and Methods. The number next to the fusion protein represents the amino acid residue N terminal to the site of fusion. The sequence of the *drrA* or *drrB* gene at the fusion junction is underlined.

[b] Activity/synthesis units. The synthesis units for the fusion proteins were determined by densitometric scanning of Western blots. NA, not applicable.

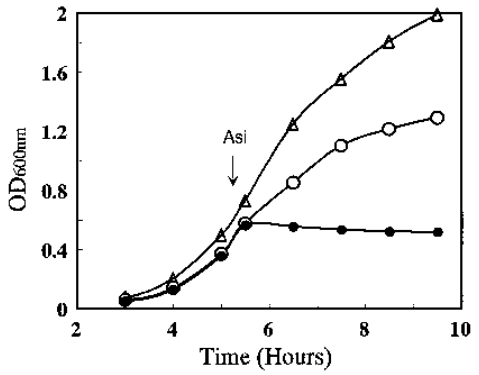

FIG. 8. Effect of induction of ArsD-DrrA or ArsD-DrrB fusion proteins. *E. coli* cells containing the indicated plasmids were grown to exponential phase and induced with 10 μM sodium arsenite. Growth was monitored by recording the OD_{600} of the cultures. △, pJHW101; ○, pDx104; ●, pDx105.

gether, DrrA and DrrB might form an ATP-dependent efflux pump that carries out transport of the drug, resulting in resistance (5).

Subcloning and expression of the *drrAB* operon in *E. coli* demonstrate that *drrA* codes for the DrrA protein. A band of 36 kDa corresponding to DrrA was visualized upon SDS-PAGE in lysates prepared from cells carrying the *drrAB* insert on a plasmid. After fractionation of the cells into cytosolic and membrane fractions, a significant proportion of the DrrA protein was found to be associated with the membrane fraction, indicating that it might be a peripheral membrane protein. Expression of the DrrB protein could not be detected on Coomassie blue-stained gels, as it migrates with the same mobility as another heavily expressed protein. However, it could be detected by probing with an antiserum generated against the 24-residue DrrB peptide and was found to be localized primarily to the membrane.

From the deduced amino acid sequence, DrrB is predicted to be an integral membrane protein. Several lines of evidence presented in this report provide support for this prediction. Cell fractionation experiments suggest that >92% of DrrB is membrane bound. The presence of DrrB in the cytosolic fraction can be attributed to partial contamination of the cytosol with the membrane; about 5% SecY, a marker for the membrane proteins, was detected in the cytosolic fraction. Overexpression of a membrane protein can be toxic to the cell. Induction of ArsD-DrrA fusion with sodium arsenite had no effect on growth of cells, whereas induction of the ArsD-DrrB fusion was growth inhibitory (Fig. 8). These data indirectly suggest that DrrB is a membrane protein, expression of which by itself is toxic. It should be mentioned that overexpression of DrrB in cells containing pDx101 was not growth inhibitory, most likely due to simultaneous expression of DrrA. Experiments carried out by random mutagenesis with λTn*phoA* indicate that at least two *phoA* insertions in the *drrB* gene have high AP activity, suggesting a membrane location of the DrrB protein. Studies to determine the topological map of DrrB in the cell membrane are in progress. Finally, consistent with the hydrophobic nature of DrrB and its membrane location is also the observation that DrrB aggregates upon heating to 90°C. When the DrrB-containing samples were heated to 90°C and then analyzed by SDS-PAGE and Western blotting, the signal due to DrrB was greatly reduced. This could be due to aggregation of DrrB and its inability to migrate through the acrylamide gel as is commonly seen with hydrophobic integral membrane proteins.

