Multidrug resistance in Lactococcus lactis

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The gene encoding the secondary multidrug transporter LmrP of Lactococcus lactis was heterologously expressed in Escherichia coli. The energetics and mechanism of drug extrusion mediated by LmrP were studied in membrane vesicles of E. coli. LmrP mediated extrusion of tetraphenyl phosphonium (TPP⁺) from right-side-out membrane vesicles and uptake of the fluorescent membrane probe TMA-DPH into inside-out membrane vesicles are driven by the membrane potential (Δψ) and the transmembrane proton gradient (ΔpH), pointing to an electrogenic drug/proton antiport mechanism. Ethidium bromide, a substrate for LmrP, inhibited the LmrP mediated TPP⁺ extrusion from right-side-out membrane vesicles, showing that LmrP is capable of transporting structurally unrelated drugs. Kinetic analysis of LmrP-mediated TMA-DPH transport revealed a direct relation between the transport rate and the amount of TMA-DPH associated with the cytoplasmic leaflet of the lipid bilayer. This observation indicates that drugs are extruded from the inner leaflet of the cytoplasmic membrane into the external medium. This is the first report that shows that drug extrusion by a secondary MDR transporter occurs by a “hydrophobic vacuum cleaner” mechanism in a similar way as was proposed for the primary lactococcal MDR transporter, LmrA.
Introduction

Infections by pathogenic bacteria can often successfully be treated with antibiotics. A major drawback of the widespread use of antibiotics, however, is posed by the selection of antibiotic resistant strains. Different mechanisms of antibiotic resistance have evolved which comprise: (i) the enzymatic inactivation of the antibiotics, (ii) the alteration of the drug target, (iii) the prevention of drug entry by alterations in the cell envelope, and (iv) the active extrusion of the drugs from the cell (Nikaido, 1994). Active drug extrusion can be mediated by specific drug resistance (SDR) transporters as well as by multidrug resistance (MDR) transporters; the latter systems confer resistance to a broad range of unrelated toxic compounds (Endicott and Ling, 1989).

To date, several bacterial drug extrusion systems have been identified and characterized at the genetic level (for reviews see Nikaido, 1994, van Veen et al., 1996). The bacterial drug extrusion systems can be divided into: (i) secondary drug transporters which mediate drug extrusion in a coupled exchange with protons (Paulsen and Skurray, 1993), and (ii) ATP-binding cassette (ABC)-type drug transporters which utilize the free energy of ATP hydrolysis to extrude cytotoxic substrates (Fath and Kolter, 1993, Higgins, 1992). The secondary drug transporters are subdivided into two groups on the basis of their similarity in size and secondary structure (Schuldiner et al., 1995). The largest subgroup, termed TEXANs (Toxin EXtruding ANtiporter), consists of integral membrane proteins with 12 to 14 putative transmembrane spanning segments and an average molecular weight of 45 to 50 kDa (van Veen et al., 1996; Lewis, 1994; Marger and Saier, 1993; Saier et al., 1994). The second subgroup comprises the mini TEXANs which share functional similarity with the TEXANs but are much smaller (12-15 kDa) and form only 4 putative transmembrane α-helices (Yerushalmi et al., 1995; Grinius and Goldberg, 1994).

In Lactococcus lactis, two distinct transport systems (LmrP and LmrA) have been identified which mediate active extrusion of multiple cationic drugs (Chapter II). LmrP is a secondary drug transporter comprising 408 amino acid residues with 12 putative membrane spanning segments (Chapter III). The protein is homologous to several drug transporters belonging to the group of TEXANs. The second multidrug transporter, LmrA, is a 589-amino acid integral membrane protein and belongs to the ATP-binding cassette (ABC) family of drug transporters (Chapter V). Most strikingly, LmrA is the first example of a bacterial ATP-dependent multidrug extrusion system in which both functional and structural properties of the human MDR1 gene encoded MDR transporter P-glycoprotein (P-gp) are united.
The involvement of the \( \Delta p \) as a driving force for multidrug extrusion by TEXAN members has not been studied extensively. Instead, this characteristic of TEXANs is based on the inhibition of drug transport by protonophores like CCCP, and on their structural similarity to other secondary transport systems (Neyfakh et al., 1991; Yoshida et al., 1990; Lomovskaya and Lewis, 1992). We have now studied the energetics and mechanism of drug transport by LmrP in membrane vesicles of \( E. coli \) expressing the protein. Evidence is presented that LmrP mediates drug transport via an electrogenic mechanism in which drugs are expelled from the inner leaflet of the phospholipid bilayer.

