THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 39, Issue of September 27, pp. 24123–24128, 1996

Printed in U.S.A.

Energetics and Mechanism of Drug Transport Mediated by the Lactococcal Multidrug Transporter LmrP*

(Received for publication, May 17, 1996)

Henk Bolhuis, Hendrik W. van Veen, Jan Roel Brands, Monique Putman, Bert Poolman, Arnold J. M. Driessen, and Wil N. Konings‡

From the Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands

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 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH) into inside-out membrane vesicles are driven by the membrane potential ($\Delta\psi$) 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 multidrug resistance (MDR) transporter occurs by a "hydrophobic vacuum cleaner" mechanism in a similar way as was proposed for the primary lactococcal MDR transporter, LmrA.

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 (1). Active drug extrusion can be mediated by specific drug resistance (SDR)[1] transporters as well as by multidrug resistance (MDR) transporters; the latter systems confer resistance to a broad range of unrelated toxic compounds (2).

To date, several bacterial drug extrusion systems have been identified and characterized at the genetic level (for reviews, see Refs. 1 and 3). The bacterial drug extrusion systems can be divided into: (i) secondary drug transporters which mediate drug extrusion in a coupled exchange with protons (4) and (ii) ATP-binding cassette-type drug transporters that utilize the free energy of ATP hydrolysis to extrude cytotoxic substrates (5, 6). The secondary drug transporters are subdivided into two groups on the basis of their similarity in size and secondary structure (7). The largest subgroup, termed TEXANs (toxin-extruding antiporter), consists of integral membrane proteins with 12–14 putative transmembrane-spanning segments and an average molecular mass of 45–50 kDa (3, 8–10). The second subgroup comprises the mini-TEXANs, which share functional similarity with the TEXANs but are much smaller (12–15 kDa) and form only four putative transmembrane α-helices (11, 12).

In *Lactococcus lactis*, two distinct transport systems (LmrP and LmrA) have been identified which mediate active extrusion of multiple cationic drugs (13). LmrP is a secondary drug transporter comprising 408 amino acid residues with 12 putative membrane-spanning segments (14). 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 family of drug transporters.[2] 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 are united.

The involvement of the Δp 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 carbonyl cyanide *p*-chlorophenylhydrazone and on their structural similarity to other secondary transport systems (16–18). We have now studied the energetics and mechanism of drug transport by LmrP in membrane vesicles of *Escherichia 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 I. *L. lactis* strains were grown at 30 °C on M17 medium (Difco) supplemented with glucose (25 mM) and erythromycin (5 μg/ml) when needed. *E. coli* was grown aerobically at 37 °C on Luria Broth (19) with carbenicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside when needed.

DNA Manipulation and Construction of H-LmrP—General proce-

* This research was funded by a grant from the biotechnology (BIOTECH) program of the EC-Science Foundation (BIO2-CT93-0145). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 31-50-363-21-50; Fax: 31-50-363-21-54; E-mail: w.n.konings@biol.rug.nl.

[1] The abbreviations used are: SDR, specific drug resistance; MDR, multidrug resistance; TEXAN, toxin-extruding antiporter; ISO, inside-out; RSO, right-side-out; Ni-NTA, Ni^{2+}-nitrilotriacetic acid; TPP^+, tetraphenyl phosphonium; TMA-DPH, 1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene.

[2] van Veen, H. W., Venema, K., Bolhuis, H., Oussenko, I., Kok, J., Poolman, B., Driessen, A. J. M., and Konings, W. N. (1996) *Proc. Natl. Acad. Sci. U. S. A.*, in press.

