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Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1

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ABSTRACT **Resistance of *Lactococcus lactis* to cytotoxic compounds shares features with the multidrug resistance phenotype of mammalian tumor cells. Here, we report the gene cloning and functional characterization in *Escherichia coli* of LmrA, a lactococcal structural and functional homolog of the human multidrug resistance P-glycoprotein MDR1. LmrA is a 590-aa polypeptide that has a putative topology of six α-helical transmembrane segments in the N-terminal hydrophobic domain, followed by a hydrophilic domain containing the ATP-binding site. LmrA is similar to each of the two halves of MDR1 and may function as a homodimer. The sequence conservation between LmrA and MDR1 includes particular regions in the transmembrane domains and connecting loops, which, in MDR1 and the MDR1 homologs in other mammalian species, have been implicated as determinants of drug recognition and binding. LmrA and MDR1 extrude a similar spectrum of amphiphilic cationic compounds, and the activity of both systems is reversed by reserpine and verapamil. As LmrA can be functionally expressed in *E. coli*, it offers a useful prokaryotic model for future studies on the molecular mechanism of MDR1-like multidrug transporters.**

Multidrug resistance poses a serious clinical problem in the treatment of cancer and infectious diseases and is responsible for many tens of thousands of deaths each year (1, 2). Resistance of human cancer cells is commonly associated with high expression levels of the MDR1-encoded P-glycoprotein (3, 4). Indeed, transfection experiments demonstrate that overexpression of MDR1 alone can confer multidrug resistance to an otherwise drug-sensitive cell line (5). MDR1 and related P-glycoproteins are members of the ATP-binding cassette (ABC) superfamily of transporters (6), whose functions include the ATP-dependent extrusion of amphiphilic compounds out of the cell (7–9).

Several ABC transporters sharing homology with MDR1 have been identified in microorganisms. Yeast ABC proteins include the α-mating pheromone transporter STE6 (10) and the multidrug transporters PDR5 (11) and SNQ2 (12). Furthermore, overexpression of *Plasmodium* pfMdr1 has been implicated in chloroquine resistance of the malarial parasite (13, 14). Like MDR1, the characteristic features of these transporters include the presence of two homologous halves, each containing an ATP-binding domain, and a membrane domain composed of several (usually six) putative α-helical transmembrane segments. The notion that these two halves must cooperate to the formation of a single transporter is, amongst others, supported by the observation that the independent expression of each half of STE6 in yeast cells does not yield a functional transporter, while simultaneous expression of both halves does (15). Certain ABC proteins, such as the bacterial α-hemolysin transporter HlyB (16), are half the size of MDR1 with only a single domain of six transmembrane segments and a single ABC domain.

In various bacteria, including *Lactococcus lactis* (17), *Bacillus subtilis* (18), and *Escherichia coli* (19), genes encoding multidrug extrusion systems have been cloned, sequenced, and functionally expressed. To date, all bacterial multidrug transporters characterized use the proton motive force rather than ATP as the driving force and act as a drug/H^+ antiporter (2). In previous work, however, we discovered that one mechanism of multidrug resistance in *L. lactis* is dependent on drug efflux by an ATP-dependent transport system (20). This notion prompted us to search for a putative MDR1-like gene in *L. lactis*. Here, we describe the gene cloning and functional characterization in *E. coli* of LmrA, a lactococcal ABC-type multidrug transporter that shares both structural and functional properties with MDR1 and is able to transport multiple drugs.

MATERIALS AND METHODS

The isolation of the *L. lactis* MG1363 genomic DNA clone (6.0-kb *Sau* 3A DNA fragment in the *E. coli* cloning plasmid pUC19) containing the *apl* and *lmrA* genes will be described elsewhere. Nucleotide sequence analysis of both DNA strands was performed using the dideoxynucleotide chain-termination procedure (21). PCGENE (release 6.8; Genofit, Geneva) was used for computer-assisted analysis of nucleotide and protein sequences. Amino acid substitutions said to be conserved are: A/S/T, D/E, N/Q, R/K, I/L/M/V, and F/Y/W. Protein secondary structure was predicted from hydropathy profiling using the algorithm of Kyte and Doolittle (22) with a window size of 10 residues.

