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Functional expression of mouse Mdr1 in an outer membrane permeability mutant of *Escherichia coli*

ODED BÉJÀ AND EITAN BIBI*

Department of Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel

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ABSTRACT **Functional expression of the multidrug resistance protein P-glycoprotein (P-gp) in *Escherichia coli* is providing an appropriate system for structure/function studies and might provide an invaluable tool to screen potential P-gp substrates and inhibitors. The major problem encountered in such studies, however, is the impermeability of the outer membrane of Gram-negative bacteria, which protects microorganisms against the cytotoxic effects of many lipophilic cancer drugs and blocks accessibility of P-gp reversal agents. In the present study we have constructed, by mutagenesis, a "leaky" (containing a permeable outer membrane) strain of *E. coli*, which is significantly more susceptible to the toxic effect of known P-gp substrates and cytotoxic agents. Expression of mouse Mdr1 in the mutant confers cross-resistance to daunomycin, quinidine, chloroquine, rhodamine 6G, and puromycin. Most importantly, reserpine and doxorubicin completely abolish Mdr1-mediated rhodamine resistance. The results provide strong support for previous observations, suggesting that Mdr1 can be expressed functionally in *E. coli* and indicate that the leaky mutant will be useful for further structure/function studies of the heterologously expressed eukaryotic drug efflux protein.**

The simultaneous emergence of resistance of cultured cells *in vitro* and tumor cells *in vivo* to many unrelated hydrophobic chemotherapeutic drugs is termed multidrug resistance (MDR; reviewed in ref. 1). A major form of MDR (reviewed in refs. 2–4) is caused by the overexpression of a 170-kDa membrane protein that belongs to the ATP-binding cassette or traffic-ATPase superfamily of transport proteins (5, 6) and is called Mdr or P-glycoprotein (P-gp). P-gp binds analogs of ATP (7, 8) and cytotoxic drugs (9, 10) and exhibits ATPase activity (11–13). The MDR phenotype can be overcome by chemosensitizers. These reversal agents are also unrelated structurally to each other and include calcium channel blockers such as verapamil (14), calmodulin inhibitors (15), immunosuppressants (16), reserpine (17), the anti-arrhythmic agent quinidine (18), and lysosomotropic amines such as chloroquine (19). The ability of *mdr* genes to directly confer MDR has been established in transfection experiments (8, 20), and its transport activity has been investigated with whole cells, with plasma membrane preparations, and also recently with reconstituted liposomes (21, 22) and with heterologous secretory vesicles from yeast (23). In this regard, we believe that heterologous expression systems in microorganisms may be advantageous for studying P-gp and other membrane proteins (for review see ref. 24).

Heterologous expression systems for P-gp studies in yeast (25, 26) and in insect cells (11, 27) have been developed in recent years. In addition, it has also been shown that the mouse Mdr1 protein can be expressed functionally in *Escherichia coli* (28). Briefly, the protein expressed in *E. coli* is found in the membrane in relatively stable form with an apparent molecular weight similar to that of the unglycosylated P-gp (120–140 kDa). The protein is recognized by monoclonal antibodies directed against the putative nucleotide binding fold of P-gp (C219). Cells expressing Mdr1 acquire resistance against the lipophilic cations tetraphenylphosphonium (TPP^+) and tetraphenylarsonium (TPA^+), known substrates of P-gp (29). Moreover, transport experiments with radiolabeled TPA^+ demonstrate that resistance is probably due to low levels of accumulation in cells expressing Mdr1.

One advantage of this heterologous expression system for eukaryotic integral membrane proteins is the possibility to apply well-characterized genetic methods to analyze membrane protein topology as demonstrated recently (30, 31), taking into account that the topological signals in *E. coli* and in eukaryotic systems are probably similar (32). Another advantage is the simple and rapid life cycle of prokaryotic microorganisms, which makes this heterologous expression system attractive for drug susceptibility assays and screening for P-gp reversal agents. Unfortunately, however, the outer membrane of Gram-negative bacteria represents a strong permeability barrier to many cancer drugs, as well as to P-gp inhibitors, primarily because of the lipophilic nature of these compounds. Consequently, we have not been able to detect substantial toxic effects of known cancer drugs or P-gp substrates other than TPP^+ and TPA^+. In fact, we observed that the concentrations needed to inhibit growth of *E. coli* are beyond the solubility limit of many P-gp-related cytotoxic agents (E.B., unpublished data).