**Materials and methods**

Growth of the organisms. Bacterial strains and plasmids used in this study are listed in Table 1. Lactococcus lactis strains were grown at 30°C on M17 medium (Difco) supplemented with glucose (25 mM) and erythromycin (5 \( \mu \)g/ml) when needed. \( E. coli \) was grown aerobically at 37°C on Luria Broth (Sambrook et al., 1989) with carbenicillin (50 \( \mu \)g/ml) and IPTG when needed.

DNA manipulation and construction of H-LmrP. General procedures for cloning and DNA manipulations were performed essentially as described by Sambrook et al. (1989). In order to subclone \( \text{ImrP} \), the gene was amplified from the chromosome of \( L. lactis \) MG1363 via the polymerase chain reaction as described previously (Chapter III). The amino-terminal polyhistidine (His)-tag containing LmrP derivative H-LmrP was constructed by subcloning the \( \text{ImrP} \) containing PCR fragment in frame with a His\(_6\)-tag encoding sequence in the \( E. coli \) expression vector pET302 (Manting, van der Does and Driessen, submitted), and transformation of ligated DNA to \( E. coli \) DH5\( \alpha \). The different cloning steps were checked by restriction analysis and double-stranded DNA sequencing using the dideoxy chain-termination procedure (Sanger et al., 1977) and the T7 DNA sequencing kit (Pharmacia).

Preparation of membrane vesicles. \( E. coli \) DH5\( \alpha \) was grown aerobically at 37°C to an \( A_600 \) of about 0.8 on Luria Broth supplemented with carbenicillin (50 \( \mu \)g/ml) plus IPTG (100 \( \mu \)g/ml) when appropriate. Inside-out (ISO) (Ambudkar et al., 1984) and right-side-out (RSO) (Kaback, 1971) membrane vesicles were prepared as described and resuspended in 50 mM potassium HEPES (pH 7.0) containing 5 mM MgSO\(_4\) (ISO), or in 50 mM potassium phosphate (pH 7.0) containing 5 mM MgSO\(_4\) (RSO). Membrane vesicles were stored in liquid nitrogen.
Purification of H-LmrP. H-LmrP was partially purified via Ni-NTA agarose affinity chromatography (QIAGEN Inc., USA). Membrane vesicles (5 mg of protein) were solubilized in 3.5 ml buffer A [50 mM sodium phosphate (pH 8.0) plus 600 mM NaCl, 10 mM Tris/HCl, and 20% v/v glycerol] plus Triton X-100 (1% w/v) for 30 min at room temperature. Subsequently, the mixture was centrifuged (15 min, 350,000 × g at 4 °C) and the supernatant was gently mixed with 200 µl of a 50% Ni-NTA resin slurry (100 µl column material) for 30 min at room temperature, after which the suspension was poured into a column. The column was subsequently washed with 20 column volumes of buffer A plus 0.5% (w/v) Triton X-100 (buffer B), followed by 50 column volumes of buffer B plus 10 mM imidazole, and 5 column volumes of buffer B plus 25 mM imidazole. Finally, H-LmrP was eluted from the column with 5 volumes of buffer B plus 250 mM imidazole and the total fraction (500 µl) was collected. The fractions were analyzed by SDS-PAGE (Laemmlli, 1970).

TMA-DPH transport in ISO membrane vesicles. The amount of membrane associated TMA-DPH (Molecular Probes Inc., Eugene, OR, USA) was measured fluorimetrically as described (Chapter VI). ISO membrane vesicles prepared from E. coli DH5α (0.4 mg protein/ml) were suspended in oxygen-saturated 50 mM potassium HEPES (pH 7.5) containing 25 mM KSO₄ plus 5 mM MgSO₄. A Δp (inside positive and acidic) was generated by the oxidation of D-lactate via a constitutively expressed, membrane associated lactate dehydrogenase (van Schie et al., 1985). The fluorescence development upon addition of TMA-DPH (100 nM, final concentration) was recorded in time using excitation and emission wavelengths of 350 and 425 nm, and slit widths of 5 and 10 nm, respectively.