Plasmid pGKLmrA was constructed by subcloning the 2.3-kb *Sph*I–*Pvu*II fragment, containing *lmrA*, into the plasmid pGK13 harboring a chloramphenicol resistance marker for positive selection (23). *E. coli* CS1562 (*tolC6*::Tn10; ref. 24) was transformed with plasmid DNA by electroporation (21). Transformants were selected on Luria broth supplemented with 25 mM glucose, 16 μg of tetracycline per ml, and 9 μg of chloramphenicol per ml. The sensitivity of transformants to various drugs was assessed by inoculating exponentially growing cultures (1:100) into 96-well plates containing serial dilutions of the drugs in the liquid medium described above. The growth rate at a given drug concentration relative to growth in its absence was determined as a function of the drug concentration. For Northern blot analysis, total RNA of transformants was isolated as described (21), 30 μg of which was fractionated on a 2.2 M formaldehyde/1.2% (wt/vol) agarose gel, transferred to Qiabrane membrane (Qiagen, Westburg, the Netherlands), and hybridized to the 2.3-kb *Sph*I–*Pvu*II

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Abbreviations: ABC, ATP-binding cassette; TPP^+, tetraphenylphosphonium.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U63741).

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DNA fragment. Transcript sizes (in kb) were estimated using a 0.24- to 9.5-kb RNA ladder (GIBCO/BRL).

To study LmrA-mediated drug transport, cells were harvested in the mid-exponential phase, washed, and resuspended at a protein concentration of 0.2 mg/ml in 50 mM potassium-Hepes (pH 7.5) supplemented with 3 mM $MgSO_4$. Inside-out membrane vesicles were prepared as described (25) and diluted to a protein concentration of 1.0 mg/ml in the Hepes buffer described above. Ethidium transport in cells and membrane vesicles was measured using fluorescence spectrometry (20). The uptake of [^{3}H(G)]daunomycin (96.2 GBq/mmol; New England Nuclear) and *N*-(4′,4′-azo-*n*-pentyl)-21-deoxy-[21-^{3}H]ajmalinium (46 GBq/mmol) in membrane vesicles was assayed via the filtration method (8). The transmembrane potential ($\Delta\psi$) in cells was measured using a tetraphenylphosphonium (TPP^+)-selective electrode (26) in the presence of 50 μM reserpine to inhibit the LmrA-mediated extrusion of TPP^+. In inside-out membrane vesicles, the $\Delta\psi$ was measured using the fluorescent probe bis-(3-phenyl-5-oxoisoxazol-4-yl)-pentamethine oxonol (Oxonol V; Molecular Probes). The transmembrane pH gradient (ΔpH) in cells and inside-out membrane vesicles was estimated from the increase in $\Delta\psi$ upon the addition of nigericin at a concentration of 1 nmol per mg of protein. All experiments were performed at least in triplicate. Standard deviations were calculated where possible, and these are indicated as $\pm$SD or as error bars in the figures.

RESULTS

The lactococcal MDR1 homolog was discovered in the course of work on the *apl* gene of *L. lactis* MG1363, which encodes an alkaline phosphatase-like enzyme. Analysis of a chromosomal DNA fragment containing *apl* revealed a convergently transcribed, 3′ adjacent open reading frame of 1770 bp, designated *lmrA*. The *lmrA* gene encodes a polypeptide of 590 aa with a calculated molecular mass of 64,613 Da. Hydropathy analysis of LmrA suggests the presence of an N-terminal hydrophobic domain with six putative α-helical transmembrane segments and a C-terminal hydrophilic domain (data not shown). This latter domain contains features diagnostic of an ABC-type ATPase, such as the ABC signature sequence and the Walker A and B motifs (27).