In this study, we describe the construction of an *E. coli* strain with a "leaky" outer membrane that is susceptible to the toxic effect of P-gp-related lipophilic drugs. Expression of mouse Mdr1 in these cells confers MDR. In addition, we demonstrate that P-gp-mediated drug resistance is reversed by reserpine and doxorubicin. In brief, the observations provide further support for the argument that heterologous expression in *E. coli* can be used to study structural and functional properties of P-gp.

EXPERIMENTAL PROCEDURES

Materials. TPP^+ (bromide salt) and TPA^+ (chloride salt) were purchased from Aldrich. 5-bromo-4-chloro-3-indolyl phosphate (X-P), verapamil, rhodamine 6G, chloroquine, quinidine, daunomycin, reserpine, doxorubicin, vinblastine, vincristine, colchicine, actinomycin D, and nalidixic acid were all purchased from Sigma. Monoclonal antibodies C219 were obtained from Centocor. Affinity purified goat anti-mouse antibodies conjugated to horseradish peroxidase were obtained from Bio-Rad. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. *E. coli* UT5600[*ompT*$^-$], obtained from the *E. coli* Genetic Stock Center at Yale

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Abbreviations: MDR, multidrug resistance; TPP, tetraphenylphosphonium; TPA, tetraphenylarsonium; LB, Luria broth; X-P, 5-bromo-4-chloro-3-indolyl phosphate; P-gp, P-glycoprotein.

*To whom reprint requests should be addressed.

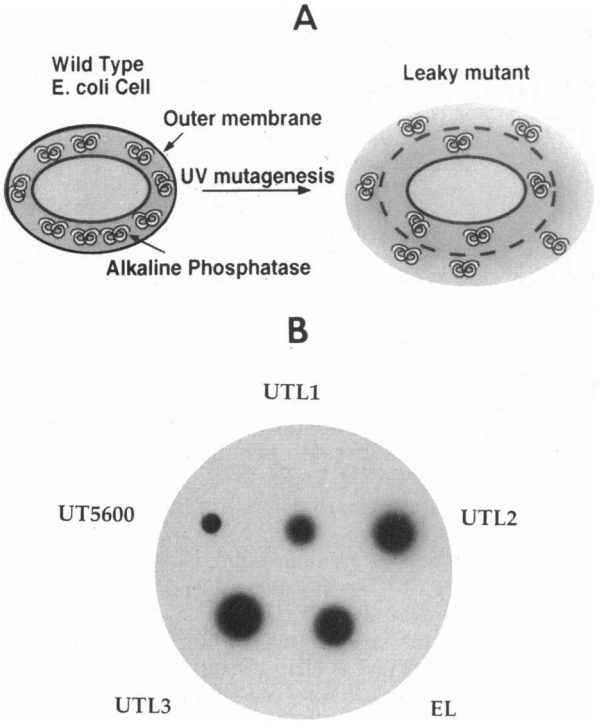

FIG. 1. (*A*) Schematic representation of the use of alkaline phosphatase to detect leaky mutants of *E. coli* with permeable outer membranes. (*B*) Demonstration of the blue halos produced by the leaky mutants UTL1, UTL2, and UTL3. Cells harboring pB*tac*(*phoA*) were grown at 30°C for 8 hr and induced by 0.5 mM isopropyl β-D-thiogalactopyranoside for 1 hr. Each culture (1.5 ml) was collected by centrifugation and resuspended in 50 μl of LB. Concentrated cells (2.5 μl) were then applied on LB agar plate containing 100 μg of ampicillin per ml and 200 μg of X-P per ml. Plates were photographed after 16 hr at 30°C. EL is a leaky *E. coli* K12 mutant (E.B. and N. Citri, unpublished results) that was served as a positive control.

University (strain 7092), was used for mutagenesis and expression studies. *E. coli* UTL1, UTL2, and UTL3 were constructed in this study. Plasmid pT7-5(*mdr1*) encoding mouse Mdr1 under the *lac* promoter was described elsewhere (28) and plasmid pT7-5(*lacY*) encoding *lac* permease under the same promoter as *mdr1* in pT7-5(*mdr1*) was used as a control. Plasmid pB*tac*(*phoA*) encoding alkaline phosphatase was kindly provided by Colin Manoil (University of Washington, Seattle).