TABLE I
Bacterial strains and plasmids

Bacterium/plasmid	Relevant characteristics	Source/Refs.
Bacterium		
L. lactis		
MG1363	ML3, plasmid free, *Lac*$^-$, *Prt*	(47)
E. coli		
DH5α	*supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *rel*A1, ΔLacU169 (*80 LacZΔM15)	Life Technologies, Inc.
Plasmids		
pBluescript SKII$^-$	Ap^R, expression vector	Stratagene
pSKLMR3.2	pBluescript SKII$^-$, carrying *ImnP* of *L. lactis* on a 3.2-kb *Hin*dIII fragment	(14)
pGK13	Em^R, Cm^R, *E. coli-L. lactis* shuttle vector	(15)
pGKLMR3.2	pGK13, carrying *ImnP* of *L. lactis* on a 3.2-kb *Hin*dIII fragment	(14)
pTRC99A	Ap^R, pBR322 derivative containing *trc* promoter	Pharmacia
pET302	pTRC99A carrying a His-tag coding region and *trc* promoter	van der Does, submitted for publication
pHLP1	pET302 carrying *ImnP* on a 1.390-kb *Nco*I-*Xba*I fragment	This work

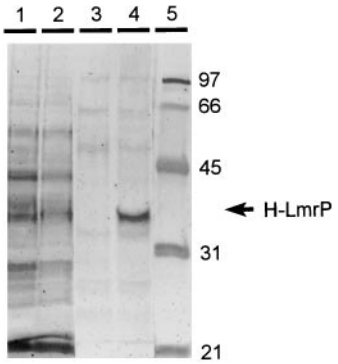

FIG. 1. **Expression of H-LmrP in *E. coli* and identification of the protein.** Silver-stained 10% SDS-PAGE containing samples of total membranes (*lanes 1* and *2*) and Ni-NTA eluates (*lanes 3* and *4*) from cells harboring the control vector pET302 (*lanes 1* and *3*) or the H-LmrP-encoding vector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

dures for cloning and DNA manipulations were performed essentially as described by Sambrook *et al*. (1989). In order to subclone *lmrP*, the gene was amplified from the chromosome of *L. lactis* MG1363 via the polymerase chain reaction as described previously (14). The amino-terminal polyhistidine (His_6)-tag containing LmrP derivative H-LmrP was constructed by subcloning the *lmrP* containing polymerase chain reaction 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* DH5α. The different cloning steps were checked by restriction analysis and double-stranded DNA sequencing using the dideoxy chain-termination procedure (20) and the T7 DNA sequencing kit (Pharmacia Biotech Inc.).

Preparation of Membrane Vesicles—E. coli DH5α was grown aerobically at 37 °C to an A_{660} of about 0.8 on Luria Broth supplemented with carbenicillin (50 μg/ml) plus isopropyl-β-D-thiogalactopyranoside (100 μg/ml) when appropriate. Inside-out (ISO) (21) and right-side-out (RSO) (22) 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 (Ni^{2+}-nitrilotriacetic acid)-agarose affinity chromatography (Qiagen Inc.). Membrane vesicles (5 mg of protein) were solubilized in 3.5 ml of 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 of 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 (23).