Wild-type *E. coli* K-12 could not be used to study the resistance phenotype due to its intrinsic resistance to Dox. *E. coli* N43 (*acrA*) and HN818 (Δ*acrAB*) were found to be Dox sensitive. Transformation of these strains with pDx101 resulted in resistance to Dox. Resistance to Dnr was not observed in *E. coli* transformed with this plasmid (not shown). pDx102 or pDx103 was found to be unable to confer Dox resistance. While expression of DrrA was seen in pDx102-containing cells, DrrB was not produced at levels high enough to be detected in pDx103-containing cells (data not shown). Hence, it cannot be concluded from the data presented here that DrrB by itself is insufficient to confer Dox resistance. It appears that the DrrA and DrrB proteins form a complex, and formation of such a complex is required for expression and/or stable maintenance of DrrB in the cell. It is interesting that efforts to express DrrB alone as a translational fusion to ArsD proved growth inhibitory, emphasizing the need for a tight regulation of expression of these two proteins.

Nucleotide sequence analysis of the *drrA* gene shows the presence of a consensus nucleotide binding domain (20) with homology to the ABC family of transporters (3). Hence, resistance to Dox might be conferred by an ATP-driven efflux mechanism. In this report, DrrA protein has been shown to bind ATP in a UV cross-linking assay (Fig. 4). ATP binding was Mg^{2+} dependent. Moreover, addition of Dox enhanced Mg^{2+}-ATP binding, indicating that the activity of DrrA is regulated by the substrate of the pump. Hence, DrrA appears to be an allosteric protein where ATP binding is influenced by interaction of Dox at a binding site in DrrA. Alternatively, the effect on ATP binding to DrrA could be an indirect effect of Dox binding to DrrB. ATP binding was also enhanced in the presence of Dnr, which is structurally quite similar to Dox and is produced by the same metabolic pathway. The stimulation of ATP binding was greater in the presence of Dox than in the presence of Dnr. Since a 10-fold excess of nonradioactive GTP in competition experiments eliminated ATP binding, UV-activated [α-^{32}P]GTP binding to DrrA was examined. DrrA bound GTP as efficiently as ATP in the presence of Mg^{2+}. In addition, GTP binding was also enhanced upon addition of Dox or Dnr to the reaction mixture. These data indicate that DrrA is a Dox-stimulated Mg^{2+}-dependent ATPase and/or GTPase. The *E. coli* expression system for DrrA and DrrB described in this report provides a functional system for biochemical characterization of the DrrA and DrrB proteins.

Data shown in this report support the hypothesis that the *drrAB* operon of *S. peucetius* confers resistance to Dox by an ATP-dependent efflux mechanism similar to the one used by the MDR protein in human cancer cells. The DrrB protein is expected to form the pore or the channel in the membrane, and the DrrA protein appears to be the energy-transducing subunit that forms a complex with DrrB. Since most ABC transporters consist of at least four functional domains with two nucleotide binding domains and two membrane-bound domains of six transmembrane α helices each (3), the functional form of the Dox transporter likely consists of an oligomeric complex of the two subunits. Hence, a complex of monomers of DrrA and DrrB would be equivalent to a half molecule of MDR consisting of an ABC domain of roughly 200 amino acids and a transmembrane domain of six α helices.

Studies are in progress to determine if the *drrAB* operon codes for an efflux pump coupled to hydrolysis of ATP. Whether this pump has a narrow or broad substrate specificity has special relevance to studies of MDR. The presence of the Drr system in the antibiotic-producing organism also raises a very important question regarding the evolution of MDR. The existence of MDR-like transport proteins that confer resis-

tance to a variety of structurally unrelated substrates has been shown in bacteria (10, 12). However, these transporters do not serve as good model systems for MDR, as they do not show homology to the ABC family of transporters, and they energize transport by coupling to the proton motive force (10). The Drr system is the only ABC transporter described so far that carries out the same function, namely, transport of the anticancer drugs Dox and Dnr. A recent report describes the presence of an ATP-dependent MDR-like protein in *Lactobacillus lactis* (1); however, direct evidence for ATP binding or ATP requirement for transport by this protein is not available.