Growth of Cells, Expression, and Immunoblotting of Mdr1. *E. coli* UT5600 or UTL2 harboring pT7-5(*lacY*) or pT7-5(*mdr1*) plasmids were grown at 30°C in Luria broth (LB) supplemented with 10 μg of streptomycin per ml and 100 μg of ampicillin per ml. Overnight cultures were diluted 1:50 and grown to an OD_{600} of 0.4. Cells were then induced with 1.0 mM isopropyl β-D-thiogalactopyranoside, and incubation was continued for 4 hr. Cultures were harvested and membranes were prepared as described (28). Immunoblotting was carried out with the monoclonal antibody C219 and a secondary horseradish peroxidase-conjugated goat anti-mouse antibody.

Mutagenesis. Competent *E. coli* UT5600 cells (500 μl) were spread on sterile 30 × 10 mm Petri dish on ice and exposed to UV light (≈80 J/m²). Immediately after UV irradiation, cells were transformed with pB*tac*(*phoA*) and plated over LB plates containing 100 μg of ampicillin per ml and 200 μg of X-P per ml.

Resistance Assays. Resistance of cells without plasmids or harboring pT7-5(*lacY*) or pT7-5(*mdr1*) against various compounds was assayed in both solid and liquid media. When tested on solid media, overnight cultures were diluted to an OD_{600} of 0.1 and grown at 30°C for a few hours. Cultures (200 μl; OD_{600} = 0.5) were mixed with 3 ml of soft agar (LB with 0.8% agar) at 45°C and poured over 1.5% agar LB square Petri dishes. Bistris propane (60 mM) was used to maintain pH 7.4 in experiments with chloroquine and quinidine. Fifteen minutes later, antibiotic filter disks were applied on each lawn, and the appropriate amount of tested antibiotic material was carefully loaded on each filter disk. Inhibition zones were measured after overnight growth at 30°C. When tested in liquid medium, overnight cultures were diluted into fresh LB containing ampicillin (100 μg/ml) and grown up to an OD_{600} of 0.6. Cells were then diluted again and aliquoted (50 μl) into 96-well microplates containing 50 μl of various concentrations of the drugs. At the beginning of a typical experiment, the cell density (OD_{600} = 0.03) in the wells was measured in a microplate autoreader (model EL309; Bio-Tek, Burlington, VT). Plates were incubated at 30°C shaker, and cell density was monitored by following the absorption at 600 nm every 6 hr. Concentrations required to inhibit 50% of the growth (D_{50}) were determined. In experiments with chloroquine or quinidine, the LB medium was supplemented with 60 mM Bistris propane to maintain pH 7.4.

RESULTS

Construction of Leaky *E. coli* strains. We have observed previously that *E. coli* UT5600 (*OmpT*⁻) expresses Mdr1 in a relatively stable state (28). When Mdr1–alkaline phosphatase hybrids are analyzed by comparative immunoprecipitation experiments in *E. coli* UT5600 or CC181(*OmpT*⁺), it is apparent that the hybrids are expressed at a much higher level in the *OmpT*⁻ strain (30). Therefore, we chose to use *E. coli*

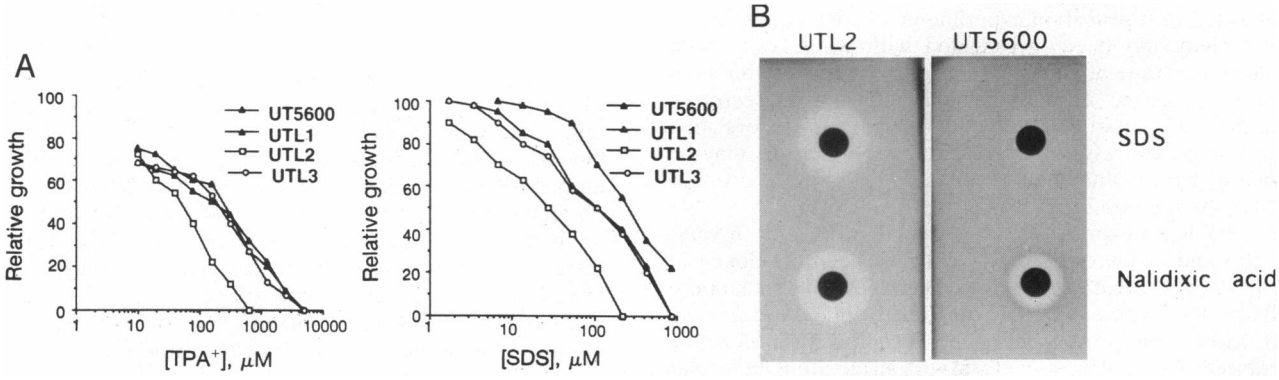

FIG. 2. Effect of hydrophobic agents on *E. coli* UT5600 and UTL2. (*A*) Resistance of *E. coli* UT5600 and UTL strains to SDS and to TPA^+. Cells were grown on LB in the presence of different concentrations of SDS or TPA^+. Relative growth is plotted as a function of drug concentration. (*B*) Resistance of *E. coli* UT5600 and UTL2 to SDS and to nalidixic acid. A lawn of *E. coli* UT5600 or UTL2 was created on solid LB. A filter paper disk saturated with 2 mg of SDS or 25 μg of nalidixic acid was placed on the plate. Plates were photographed after overnight growth at 30°C.