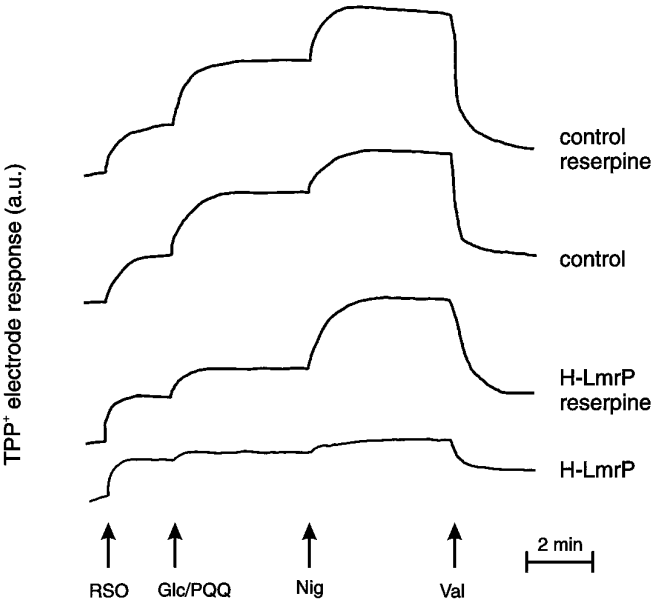

FIG. 2. **TPP^+ accumulation in right-side-out membrane vesicles of *E. coli* DH5α.** The accumulation of TPP^+ in RSO membrane vesicles of *E. coli* DH5α/pET302 (control) and DH5α/pHLP1 (H-LmrP) was followed by recording the external free TPP^+ concentration with an ion selective electrode. Measurements were carried out in potassium phosphate (50 mM (pH 6.8)) containing 5 mM $MgSO_4$ plus 4 μM TPP^+ at 30 °C. The additions of RSO membrane vesicles (0.3 mg of protein/ml), 2 μM of pyrroloquinoline quinone plus 10 mM of glucose, 1 μM of nigericin, and 1 μM of valinomycin are indicated by *arrows*. Reserpine was included in the phosphate buffer at a final concentration of 10 μg/ml when indicated in the figure.

TMA-DPH Transport in ISO Membrane Vesicles—The amount of membrane associated TMA-DPH (Molecular Probes Inc., Eugene, OR) was measured fluorimetrically as described (24). ISO membrane vesicles prepared from *E. coli* DH5α (0.4 mg of protein/ml) were suspended in oxygen-saturated 50 mM potassium HEPES (pH 7.5) containing 25 mM K_2SO_4 plus 5 mM $MgSO_4$. A Δp (inside positive and acidic) was generated by the oxidation of D-lactate via a constitutively expressed, membrane-associated lactate dehydrogenase (25). 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—TPP^+ accumulation in RSO membrane vesicles was calculated from the external free probe concentration as recorded by a TPP^+-selective electrode (26). 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 $MgSO_4$ to a final concentration of 0.3 mg of protein/ml. A Δp (inside negative and alkaline) was generated by oxidation of glucose (10 mM) via the membrane bound pyrroloquinoline quinone-dependent glucose dehydrogenase of *E. coli* (25). Pyrroloquinoline quinone was added to a final concentration of 1 μM.

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.

H-LmrP-mediated TPP$^+$ Extrusion—To asses the functional expression of H-LmrP in *E. coli*, TPP$^+$ efflux by isolated RSO membrane vesicles was monitored using an ion selective electrode. Generation of a $\Delta\psi$ (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 $\Delta\psi$-driven passive influx of TPP$^+$ was much lower in RSO membrane vesicles containing H-LmrP (Fig. 2; pHLP1). Importantly, measurements of $\Delta\psi$ by the fluorescent probe $DiSC_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide) 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. An enhanced $\Delta\psi$-driven uptake of TPP$^+$ was observed in pHLP1 RSO membrane vesicles in the presence of reserpine (Fig. 2), a known inhibitor of LmrP and other MDR transporters (14, 27). The TPP$^+$ accumulation in the presence of reserpine was similar for the control and H-LmrP containing RSO membrane vesicles. 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 (14). 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*.