DrrA shows strong homology to another ABC transporter, ABC1, which was recently cloned and sequenced from human and mouse DNAs (11). ABC1 belongs to a subfamily of MDR proteins and has the same domain arrangement and size as MDR. Interestingly, however, it is evolutionarily closer to the bacterial ABC transporters, DrrA and NodI, than to MDR. The amino acid alignment of DrrA and ABC1 showed that each 200-amino-acid-long ABC domain in ABC1 has roughly 36% identity and 54% similarity to DrrA. The physiological function of ABC1 has not been elucidated. However, its similarity with DrrA makes DrrAB a very attractive model system for the study of ABC transporters of both prokaryotic and eukaryotic origin.

ACKNOWLEDGMENTS

This work was supported in part by an initiation grant from Georgia State University. The core facility of the Department of Biology at Georgia State University was supported by Georgia Research Alliance.

I am grateful to Richard Hutchinson for providing the cosmid clone pWHM612, to Colin Manoil and Jon Beckwith for providing *E. coli* CC118 cells and the λTn*phoA* phage, to Hiroshi Nikaido for providing *E. coli* HN818, and to P. C. Tai for a gift of anti-SecY and anti-EFG antibodies. Thanks are due to Barry Rosen for his suggestion on the use of *acr* strains of *E. coli* for studying Dox resistance and to Barry Rosen and P. C. Tai for critical reading of the manuscript.

REFERENCES

1. **Bolhuis, H., H. W. V. Veen, D. Molenaar, B. Poolman, A. J. M. Driessen, and W. N. Konings.** 1996. Multidrug resistance in *Lactococcus lactis*: evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane. EMBO J. **15:**4239–4245.
2. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96:**307–316.
3. **Fath, T. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
4. **Gottesman, M. M., and I. Pastan.** 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. **62:**385–427.
5. **Guilfoile, P. G., and R. Hutchinson.** 1991. A bacterial analog of the *mdr* gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of doxorubicin and daunorubicin. Proc. Natl. Acad. Sci. USA **88:**8553–8557.
6. **Hancock, D. C., and G. I. Evan.** 1992. Synthesis of peptides for use as immunogens. Method Mol. Biol. **10:**23–41.
7. **Kaur, P., and B. P. Rosen.** 1993. Complementation between nucleotide binding domains in an anion translocating ATPase. J. Bacteriol. **175:**351–357.
8. **Kaur, P., and B. P. Rosen.** 1994. In vitro assembly of an anion-translocating ATPase from peptide fragments. J. Biol. Chem. **269:**9698–9704.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
10. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biochem. Sci. **19:**119–123.
11. **Luciani, M. F., F. Denizot, S. Savary, M. G. Mattei, and G. Chimini.** 1994. Cloning of two novel ABC transporters mapping on human chromosome 9. Genomics **21:**150–159.
12. **Ma, D., D. N. Cook, M. Alberti, H. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
13. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
14. **Miller, J.** 1992. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Nakamura, H.** 1965. Gene-controlled resistance to acriflavine and other basic dyes in *Escherichia coli*. J. Bacteriol. **90:**8–14.
16. **Reizer, J., A. Reizer, and M. H. Saier.** 1992. A new subfamily of bacterial ABC-type transport systems catalyzing export of drugs and carbohydrates. Protein Sci. **1:**1326–1332.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Senior, A. E., M. K. Al-Shawi, and I. L. Urbatsch.** 1995. ATP hydrolysis by multidrug-resistance protein from Chinese hamster ovary cells. J. Bioenerg. Biomembr. **27:**31–36.
19. **Shapiro, A. B., and V. Ling.** 1995. ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells. J. Biol. Chem. **270:**16167–16175.
20. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distinctly related sequences in the α- and β-subunits of the ATP synthase, myosin kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
21. **Wu, J., L. S. Tisa, and B. P. Rosen.** 1992. Membrane topology of the ArsB protein, the membrane component of an anion-translocating ATPase. J. Biol. Chem. **267:**12570–12576.
22. **Yue, V. T., and P. R. Schimmel.** 1977. Direct and specific photochemical cross-linking to an aminoacyl-tRNA synthetase. Biochemistry **16:**4678–4684.