TPP⁺ accumulation in RSO membrane vesicles. Tetraphenyl phosphonium (TPP⁺) accumulation in RSO membrane vesicles was calculated from the external free probe concentration as recorded by a TPP⁺-selective electrode (Shinbo et al., 1978). In competition experiments, ethidium bromide was added prior to TPP⁺ to correct for changes in the electrode output. RSO membrane vesicles were resuspended in potassium phosphate (pH 6.8) containing 5 mM MgSO₄ to a final concentration of 0.3 mg protein/ml. A Δp (inside negative and alkaline) was generated by oxidation of glucose (10 mM) via the membrane bound pyrrolo-quinoline quinone (PQQ)-dependent glucose dehydrogenase of E. coli (van Schie et al., 1985). PQQ was added to a final concentration of 1 µM.
## Table 1

**Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Bacterium/plasmid</th>
<th>Relevant characteristics</th>
<th>Source/refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. lactis</td>
<td>ML3, plasmid free, Lac; Prt&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Gasson (1983)</td>
</tr>
<tr>
<td>MG1363</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1, ΔlacU169 (*80LacΔM15)</td>
<td>BRL Inc., Bethesda</td>
</tr>
<tr>
<td>DH5α</td>
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<tr>
<td><strong>Plasmids</strong></td>
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</tr>
<tr>
<td>pBluescript SKII&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; expression vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pSKLMR3.2</td>
<td>pBluescript SK II; carrying LmrP of L. lactis on a 3.2 kb HindIII fragment</td>
<td>(Chapter III)</td>
</tr>
<tr>
<td>pGK13</td>
<td>Em&lt;sup&gt;R&lt;/sup&gt; Cm&lt;sup&gt;R&lt;/sup&gt; E. coli-L. lactis shuttle vector</td>
<td>Kok et al. (1984)</td>
</tr>
<tr>
<td>pGKLMR3.2</td>
<td>pGK13, carrying LmrP of L. lactis on a 3.2 kb HindIII fragment</td>
<td>(Chapter III)</td>
</tr>
<tr>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; pBR322 derivative containing trc promoter</td>
<td>Pharmacia</td>
<td></td>
</tr>
<tr>
<td>pET302</td>
<td>pTRC99A carrying a His-tag coding region and trc promoter</td>
<td>van der Does (submitted)</td>
</tr>
<tr>
<td>pHLP1</td>
<td>pET302 carrying LmrP on a 1.390 kb Ncol-Xbal fragment</td>
<td>This work</td>
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Chapter IV

Results

Heterologous expression of LmrP and identification of the protein. LmrP was heterologously expressed in E. coli strain DH5α using the plasmid vector pET302 on which LmrP was transcribed from the trc promoter. To evaluate the expression of LmrP in E. coli, a His-tag was engineered at the amino-terminus of the protein, yielding H-LmrP, which allows detection of the protein after purification by Nickel-NTA chromatography. Fig. 1 shows that H-LmrP corresponds to a protein with an apparent molecular mass of 35 kDa on SDS-PAGE.

![Fig. 1. Expression of H-LmrP in E. coli and identification of the protein. Silver stained 10% SDS-PAGE containing samples of total membranes (lanes 1 & 2) and Ni-NTA eluates (lanes 3 & 4) from cells harboring the control vector pET302 (lane 1 & 3) or the H-LmrP encoding vector pHLP1 (lane 2 & 4). Lane 5, molecular weight markers; the arrow indicates the position of H-LmrP.](image)

H-LmrP mediated TPP⁺ extrusion. To assess the functional expression of H-LmrP in E. coli, tetrphenyl phosphonium (TPP⁺) efflux by isolated RSO membrane vesicles was monitored using an ion selective electrode. Generation of a Δψ (inside negative) by glucose oxidation, in the presence or absence of the potassium/proton ionophore nigericin, resulted in a high accumulation of TPP⁺ in control RSO membrane vesicles (Fig. 2; pET302). The Δψ-driven passive influx of TPP⁺ was much lower in RSO membrane vesicles containing H-LmrP (Fig. 2; pHLP1). Importantly, measurements of Δψ by the fluorescent probe DiSC₅(5) revealed comparable values for the control and LmrP containing RSO membrane vesicles (data not shown), indicating that differences in TPP⁺
accumulation are most likely due to active extrusion of TPP\(^+\) via H-LmrP.