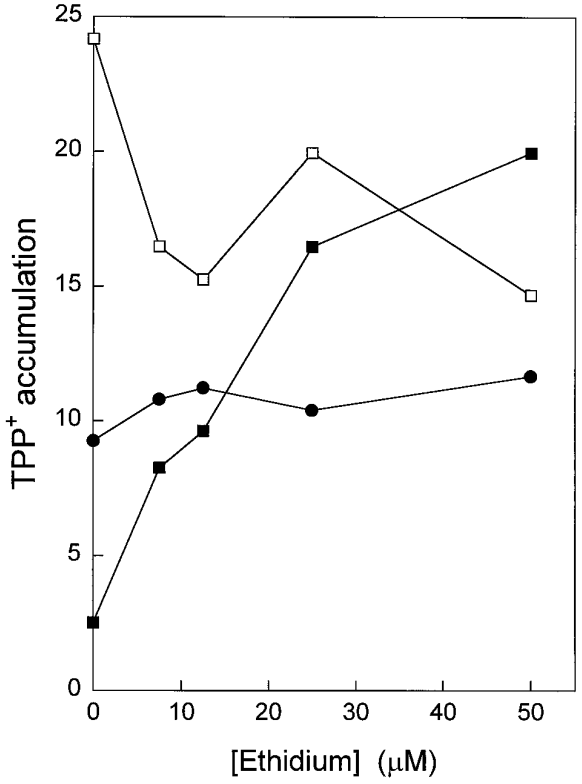

FIG. 3. **Ethidium bromide and TPP$^+$ compete for LmrP-mediated extrusion.** TPP$^+$ accumulation as function of the ethidium concentration was assayed in H-LmrP containing pHLP1 RSO membrane vesicles (■), pHLP1 RSO membrane vesicles plus reserpine (□), and in the control pET302 RSO membrane vesicles (●) 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.

Energetics of LmrP-mediated Drug Transport—The H-LmrP-mediated extrusion of TPP$^+$ in the presence of nigericin (Fig. 2) indicates that the $\Delta\psi$ 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 $2.4 * 10^5$), which is only fluorescent when present in the membrane (28). 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 (29). 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

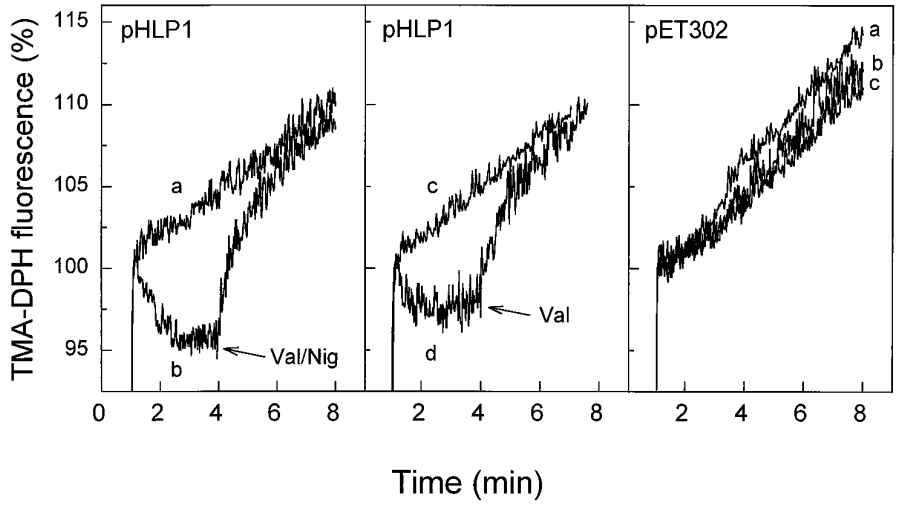

FIG. 4. **TMA-DPH transport in inside-out membrane vesicles of *E. coli* DH5α.** Uptake of TMA-DPH (100 nM, final concentration) was followed fluorimetrically in an aerated buffer containing ISO membrane vesicles (0.4 mg of protein/ml) of *E. coli* DH5α harboring pHLP1 or pET302. The fluorescence development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