Comparison of LmrA with members of the ABC protein superfamily revealed the highest overall sequence similarity to the subfamily of multidrug resistance P-glycoproteins, most notably human MDR1 and the MDR1 homolog in *Caenorhabditis elegans*. LmrA and each half of human MDR1 share 34% identical residues with an additional 16% conservative substitutions. The sequence identity between LmrA and the N- and C-terminal halves of human MDR1 is observed throughout their lengths (Fig. 1). The membrane domains of LmrA (residues 1–361) and the N- and C-terminal halves of MDR1 are 23% and 27% identical, respectively, whereas the ABC domains of the proteins are 48% and 43% identical, respectively. The overall sequence similarity between LmrA and ABC transporters associated with (*i*) the uptake of solutes, (*ii*) antigen presentation, (*iii*) the excretion of competence or mating factors, or (*iv*) the excretion of bacterial antibiotics, toxins, or polysaccharides is <25% and is mostly confined to the hydrophilic ABC domains. Interestingly, LmrA shares overall sequence identity with the *E. coli* MsbA protein (28% identical residues), the function of which is unknown at the

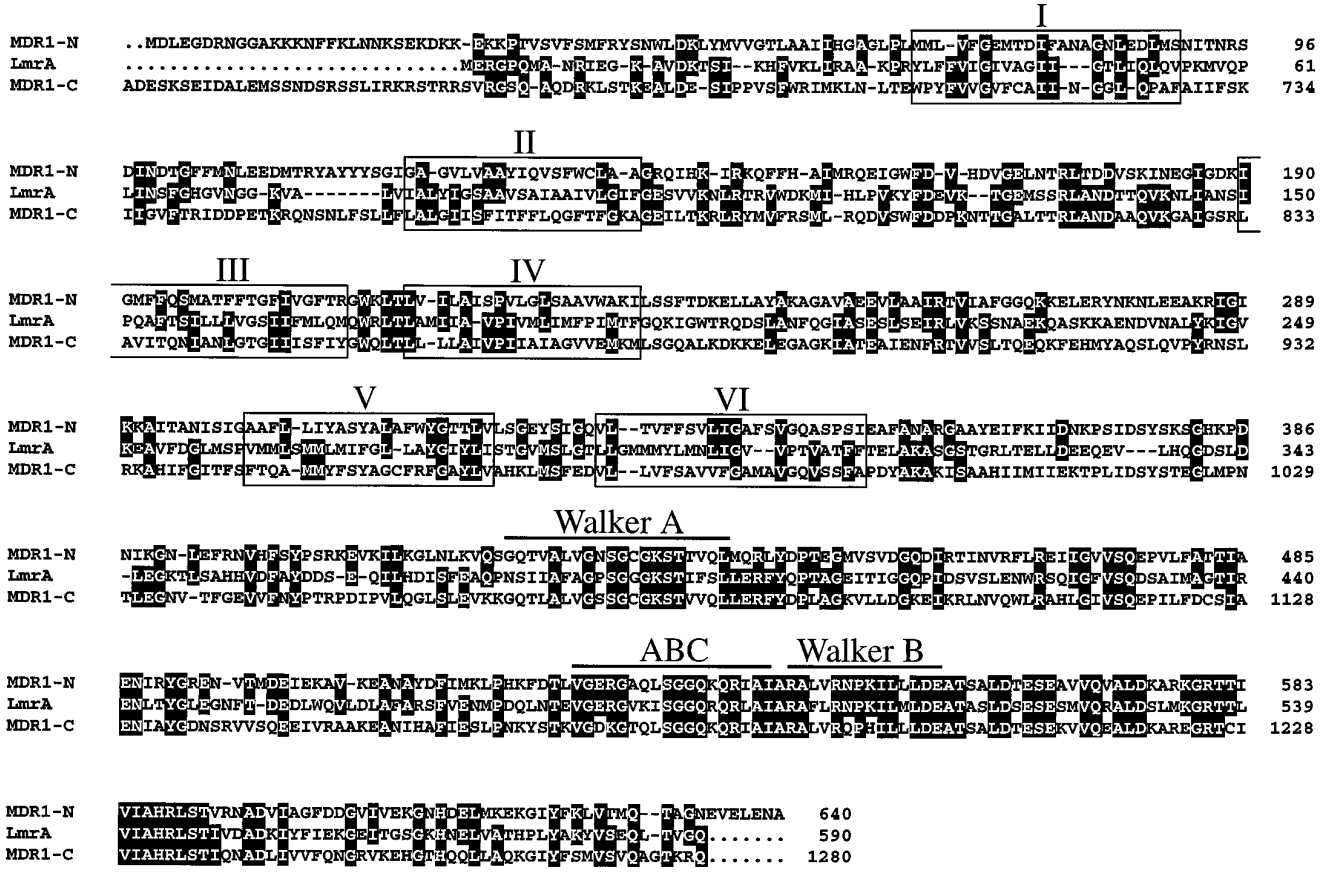

FIG. 1. Comparison of the amino acid sequence of LmrA and the N- and C-terminal halves of the human multidrug resistance P-glycoprotein MDR1 (3). MDR1-N and MDR1-C represent amino acid residues 1–640 and 641-1280 of MDR1, respectively. The last residue in each row is numbered. A dark background indicates identical residues. The roman numbers refer to the predicted transmembrane α-helices of LmrA. The ABC signature sequence and Walker A and B motifs are indicated. Gaps introduced to optimize the alignment are indicated by —.

present (28), and with the product of an unidentified open reading frame in *B. subtilis* (31% identical residues) and *Staphylococcus aureus* (33% identical residues) (GenBank accession nos. P45861 and U29478, respectively). The statistical significance of each alignment score was evaluated using the Dayhoff MDM-78 comparison matrix (29). If an alignment score is >9 SD above the mean of randomly permuted sequences ($P \leq 10^{-19}$), the sequence similarity is generally considered to be too large to have arisen either by chance or by a convergent evolutionary process (30). The significance of the scores for the alignments of the ABC domain of LmrA with the ABC domains of other ABC proteins is >9 SD. For alignments of the membrane domain of LmrA with the membrane domains of functionally verified ABC-type transporters, a significance of >9 SD is only observed for the subfamily of P-glycoproteins, most notably human MDR1 and the MDR1 homolog in *C. elegans*, but not for specific drug extrusion systems such as HlyB, STE6, and the doxo- and daunorubicin transporter DrrAB from *Streptomyces peuceticus* (ref. 31; Table 1).

Having established the structural similarity between LmrA and MDR1, we began to explore the function of LmrA. For this purpose, *lmrA* was subcloned into the *E. coli*/*L. lactis* shuttle vector pGK13 (23), giving pGKLmrA. Control and *lmrA* containing plasmids were transferred to *E. coli* strain CS1562, which is hypersensitive to drugs due to a deficiency in the TolC protein (24). Northern blot analysis was performed to confirm the expression of *lmrA* in this host. Using an *lmrA* gene-specific DNA probe, the 1.8-kb *lmrA* messenger was readily detectable in cells harboring pGKLmrA. The signal was absent in the parental vector control (data not shown).

Two approaches were used to assess the ability of heterologously expressed LmrA to act as a multidrug extrusion system: (*i*) *in vivo* resistance to growth inhibition by lipophilic cations, and (*ii*) transport of lipophilic cations. *E. coli* CS1562/pGK13 is unable to grow on solid media containing ethidium at concentrations >20 μM. Strikingly, cells harboring pGKLmrA are able to form colonies on plates containing 60 μM ethidium after overnight incubation at 37°C. This difference in *in vivo* drug resistance was studied more extensively in liquid cultures in the presence of various drugs that are known substrates of MDR1 (4). The results, depicted in Table 2, show that the expression of LmrA in *E. coli* CS1562 increases resistance to ethidium, daunomycin, rhodamine 6G, and TPP^+.