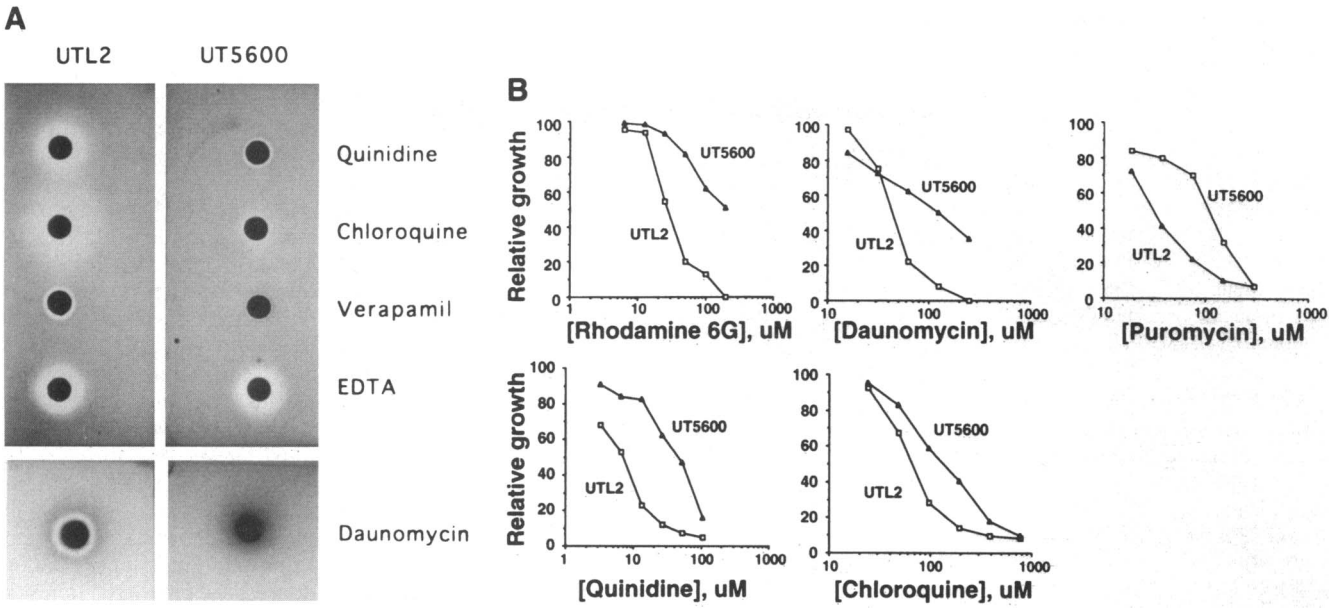

FIG. 3. Effect of P-gp agents on *E. coli* UT5600 and UTL2. (*A*) Resistance of *E. coli* UT5600 and UTL2 to the cytotoxic agents on solid medium. A lawn of *E. coli* UT5600 or UTL2 was created on solid LB plates. Filter paper disks saturated with 2 mg of chloroquine, 0.5 mg of quinidine, 105 μg of daunomycin, 0.5 mg of Verapamil, or 370 μg of EDTA were placed on each lawn. Plates were photographed after overnight growth at 30°C. (*B*) Resistance of *E. coli* UT5600 and UTL2 to P-gp-related cytotoxic agents in liquid medium. Cells were grown in LB in the presence of different concentrations of various drugs. Relative growth is plotted as a function of drug concentration.