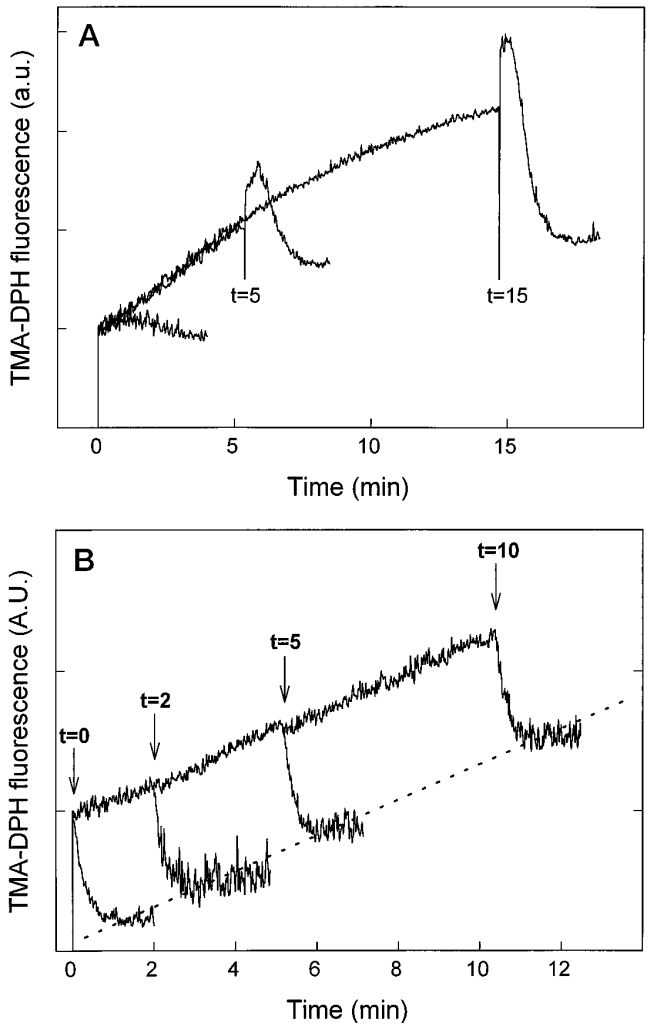

FIG. 5. **Rate of energy-dependent TMA-DPH extrusion in the course of TMA-DPH partitioning in the phospholipid bilayer.** Energy-dependent TMA-DPH fluorescence development in cells of *L. lactis* MG1363/pGKLMR3.2 (*A*) and in ISO membrane vesicles of *E. coli* DH5α/pHLP1 (*B*). Cells and ISO membrane vesicles were energized with 25 mM of glucose or 10 mM of D(L)-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.

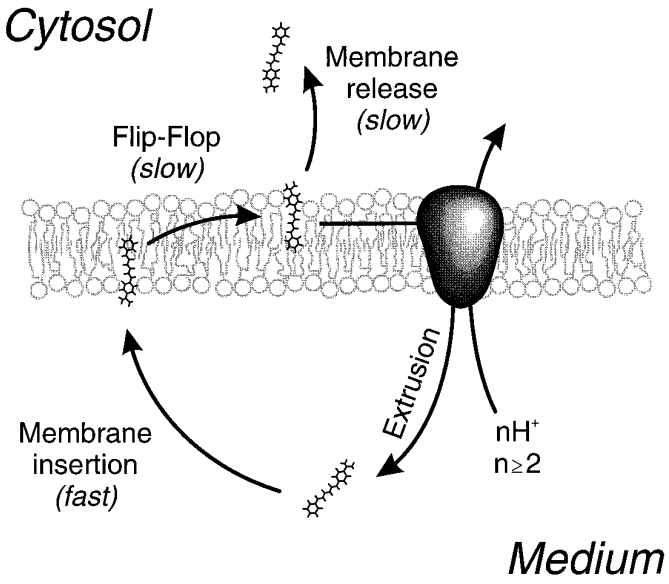

FIG. 6. **Proposed mechanism of LmrP-mediated TMA-DPH extrusion.**

transport into the intravesicular space was partially inhibited upon dissipation of the ΔpH by nigericin or by dissipation of the $\Delta\psi$ 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 $\Delta\psi$ and the ΔpH function as a driving force of LmrP-mediated drug transport.