Fig. 2. TPP\(^+\) accumulation in right-side-out membrane vesicles of E. coli DH5\(\alpha\). The accumulation of TPP\(^+\)in RSO membrane vesicles of E. coli DH5\(\alpha\)/pET302 (control) and DH5\(\alpha\)/pHLIP1 (H-LmrP) was followed by recording the external free TPP\(^+\) concentration with an ion selective electrode. Measurements were carried out in potassium phosphate (50 mM, pH 6.8) containing 5 mM MgSO\(_4\) plus 4 \(\mu\)M TPP at 30°C. The additions of RSO membrane vesicles (0.3 mg of protein/ml), 2 \(\mu\)M of PQQ plus 10 mM of glucose, 1 \(\mu\)M of nigericin, and 1 \(\mu\)M of valinomycin are indicated by arrows. Reserpine was included in the phosphate buffer at a final concentration of 10 \(\mu\)g/ml when indicated in the figure.

An enhanced \(\Delta\psi\)-driven uptake of TPP was observed in pHLIP1 RSO membrane vesicles in
the presence of reserpine (Fig. 2), a known inhibitor of LmrP and other MDR transporters (Chapter III, Ahmed et al., 1993). The TPP⁺ accumulation in the presence of reserpine was similar for the control and H-LmrP containing RSO membrane vesicles.

Fig. 3. Ethidium bromide and TPP⁺ compete for LmrP-mediated extrusion. TPP⁺ accumulation as function of the ethidium concentration was assayed in H-LmrP containing pHLP1 RSO membrane vesicles (■), pHLP1 RSO membrane vesicles plus reserpine (□) and in the control pET302 RSO membrane vesicles (●) as described in the legend to Fig. 2. The maximal TPP⁺ accumulation was calculated from the difference in recorder output before and after dissipation of the membrane potential with valinomycin.

These data show that the decreased TPP⁺ accumulation in pHLP1 RSO membrane vesicles results from LmrP-mediated TPP⁺ extrusion. In previous experiments we have shown that ethidium bromide is a substrate for LmrP (Chapter III). In accordance with this observation, the accumulation of TPP⁺ in pHLP1 RSO membrane vesicles was enhanced in the presence of increasing concentrations of ethidium bromide, whereas TPP⁺ accumulation was not affected by ethidium bromide in control RSO membrane vesicles or in pHLP1 RSO membrane vesicles plus reserpine (Fig. 3). These experiments demonstrate that H-LmrP is functionally expressed in E. coli.
Energetics and Mechanism of LmrP

Fig. 4. TMA-DPH transport in inside-out membrane vesicles of *E. coli* DH5α. Uptake of TMA-DPH (100 nM, final concentration) was followed fluorometrically in an aerated buffer containing ISO membrane vesicles (0.4 mg of protein/ml) of *E. coli* DH5α harboring pHLP1 or pET302. The fluorescence development was assayed in: (a) non-energized ISO membrane vesicles, (b) ISO membrane vesicles energized with 10 mM of D-lactate, (c) as b plus 10 µg of reserpine/ml, and (d) as b preincubated with 1 µM of nigericin. Valinomycin was added to a final concentration of 1 µM as indicated (val).

Energetics of LmrP-mediated drug transport. The H-LmrP mediated extrusion of TPP in the presence of nigericin (Fig. 2) indicates that the ΔΨ can function as the sole driving force for efflux. To study the energetics of LmrP in greater detail, TMA-DPH transport was measured in ISO membrane vesicles of *E. coli* DH5α in which an inversed Δp (inside acidic and positive) was generated by the oxidation of D-lactate. TMA-DPH is an amphiphilic, cationic and hydrophobic membrane probe (partition coefficient in octanol/water of 2.4 × 10⁵), which is only fluorescent when present in the membrane (Prendergast et al., 1981).