UT5600 as the parental strain for further manipulations. The main goal was to obtain leaky mutants of *E. coli* UT5600, thus allowing accessibility of lipophilic drugs to the cytoplasmic membrane. Mutants exhibiting a leaky phenotype have been described in the past, and they are usually defective in constituents of the outer membrane, such as lipoproteins (33). Such mutants are easy to obtain using simple screening procedures. One useful procedure is based on enzymatic reactions mediated by periplasmic enzymes. In leaky mutants, the soluble periplasmic enzymes diffuse away from the cell (via the defective outer membrane), and the enzymatic activities are detected in the growth medium. In this manner, leaky mutants have been isolated using β-lactamase as a reporter (E.B. and N. Citri, unpublished results; see Fig. 1*B*, EL). In this study, alkaline phosphatase is used as the reporter (34). Briefly, cells expressing native alkaline phosphatase grow as blue colonies on agar plates containing X-P. The blue hydrolysis product from X-P is insoluble and retained within the colonies. However, if the enzyme is able to diffuse away from the colonies into the agar, the blue product generated appears as a halo around the colonies (Fig. 1*A*). To obtain leaky mutants, competent *E. coli* UT5600 cells were exposed to UV light and immediately transformed with a plasmid encoding alkaline phosphatase. Transformants were plated on agar containing 200 μg of X-P per ml and 100 μg of ampicillin per ml. After an 18-hr incubation at 37°C, three colonies (UTL1, UTL2, and UTL3) of ≈10,000 transformants were found to form large blue halos (Fig. 1*B*), indicating that alkaline phosphatase from these mutants is liberated into the medium.

Characterization of the Leaky Mutants. Mutations that cause significant alteration in the outer membrane permeability barrier result in leakage of periplasmic proteins into the medium (35) and in higher susceptibility to hydrophobic antibiotics (36) and detergents (37). To examine the permeability of the outer membrane to hydrophobic agents, the sensitivity of the mutants to SDS or TPA^+ was tested (Fig. 2*A*). As shown, the data demonstrate clearly that mutant UTL2 is significantly more sensitive to both compounds. In addition, by using a semiquantitative disk assay, the inhibition zones (clear halos) caused by the hydrophobic quinolone derivative nalidixic acid or by SDS are bigger with UTL2 than with wild-type UT5600 (Fig. 2*B*). It is important to note that hydrophilic molecules such as EDTA cause similar growth inhibition zones with the wild-type and with the mutant UTL2 (Fig. 3*B*). As a result of these experiments, UTL2 was chosen for further characterization.

***E. coli* UTL2 Cells Are Susceptible to the Toxic Effect of P-gp Related Agents.** Wild-type *E. coli* is insensitive to most cancer drugs, probably because of the outer membrane permeability barrier. To examine cross-sensitivity of the leaky mutant to various cytotoxic agents that are substrates for P-gp, *E. coli* UT5600 and the permeable mutant UTL2 were exposed to various drugs using filter disks on agar plates (Fig. 3*A*) or in liquid media (Fig. 3*B*). On plates, inhibition was observed with certain drugs only, probably because other drugs are either not toxic to *E. coli* or absorbed to the agar. The results obtained

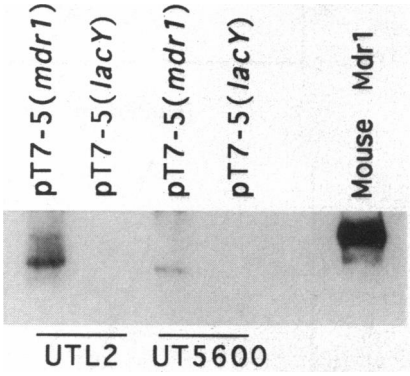

FIG. 4. Comparison of the levels of Mdr1 expression in *E. coli* UT5600, UTL2, or transfected hamster LR73 ovary cells. *E. coli* UT5600 or UTL2 transformed with pT7-5(*mdr1*) or pT7-5(*lacY*) as control were induced with isopropyl β-D-thiogalactopyranoside. Membranes were prepared and 15 μg of membrane proteins or 2.8 μg of membrane proteins from LR73 cells overexpressing Mdr1 were subjected to SDS/6% PAGE and electroblotted, and the nitrocellulose paper was incubated with monoclonal antibodies C219. After incubation with horseradish peroxidase-conjugated rabbit anti-mouse antibodies, followed by a short incubation with luminescent substrate (Amersham), the nitrocellulose paper was exposed to film for 10 min.