Kinetics of LmrP-mediated TMA-DPH Transport—The kinetics of TMA-DPH fluorescence development upon the addition to cells is biphasic (24) (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^R cells overexpressing the ATP-dependent MDR-transporter, the initial TMA-DPH extrusion rate correlates with the amount of probe associated with the cytoplasmic leaflet (24), suggesting that LmrA extrudes the TMA-DPH from the inner leaflet of the lipid bilayer. As shown in Fig. 5*A*, 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. 5*A*). 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. 5*A*). 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. 5*B*). 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^+ accumulation in pHLP1 RSO membrane vesicles upon generation of a Δp and the restoration of TPP^+ accumulation, up to levels observed in the control RSO membrane vesicles, upon addition of reserpine (Fig. 2) demonstrate that TPP^+ extrusion is LmrP-mediated. In addition, the competition between TPP^+ 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 (14). The slight enhancement of TPP^+ accumulation in the control RSO membrane vesicles upon addition of reserpine points to the presence of a low endogenous TPP^+ transport activity in *E. coli*. Indeed, genes specifying MDR transporters such as the mini-TEXAN EmrE (11) and the TEXANs EmrAB (18) and AcrAB (30) 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 (31), 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 (Fig. 5*A*).[3] 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 $\Delta\psi$ (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^+ ($n \geq 2$) antiport mechanism. A similar mechanism was proposed for the mini-TEXANs Smr (12) and EmrE (11). 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 (32, 33), despite the fact that LmrP confers resistance to tetracycline as well.[3]

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 (24, 28, 29). 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. As was shown previously, the passive release of TMA-DPH from the membrane into the aqueous space is a slow process (24), 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 (34). In terms of MDR, the slow passive release from the membrane of amphiphilic cationic drugs would favor 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. 5*B*). 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 (35–39) 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 (40, 41). Interestingly, the mechanism of drug extrusion by LmrP is similar to that of the ATP-dependent drug transporter LmrA of *L. lactis* (24). This shows that despite of a different mechanism of energy coupling to drug transport (Δp *versus* ATP hydrolysis), these nonhomologous 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 (42–46).

Acknowledgment—We thank C. van der Does for providing plasmid pET302.

[3] H. Bolhuis, H. W. van Veen, J. R. Brands, M. Putman, B. Poolman, A. J. M. Driessen, and W. N. Konings, unpublished results.