To elucidate the mechanism of LmrA-associated drug resistance, fluorimetric ethidium transport assays were performed. Washed cell suspensions of *E. coli* CS1562 containing pGKLmrA or pGK13 accumulated ethidium at the same initial rate (Fig. 2). In the control cells, this $\Delta\psi$-driven passive influx of the lipophilic cation was enhanced upon energization with glucose, due to the increase of the $\Delta\psi$ (interior negative) and ΔpH (interior alkaline) from −67 to −90 mV and from −5 to −9 mV, respectively (data not shown). Although comparable changes in $\Delta\psi$ and ΔpH were observed in LmrA-expressing cells, energization with glucose resulted in the extrusion of ethidium rather than uptake (Fig. 2). Hence, drug resistance in LmrA-expressing cells is based on active drug efflux.

Table 1. Statistical significance of alignments of the membrane domain of LmrA with the membrane domains of ABC-type (multi)drug transporters

Transporter	Statistical significance
MDR1-N	11.6
MDR1-C	10.8
CE MDR1-C	9.8
CE MDR1-N	9.0
HlyB	7.3
STE6-C	7.3
STE6-N	5.3
DrrB	−0.5

The GenBank data base accession nos. are indicated in parentheses: human MDR1 (P08183), CE MDR1 in *C. elegans* (P34712), HlyB in *E. coli* (M81823), STE6 in *Saccharomyces cerevisiae* (P12866), and DrrB in *Streptomyces peuceticus* (M73758). The N- and C-terminal halves of MDR1, CE MDR1, and STE6 are indicated by the extensions N and C, respectively. The sequence comparisons were repeated with 150 permutations using a gap penalty of 80.

Table 2. Effect of *lmrA* gene expression on the relative resistance to drugs of *E. coli* CS1562

Drug	Relative resistance
Ethidium	41 ± 6
Daunomycin	32 ± 5
Rhodamine 6G	45 ± 8
TPP^+	54 ± 5

Relative resistances were determined by dividing the IC_{50} (the drug concentration required to inhibit the growth rate by 50%) for cells harboring pGKLmrA by the IC_{50} for control cells harboring pGK13. The latter values varied between 4 and 5 μM for the drugs tested.

The energetics and specificity of LmrA-mediated drug transport were studied in more detail in inside-out membrane vesicles. Daunomycin uptake above equilibration levels was observed in membrane vesicles of LmrA-expressing cells in the presence of ATP, an ATP-regenerating system, and the ionophores valinomycin plus nigericin that selectively dissipate the $\Delta\psi$ (interior positive) and ΔpH (interior acidic), respectively (Fig. 3*A*). The dissipation of the components of the proton motive force by the ionophores was confirmed in experiments in which the fluorescent probe Oxonol V was used to monitor the $\Delta\psi$. Daunomycin was not accumulated in these membrane vesicles in the presence of ATPγS, a non-hydrolyzable ATP analog, indicating that ATP hydrolysis is required for transport. This conclusion was confirmed by the inhibition of active daunomycin uptake by *ortho*-vanadate, an inhibitor of ABC