Table 1. Drug resistance of *E. coli* UT5600, UTL2, and UTL2 expressing Mdr1 or lactose permease

Drug	D_{50}, μM			
	UT5600	UTL2	UTL2 pT7-5(*lacY*)	UTL2 pT7-5(*mdr1*)
EDTA	57	42		
Erythromycin	6	7		
SDS	267	26		
Daunomycin	125	45		
Chloroquine	136	67	34	232
Puromycin	115	32	35	100
Quinidine	475	66	40	224
TPA†	240	50	90	340
Rhodamine 6G	200	27	20	75

D_{50} is the concentration needed to inhibit growth by 50%. It was calculated from triplicates of growth experiments as described in Fig. 2*A*, 3*A*, and 5.

on agar plates clearly demonstrate that UTL2 is significantly more sensitive to the toxic effect of chloroquine and quinidine and to a lesser extent to daunomycin (Fig. 3*B*). High concentrations of verapamil are also more toxic to UTL2 than UT5600 (Fig. 3*B*). By using a more quantitative growth analysis in liquid media, it is clear that UTL2 is also dramatically more sensitive to rhodamine, daunomycin, and puromycin (Fig. 3*A*). The concentrations needed to inhibit growth by 50% (D_{50} values) are summarized in Table 1. Other drugs such as doxorubicin, vinblastine, vincristine, actinomycin D, and colchicine have no effect on either *E. coli* UT5600 or UTL2 at the highest concentrations that can be tested (data not shown).

Expression of Mdr1 in *E. coli* UTL2 Confers Multidrug Resistance. An obvious prerequisite for P-gp studies is the ability of the mutant to express Mdr1 to a level that is at least comparable to that observed in UT5600. Thus, membranes from *E. coli* UT5600 or UTL2 harboring pT7-5/*mdr1* were examined for Mdr1 expression by Western blotting with anti-P-gp monoclonal antibody C219 (Fig. 4). Surprisingly, the level of Mdr1 in UTL2 cells is even higher (about 8-fold) than in UT5600. As of yet we do not know the reason for this improvement in the expression level of Mdr1 in UTL2 cells. The higher level of Mdr1 expression, though, enables the following investigation of Mdr1 in UTL2. In a previous study (28), it was suggested that mouse Mdr1 is functional when expressed in *E. coli*. However, it was not possible to demonstrate Mdr1-mediated resistance to its known chemotherapeutic substrates in the heterologous system, mainly because of the impermeable outer membrane. This problem has now been resolved with the new leaky strain of *E. coli* UTL2. When UTL2 cells harboring pT7-5/*mdr1* or vector without *mdr1*, were exposed to various P-gp-related compounds in liquid medium (Fig. 5), it is readily apparent that Mdr1 confers significant resistance against quinidine, chloroquine, puromycin, and rhodamine (Fig. 5). Despite the high concentrations of drug needed to inhibit growth of UTL2 (≈10–100 times the concentrations needed to inhibit growth of mammalian cells), Mdr1 is able to confer significant resistance. The calculated D_{50} of various drugs in UTL2 with or without Mdr1 are summarized in Table 1. Although data are not shown, disk assays on plates demonstrate that UTL2 expressing Mdr1 is also resistant to daunomycin.

Reversal of Mdr1-Mediated Drug Resistance in *E. coli*. In addition to its effect on the susceptibility of *E. coli* to P-gp-related drugs, the outer membrane permeability barrier also prevents access of MDR reversal agents to the cytoplasmic membrane. Consequently, we have not been able to test the