REFERENCES

1. Nikaido, H. (1994) *Science* **264,** 382–388
2. Endicott, J. A., and Ling, V. (1989) *Annu. Rev. Biochem.* **58,** 137–171
3. van Veen, H. W., Bolhuis, H., Putman, M., and Konings, W. N. (1996a) in *Handbook of Biological Physics* (Konings, W. N., Kaback, H. R., and Lolkema, J. S., eds) Vol. II, Elsevier Science Publishers B. V., Amsterdam, in press
4. Paulsen, I. T., and Skurray, R. A. (1993) *Gene* (*Amst.*) **124,** 1–11
5. Fath, M. J., and Kolter, R. (1993) *Microbiol. Rev.* **57,** 995–1017
6. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8,** 67–113
7. Schuldiner, S., Shirvan, A., and Linial, M. (1995) *Physiol. Rev.* **75,** 369–392
8. Lewis, K. (1994) *Trends Biochem. Sci.* **17,** 18–21
9. Marger, M. D., and Saier, M. H., Jr. (1993) *Trends Biochem. Sci.* **18,** 13–20
10. Saier, M. H., Jr., Tam, R., Reizer, A., and Reizer, J. (1994) *Mol. Microbiol.* **11,** 841–847
11. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1995) *J. Biol. Chem.* **270,** 6856–6863
12. Grinius, L. L., and Goldberg, E. B. (1994) *J. Biol. Chem.* **269,** 29998–30004
13. Bolhuis, H., Molenaar, D., Poelarends, G., van Veen, H. W., Poolman, B., Driessen, A. J. M., and Konings, W. N. (1994) *J. Bacteriol.* **176,** 6957–6964
14. Bolhuis, H., Poelarends, G., van Veen, H. W., Poolman, B., Driessen, A. J. M., and Konings, W. N. (1995) *J. Biol. Chem.* **270,** 26092–26098
15. Kok, J., van der Vossen, J. M. B. M., and Venema, G. (1984) *Appl. Environ. Microbiol.* **48,** 726–731
16. Neyfakh, A. A., Bidnenko, V. E., and Chen, L. B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4781–4785
17. Yoshida, H., Bogaki, M., Nakamura, S., Ubukata, K., and Konno, M. (1990) *J. Bacteriol.* **172,** 6942–6949
18. Lomovskaya, O., and Lewis, K. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8938–8942
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
21. Ambudkar, S. V., Zlotnick, G. W., and Rosen, B. P. (1984) *J. Biol. Chem.* **259,** 6142–6146
22. Kaback, H. R. (1971) *Methods Enzymol.* **31,** 698–709
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Bolhuis, H., Molenaar, D., Van Veen, H. W., Poolman, B., Driessen, A. J. M., and Konings, W. N. (1996) *EMBO J.*, in press
25. van Schie, B. J., Hellingwerf, K. J., van Dijken, J. P., Elferink, M. G. L., van Dijl, J. M., Kuenen, J. G., and Konings, W. N. (1985) *J. Bacteriol.* **163,** 493–499
26. Shinbo, T., Kama, N., Kurihara, K., and Kobataka, Y. (1978) *Arch. Biochem. Biophys.* **187,** 414–422
27. Ahmed, M., Borsch, C. M., Neyfakh, A. A., and Schuldiner, S. (1993) *J. Biol. Chem.* **268,** 11086–11089
28. Prendergast, F. G., Haugland, R. P., and Callahan, P. J. (1981) *Biochemistry* **20,** 7333–7338
29. Kuhry, J. G., Duportal, G., Bronner, C., and Laustriat, G. (1985) *Biochim. Biophys. Acta* **845,** 60–67
30. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., and Hearst, J. E. (1995) *Mol. Microbiol.* **16,** 45–55
31. Molenaar, D., Bolhuis, H., Abee, T., Poolman, B., and Konings, W. N. (1992) *J. Bacteriol.* **174,** 3118–3124
32. McMurry, L., Petrucci, R. E., and Levy, S. B. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 3974–3977
33. Yamaguchi, A., Udagawa, T., and Sawai, T. (1990) *J. Biol. Chem.* **265,** 4809–4813
34. Bielicki, J. K., Johnson, W. J., Glick, J. M., and Rothblat, G. H. (1991) *Biochim. Biophys. Acta* **1084,** 7–14
35. Mülder, H. S., van Grondelle, R., Westerhoff, H. V., and Lankelma, J. (1993) *Eur. J. Biochem.* **218,** 871–882
36. Higgins, C. F., and Gottesman, M. M. (1992) *Trends Biochem. Sci.* **17,** 18–21
37. Gros, P., Croop, J., and Housman, D. (1986) *Cell* **47,** 371–380
38. Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I., and Gottesman, M. M. (1990) *J. Biol. Chem.* **265,** 3975–3980

39. Gottesman, M. M., and Pastan, I. (1993) *Annu. Rev. Biochem.* **62,** 385–427
40. Barabas, K., Sizensky, J. A., and Faulk, W. P. (1992) *J. Biol. Chem.* **267,** 9437–9442
41. Sikkema, J., de Bont, J. A. M., and Poolman, B. (1995) *Microbiol. Rev.* **59,** 201–222
42. Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) *Cell* **75,** 451–462
43. Homolya, L., Holló, Z., Germann, U. A., Pastan, I., Gottesman, M. M., and Sarkadi, B. (1993) *J. Biol. Chem.* **268,** 21493–21496
44. Ruetz, S., and Gros, P. (1994) *Cell* **77,** 1071–1081
45. Stein, W. D., Cardarelli, C., Pastan, I., and Gottesman, M. M. (1994) *Mol. Pharmacol.* **45,** 763–772
46. Shapiro, A. B., and Ling, V. (1995) *J. Biol. Chem.* **270,** 16167–16175
47. Gasson, M. J. (1983) *J. Bacteriol.* **154,** 1–9