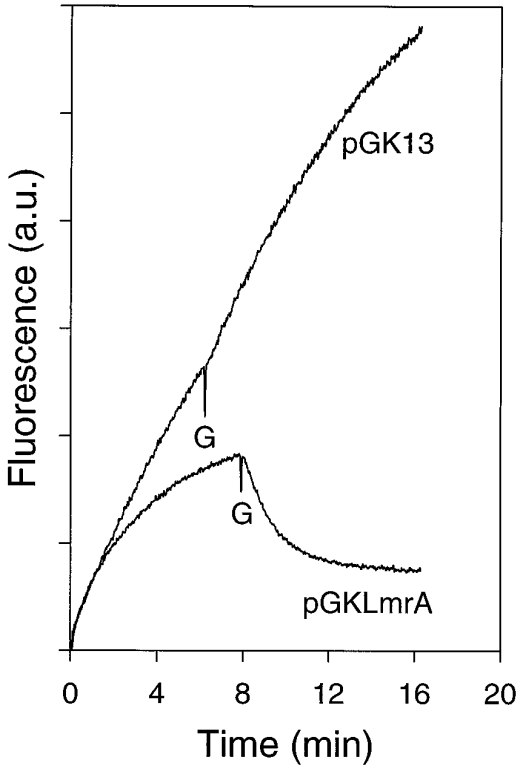

FIG. 2. Ethidium transport in *E. coli* CS1562 with (pGKLmrA) and without (pGK13) expression of LmrA. Ethidium was added to washed cell suspensions at a final concentration of 50 μM. Cells were energized by the addition of 10 mM glucose (G).