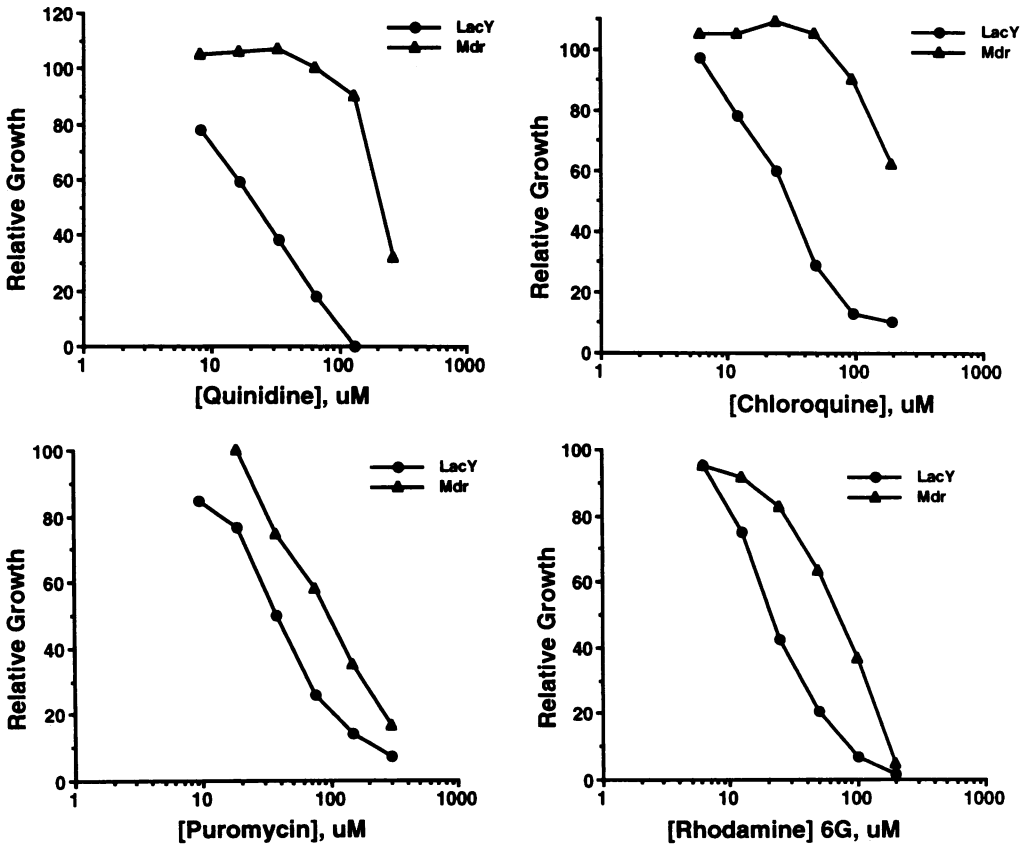

FIG. 5. Resistance of *E. coli* UTL2 cells harboring pT7-5(*mdr1*) to P-gp-related compounds in liquid medium. *E. coli* UTL2 cells expressing Mdr1 or *lac* permease as control were grown in LB in the presence of given concentrations of various drugs. Relative growth is plotted as a function of drug concentration.

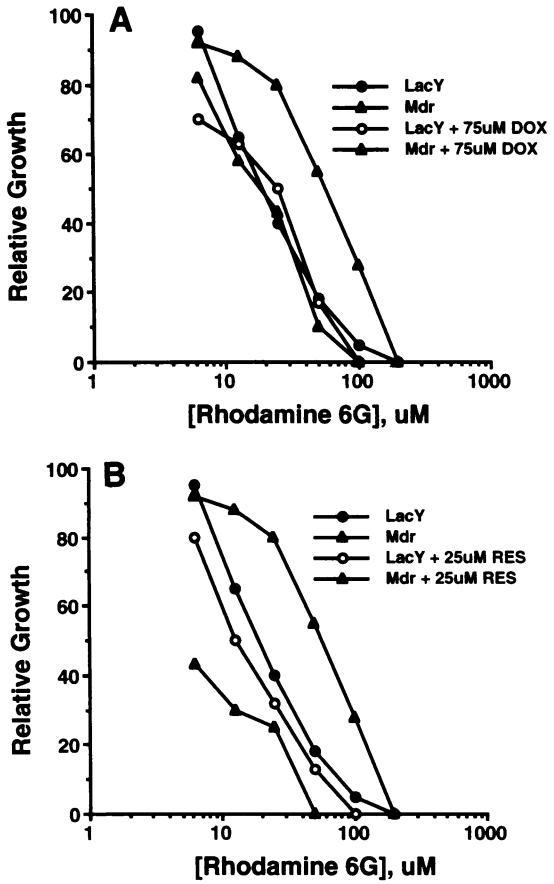

FIG. 6. Reversal of Mdr1-mediated multidrug resistance. *E. coli* UTL2 cells expressing Mdr1 or *lac* permease as control were grown in LB in the presence of different concentrations of rhodamine and with or without 75 μM doxorubicin or 25 μM reserpine. Relative growth is plotted as a function of drug concentration. These concentrations of reserpine (25 μM) or doxorubicin (75 μM) by themselves have no effect on growth.

influence of known chemosensitizers on TPP^+ or TPA^+ resistance mediated by Mdr1 expressed in *E. coli* cells. The new leaky UTL2 strain thus opens the possibility to examine inhibition of Mdr1 by various modulators *in vivo*. Two P-gp modulators were studied: reserpine, a known potent suppressor of the MDR phenotype (17), and doxorubicin, a known P-gp substrate, with which we have not been able to detect toxicity in *E. coli*. To examine the MDR reversal phenomenon *in vivo* in the heterologous system, UTL2 cells harboring pT7-5(*mdr1*) were exposed to increasing concentrations of rhodamine, with or without 25 μM reserpine or 75 μM doxorubicin. As shown in Fig. 6, reserpine and doxorubicin completely abolish Mdr1-mediated resistance against rhodamine. These observations further support the argument that *E. coli* UTL2 may serve as a model expression system to study structural and functional aspects of mouse Mdr1.

