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

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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 tetrathenyl 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 were driven by the membrane potential (ΔΨ) and the transmembrane proton gradient (ΔpH), pointing to an electrogenic drug/proton antiporter 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)³ 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 a-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. 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 (15–18). 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-thio-galactopyranoside when needed.

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

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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, Ni²⁺-nitrilotriacetic acid; TPP⁺, tetrathenyl phosphonium; TMA-DPH, 1-[4-(trimethylamino)-phenyl]-6-phenylhexa-1,3,5-triene.

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**TABLE I**

**Bacterial strains and plasmids**

| Bacterium/plasmid | Relevant characteristics | Source/Refs. |
|-------------------|--------------------------|-------------|
| **Bacterium** | | |
| *L. lactis* | ML3, plasmid free, *lac*′, *prt* | (47) |
| *E. coli* | supE44, hsdR17, recA1, endA1, gyrA96, thi-1, relA1, ΔlacU169 (*80 LacZΔM15*) | Life Technologies, Inc. |
| **Plasmids** | | |
| pBluescript SKII | Ap<sup>R</sup>, expression vector | Stratagene |
| pSKLMR3.2 | pBluescript SKII<sup>−</sup>, carrying *lmrP* of *L. lactis* on a 3.2-kb HindIII fragment | (14) |
| pGK13 | Em<sup>R</sup>, Cm<sup>R</sup>, *E. coli-L. lactis* shuttle vector | (15) |
| pSKLMR3.2 | pGK13, carrying *lmrP* of *L. lactis* on a 3.2-kb HindIII fragment | (14) |
| pTBC99A | Ap<sup>R</sup>, pBluescript SKII<sup>−</sup> derivative containing *trc* promoter | Pharmacia |
| pET302 | pTBRc22 carrying a His-tag coding region and *trc* promoter | van der Does, submitted for publication |
| pHLP1 | pET302 carrying *lmrP* on a 1.390-kb *NcoI*-XhoI fragment | This work |

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**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.

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**Fig. 2.** TPP<sup>+</sup> accumulation in right-side-out membrane vesicles of *E. coli* DH5α. The accumulation of TPP<sup>+</sup> in RSO membrane vesicles of *E. coli* DH5<sup>α</sup>/pET302 (control) and DH5<sup>α</sup>/pHLP1 (H-LmrP) was followed by recording the external free *TPP*<sup>+</sup> concentration with an ion selective electrode. Measurements were carried out in potassium phosphate (50 mM (pH 6.8)) containing 5 mM MgSO<sub>4</sub> plus 4 μM TPP<sup>+</sup> at 30 °C. The additions of RSO membrane vesicles (0.3 mg of protein/ml), 2 μM of pyrroloquinoline quinone plus 10 μM 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.

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**TMA-DPH Transport in ISO Membrane Vesicles**—The amount of membrane-associated TMA-DPH (Molecular Probes Inc., Eugene, OR) was measured fluorometrically 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<sub>2</sub>SO<sub>4</sub> plus 5 mM MgSO<sub>4</sub>. A Δp (inside positive and alkaline) was generated by the oxidation of β-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<sup>+</sup> Accumulation in RSO Membrane Vesicles**—TPP<sup>+</sup> accumulation in RSO membrane vesicles was calculated from the external free probe concentration as recorded by a TPP<sup>+</sup>-selective electrode (26). In competition experiments, ethidium bromide was added prior to TPP<sup>+</sup> to correct for changes in the electrode output. RSO membrane vesicles were resuspended in potassium phosphate (pH 6.8) containing 5 mM MgSO<sub>4</sub> 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 assess 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 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 DiSC₃(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 Δψ-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.

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 inverted 4p (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 * 10⁶), 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...
transport into the intravesicular space was partially inhibited upon dissipation of the \( \Delta p \)H by nigericin or by dissipation of the \( \Delta p \) by the potassium ionophore valinomycin. Complete inhibition of TMA-DPH transport was observed by (i) total dissipation of the \( \Delta p \) 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 \( \Delta p \) or addition of reserpine (Fig. 4). These experiments clearly demonstrate that both the \( \Delta p \) and the \( \Delta p \)H 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 \(^{\%}\) 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. 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* DH5\(\alpha\) in which LmrP was functionally expressed. The lack of TPP\(^{\%}\) accumulation in pHLP1 RSO membrane vesicles upon generation of a \( \Delta p \) 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 \( \Delta p \) (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. 5A)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 ∆ψ (inside positive) and/or ∆pH (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 ∆. In view of the charge of the transported drugs, being monovalent cationic, the data strongly suggest drug transport via an electrogenic drug/H+ (n ≥ 2) antiport mechanism. A similar mechanism was proposed for the mini-TEX-ANs Smr (12) and EmrE (11). Surprisingly, this mechanism differs from that of the LmrP homolog TetA, which mediates ANs Smr (12) and EmrE (11). This mechanism corresponds to the cytoplasmic leaflet of the membrane. Our studies suggest a "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 (∆pH 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).

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