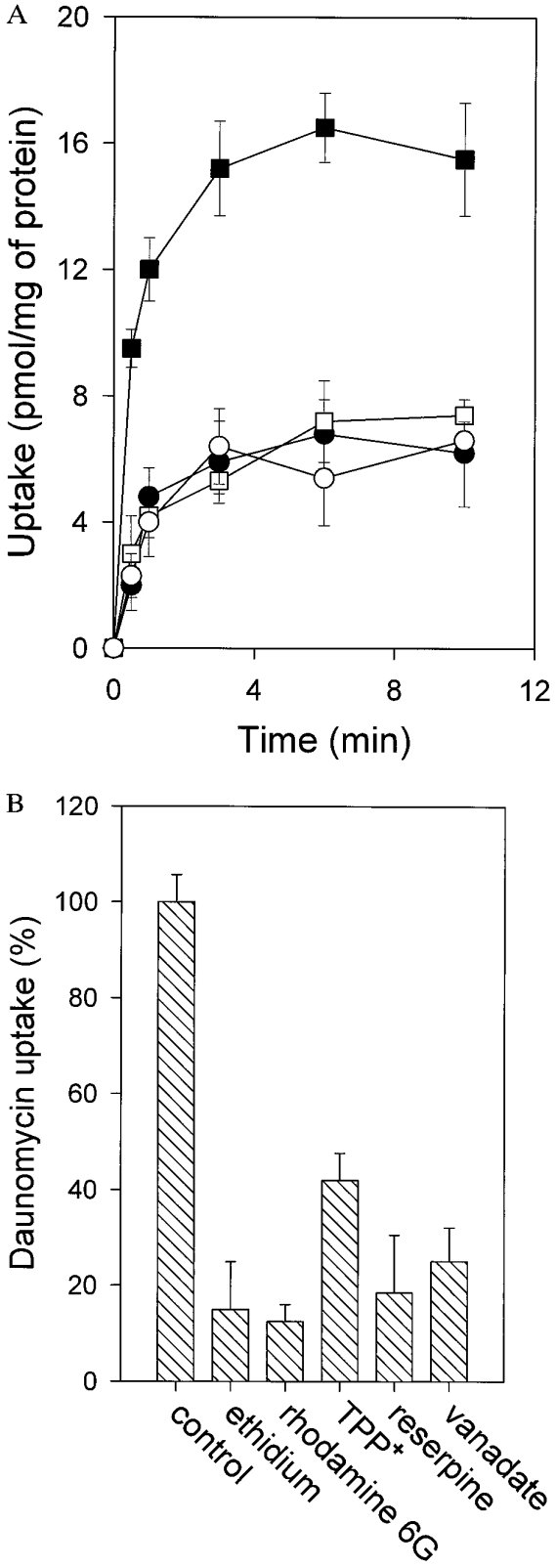

FIG. 3. Daunomycin transport in inside-out membrane vesicles. (*A*) Uptake of daunomycin (3.8 μM, final concentration) in membrane vesicles prepared from *E. coli* CS1562/pGKLmrA (●, ■) and *E. coli* CS1562/pGK13 (○, □), in the presence of valinomycin plus nigericin (each at 1 nmol per mg of protein), 5 mM creatine phosphate, and 1 mM ATPγS (●, ○) or 1 mM ATP plus 0.1 mg of creatine kinase per ml (■, □). (*B*) Effect of inhibitors on daunomycin uptake in inside-out membrane vesicles of LmrA-expressing cells. Inhibitors were included in the assay at a final concentration of 50 μM. The initial rate of ATP-dependent daunomycin uptake in membrane vesicles over the first 60 s was measured and corrected for the uptake of substrate in the presence of ATPγS. The control uptake (100%) was 7 pmol of daunomycin/min per mg of membrane protein.

transporters and P-type ATPases (Fig. 3*B*). Membrane vesicles prepared from control cells did not display the ATP-dependent uptake of daunomycin (Fig. 3*A*). Similar results were obtained for the transport of *N*-(4′,4′-azo-*n*-pentyl)-21-deoxy-ajmalinium, a high-affinity substrate of MDR1 (32) and ethidium (data not shown). The inhibition of daunomycin uptake in inside-out membrane vesicles of LmrA-expressing cells by a 12-fold excess of ethidium, rhodamine 6G, or TPP^+ points to competition between these substrates for transport by LmrA (Fig. 3*B*). Finally, LmrA-mediated drug transport was inhibited by a 12-fold excess of reserpine (Fig. 3*B*) and verapamil (data not shown). Both compounds are well-known inhibitors of human MDR1 (4).

DISCUSSION

In prokaryotes, a number of dedicated ABC-type drug export systems have been detected. A well-known example is *Streptomyces*, in which transporters such as DrrAB mediate the excretion of specific antibiotics to ensure self-resistance to the antibiotics the organism produces. To our knowledge, the lactococcal LmrA protein described in this work represents the first prokaryotic ABC transporter able to transport multiple drugs with different chemical structures and cellular targets. In view of the general organization of ABC transporters, two membrane domains and two ATP-binding domains (6), LmrA is postulated to function as a homodimer unit (or a multimeric complex derived thereof).

LmrA is a true prokaryotic homolog of MDR1. The structural similarity between the ABC and membrane domains of LmrA and the N- and C-terminal halves of MDR1 (Fig. 1) translates into a functional similarity. Both proteins mediate the extrusion of amphiphilic cationic compounds, and the activity of both transporters is reversed by reserpine, verapamil, and vanadate (Figs. 2 and 3). The observation of ATP-dependent, LmrA-mediated daunomycin transport in inside-out membrane vesicles in the absence of a proton motive force points to a direct drug transport mechanism in which the transport protein physically interacts with the drug. It has been suggested that MDR1 removes drugs from the membrane rather than from the cytoplasm (33). Recently, evidence has been obtained that LmrA expels drugs from the inner leaflet of the lipid bilayer (34). Thus, the ability of amphiphilic substrates to partition in the inner leaflet of the membrane is a prerequisite for the recognition by the multidrug transporter and is the first step in specificity. The subsequent interaction between drugs and a fairly nonspecific binding site on the transport protein will be the second determinant of drug specificity. Interestingly, the sequence conservation in the membrane domain of LmrA includes particular regions (e.g., the first cytoplasmic loop and the region comprising transmembrane segments V and VI) that have been implicated as determinants of drug recognition and binding by human MDR1 and by MDR1 homologs in other mammalian species (35).

Appreciation of the mechanisms by which eukaryotic and prokaryotic cells develop drug resistance is critical for the development of effective new drugs. Studies on the molecular mechanism of LmrA may offer a useful framework for interpreting data obtained on its medically important counterparts in humans and pathogenic microorganisms.

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