DISCUSSION

It has been shown recently (28) that Mdr1 from mouse can be expressed in *E. coli*. However, the function of heterologously expressed Mdr1 *in vivo* was only examined with the model compounds TPP and TPA (29), because of the intrinsic resistance of *E. coli* to many substrates for P-gp. In the present study, we provide a strong indication that lack of toxicity of many P-gp substrates for *E. coli* is caused by low outer membrane permeability. By using the blue halo technique (34), *E. coli* mutants with enhanced outer membrane permeability were selected, and one mutant (UTL2) was found to be substantially more sensitive to P-gp substrates. This property has enabled us to study functional aspects of mouse Mdr1 in *E. coli* using known P-gp substrates and modulators. The levels of resistance found in *E. coli* UTL2 expressing Mdr1 are similar to those in resistant tumors *in vivo*, which are usually not more than 5- to 10-fold resistant. The concentrations of P-gp related drugs required to inhibit *E. coli* UTL2 growth are much higher than those required to inhibit growth of mammalian cells. It is possible therefore that Mdr1 is unable to confer higher levels of resistance (similar to those in resistant cultured cell lines), because higher concentrations of the drugs are needed to inhibit growth of *E. coli*.

Among the various agents tested here, rhodamine (38), puromycin (39, 40), and daunomycin (41) are known P-gp substrates. Quinidine and chloroquine have been implicated in reversal of MDR phenomena only. Quinidine, an antiarrhythmic agent, is able to reverse P-gp-mediated resistance (18). In addition, it has been demonstrated that quinidine binds specifically to membrane vesicles from multidrug-resistant KB cells (42) and behaves as a competitive inhibitor of vinblastine uptake in inverted vesicles (43). Therefore, it is not surprising that Mdr1 confers quinidine resistance to *E. coli*, suggesting that the protein catalyzes export of the drug. However, unlike quinidine, chloroquine is only moderately effective in overcoming drug resistance. Chloroquine, a lysosomotropic amine, reverses drug resistance to anthracyclines and *vinca* alkaloids in multidrug-resistant cells (19, 44). Although its ability to partially block doxorubicin efflux from resistant P388R cells is significant (45), chloroquine does not inhibit *N*-(*p*-azido-[3-^{125}I]salicyl)-*N'*-(β-aminoethyl)vindesine labeling at concentrations that reverse multidrug resistance (46). Consequently, it was suggested that chloroquine may not interact with P-gp but act in a different way to reverse multidrug resistance, possibly by influencing lysosomal integrity. In this study, however, we find that *E. coli* UTL2 is sensitive to chloroquine and that Mdr1 confers chloroquine resistance. The observation suggests that chloroquine is probably a substrate for P-gp and may be translocated out of *E. coli* expressing Mdr1.

In addition to conferring drug resistance, one important aspect of the P-gp problem is the ability to reverse multidrug resistance with chemosensitizers. Experiments with the leaky mutant of *E. coli* described here also demonstrate that reserpine, a potent P-gp modulator, abolishes drug resistance in UTL2 expressing mouse Mdr1. We also initiated experiments to test whether verapamil is able to reverse Mdr1-mediated drug resistance in *E. coli*. However, the concentration of verapamil needed to inhibit Mdr1 in *E. coli* UTL2 is too high and causes inhibition of growth (data not shown). In resistant mammalian cells, the concentrations of verapamil needed to partially reverse 1–10 nM vinblastine uptake are in the micromolar range (i.e., ≈1000 times the concentration of vinblastine; ref. 41). In *E. coli*, drug concentrations in the range of 10–100 μM are needed to inhibit growth. Therefore, it is likely that toxic levels of verapamil are required for reversal.

The new expression system is potentially very powerful for large-scale screening of P-gp modulators on one hand and cytotoxic agents that are not recognized by P-gp on the other. The possibility of using known P-gp substrates in the heterologous expression system may open the way for structure/function studies by site-directed mutagenesis and selection of intragenic suppressors, operations that can be accomplished considerably more efficiently in *E. coli* than in eukaryotes. Moreover, *E. coli* UTL2 may yield inverted vesicles containing sufficient quantities of Mdr1 to study the bioenergetics of the transporter in *E. coli*. Finally, with improved expression levels, affinity purification techniques can be used to facilitate acquisition of purified Mdr1 in quantities sufficient for biochemical studies.

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