Therefore, the fluorescence properties of TMA-DPH can be directly used to follow the concentration of the probe in the lipid bilayer by means of fluorescence spectrophotometry (Kuhry et al., 1985). TMA-DPH added to pre-energized pHLP1 ISO membrane vesicles exhibited a rapid increase in TMA-DPH fluorescence, which was immediately followed by a fluorescence decrease. These results indicate that TMA-DPH inserts rapidly into the membrane and, subsequently, translocates from the membrane into the intravesicular
space (Fig. 4). Importantly, TMA-DPH transport into the intravesicular space was partially inhibited upon dissipation of the ΔpH by nigericin or by dissipation of the Δψ by the potassium ionophore valinomycin. Complete inhibition of TMA-DPH transport was observed by (i) total dissipation of the Δp by nigericin plus valinomycin, and (ii) complete inhibition of LmrP activity by reserpine. In the control ISO membrane vesicles, TMA-DPH fluorescence was not affected by dissipation of the Δp or addition of reserpine (Fig. 4). These experiments clearly demonstrate that both the Δψ and the ΔpH function as a driving force of LmrP-mediated drug transport.
Fig. 5. Rate of energy-dependent TMA-DPH extrusion in the course of TMA-DPH partitioning in the phospholipid bilayer. Energy dependent TMA-DPH fluorescence development in cells of L. lactis MG1363/pGKLMR3.2 (A) and in ISO membrane vesicles of E. coli DH5α/pHLP1 (B). Cells and ISO membrane vesicles were energized with 25 mM of glucose or 10 mM of D-lactate, respectively, 3 min prior to the addition of 100 nM of TMA-DPH, or after this addition at the time points indicated. The dashed line represents the steady state level of TMA-DPH fluorescence.
Kinetics of LmrP-mediated TMA-DPH transport. The kinetics of TMA-DPH fluorescence development upon the addition to cells is biphasic (Chapter VI) (see also Fig. 4). The initial fast phase reflects probe partitioning in the outer leaflet of the membrane, while the second and slower phase is due to the transbilayer movement of TMA-DPH into the inner leaflet of the membrane. In L. lactis Eth<sup>R</sup> cells overexpressing the ATP-dependent MDR-transporter, the initial TMA-DPH extrusion rate correlates with the amount of probe associated with the cytoplasmic leaflet (Chapter VI), suggesting that LmrA extrudes the TMA-DPH from the inner leaflet of the lipid bilayer. As shown in Fig. 5A, similar results are obtained when L. lactis cells are used that overexpress LmrP. The initial rate of TMA-DPH extrusion increases in the course of probe flipping from the outer to the cytoplasmic leaflet of the membrane, whereas the steady-state TMA-DPH fluorescence level remains the same (Fig. 5A). The initial TMA-DPH extrusion rate increased in the course of probe flipping from the external to the cytoplasmic leaflet of the membrane, whereas the steady state TMA-DPH fluorescence levels reached similar values (Fig. 5A). In pHLP1 ISO membrane vesicles, however, the initial transport rates were identical and independent of the partitioning of TMA-DPH into the internal leaflet of the inverted membrane system. The steady state TMA-DPH fluorescence in this experiment increased along with the partitioning of TMA-DPH into the internal leaflet of the membrane (Fig. 5B). The observed kinetics of TMA-DPH transport in whole cells and ISO membrane vesicles further demonstrate that the rate of TMA-DPH transport depends on the amount of probe associated with the cytoplasmic leaflet of the membrane.

Discussion.

The energetics and mechanism of LmrP-mediated drug extrusion have been studied in membrane vesicles of E. coli DH5α in which LmrP was functionally expressed. The lack of TPP<sup>+</sup> accumulation in pHLP1 RSO membrane vesicles upon generation of a Δp and the restoration of TPP<sup>+</sup> accumulation, up to levels observed in the control RSO membrane vesicles, upon addition of reserpine (Fig. 2) demonstrate that TPP<sup>+</sup> extrusion is LmrP-mediated. In addition, the competition between TPP<sup>+</sup> and ethidium bromide for LmrP-mediated transport (Fig. 3) is in agreement with our previous conclusion that multidrug resistance and transport of various unrelated drugs by L. lactis correlate with the overexpression of LmrP (Chapter III). The slight enhancement of TPP<sup>+</sup> accumulation in the control RSO membrane vesicles upon addition of reserpine points to the presence of a low endogenous TPP<sup>+</sup> transport activity in E. coli. Indeed, genes specifying MDR transporters
such as the miniTEXAN EmrE (Yerushalmi et al., 1995), and the TEXANs EmrAB (Lomovskaya and Lewis, 1992) and AcrAB (Ma et al., 1995) are present in E. coli. Low level expression of one or more of these proteins might be responsible for the extrusion of TPP⁺. Therefore, in general, data obtained from the transmembrane distribution of lipophilic cations like TPP⁺, or other probes that report changes in the Δp (Molenaar et al., 1992), have to be treated with caution as these probes might be recognized by endogenous MDR transporters.

To study the energetics and mechanism of LmrP-mediated drug transport in greater detail, the fluorescent membrane probe TMA-DPH was used as a model substrate. Preliminary studies in whole cells of L. lactis MG1363 expressing LmrP indicated that TMA-DPH is a substrate for LmrP (unpublished results; Fig. 5A). Consistent with this, LmrP-mediated TMA-DPH uptake could be measured in pHLP1 ISO membrane vesicles, while no transport was observed in the control ISO membrane vesicles (Fig. 4). The role of LmrP in the transport of TMA-DPH was confirmed by the complete inhibition of TMA-DPH transport by reserpine. The generation of a Δψ (inside positive) and/or ΔpH (inside acidic) in pHLP1 ISO membrane vesicles resulted in a significant decrease of TMA-DPH fluorescence and indicated that LmrP-mediated drug transport is driven by both components of the Δp. In view of the charge of the transported drugs, being monovalent cationic, the data strongly suggest drug transport via an electrogenic drug/nH⁺ (n≥2) antiport mechanism. A similar mechanism was proposed for the miniTEXANs Smr (Grinius and Goldberg, 1994) and EmrE (Yerushalmi et al., 1995). Surprisingly, this mechanism differs from that of the LmrP homolog TetA, which mediates the electroneutral exchange of a positively charged tetracycline-metal complex for one proton (McMurry et al., 1980, Yamaguchi et al., 1990), despite the fact that LmrP confers resistance to tetracycline as well (unpublished results).

The initial rate of LmrP-dependent TMA-DPH transport correlates with the amount of TMA-DPH in the inner membrane leaflet of whole cells, and with the amount of TMA-DPH in the outer leaflet of ISO membrane vesicles (Fig. 5). Since the outer membrane leaflet of inversely oriented ISO membrane vesicles corresponds to the cytoplasmic leaflet in vivo, both observations rule out the external leaflet of the cytoplasmic membrane as possible site of drug binding to LmrP. Fig. 6 summarizes our current view of LmrP mediated drug transport as depicted for TMA-DPH. The association of TMA-DPH with lipid bilayers and the concomitant fluorescence development has been extensively studied (Chapter VI, Prendergast et al., 1981, Kuhry et al., 1985). Due to the charged TMA-moiety, TMA-DPH is restrictively oriented perpendicular to the surface of the membrane. Since the TMA-DPH
partitioning is highly asymmetric on a time scale of several minutes, in which the fast partitioning of TMA-DPH in the external leaflet of the membrane is followed by a slow passive transbilayer movement of TMA-DPH to the trans face, it was possible to follow the redistribution of the probe over both membrane leaflets.

Fig. 6. Proposed mechanism of LmrP mediated TMA-DPH extrusion.
As was shown previously, the passive release of TMA-DPH from the membrane into the aqueous space is a slow process (Chapter VI), suggesting the presence of an energy barrier at the membrane water interface. This notion is consistent with the slow desorption of phospholipids from the vesicle surface (Bielicki et al., 1991). In terms of MDR, the slow passive release from the membrane of amphiphilic cationic drugs would favour a mechanism in which drugs are directly transported from the cytoplasmic leaflet of the membrane into the external medium. Indeed this is what is observed for the initial LmrP-mediated TMA-DPH transport into ISO membrane vesicles. A fluorescence decrease rather than an increase is observed when LmrP is active (Fig. 5B). The latter would be expected when drugs are actively translocated from the cytoplasmic to the external leaflet of the membrane followed by passive diffusion into the external medium.

LmrP mediated TMA-DPH extrusion from the inner leaflet into the external medium is consistent with the "hydrophobic vacuum cleaner" model (Mülder et al., 1993; Higgins and Gottesman, 1992; Gros et al., 1986; Raviv et al., 1990; Gottesman and Pastan, 1993) which predicts that hydrophobic drugs are most efficiently transported from the compartment where the transporter encounters the highest substrate concentration, i.e., from the membrane. Our studies suggest a role for MDR-type transporters in maintaining membrane integrity, which is important since several hydrophobic compounds are known to be toxic at the membrane level (Barabas et al., 1992, Sikkema et al., 1995). Interestingly, the mechanism of drug extrusion by LmrP is similar to that of the ATP-dependent drug transporter LmrA of L. lactis (Chapter VI). This shows that despite of a different mechanism of energy coupling to drug transport (Δp versus ATP hydrolysis), these non-homologous proteins may share similar structural features involved in drug extrusion from the cytoplasmic leaflet of the membrane. These features are likely to be present in other MDR transporters as well, including the human P-glycoprotein (Smit et al., 1993; Homolya et al., 1993; Ruetz and Gros, 1994; Stein et al., 1994; Shapiro and Ling, 1995a).

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