Facilitated Drug Influx by an Energy-uncoupled Secondary Multidrug Transporter

The majority of bacterial multidrug resistance transporters belong to the class of secondary transporters. LmrP is a proton/drug antiporter of Lactococcus lactis that extrudes positively charged lipophilic substrates from the inner leaflet of the membrane to the external medium. This study shows that LmrP is a true secondary transporter. In the absence of a proton motive force, LmrP facilitates downhill fluxes of ethidium in both directions. These fluxes are inhibited by other substrates of LmrP. The cysteine-reactive agent p-chloromercuribenzenesulfonate inhibits these fluxes in wild type LmrP but not in the cysteine-less LmrP C270A mutant. Cysteine mutagenesis of LmrP resulted in three mutants, D68C/C270A, D128C/C270A, and E327C/C270A, with an energy-uncoupled phenotype. Asp128 is located in the periplasmic domain of the major facilitator superfamily of secondary transporters and was found to play an important role in energy coupling, whereas the negatively charged residues Asp128 and Glu327 have indirect effects on the transport process. L. lactis strains expressing these uncoupled mutants of LmrP show an increased rate of ethidium influx and an increased drug susceptibility compared with cells harboring an empty vector. The rate of influx in these mutants is enhanced by a transmembrane electrical potential, inside negative. These observations suggest a new strategy for eliminating drug-resistant microbial pathogens, i.e. the design and use of modulators of secondary multidrug resistance transporters that uncouple drug efflux from proton influx, thereby allowing transmembrane electrical potential-driven influx of cationic drugs.

Multidrug resistance transporters (MDRs) play a crucial role in the resistance of prokaryotic and eukaryotic cells against cytotoxic compounds. In bacteria, including pathogens, the activities of MDRs contribute to the resistance against antibiotics (1–4). The control of spread and the prevention of selection of multidrug resistant strains has become a critical issue in the battle against contagious diseases (5). To fight antibiotic resistance efficiently, detailed knowledge of the molecular mechanisms underlying microbial drug resistance is required. Most bacterial MDRs known to date are secondary transporters. For example, LmrP is a proton motive force (pmf)-driven MDR of Lactococcus lactis (6). LmrP is a member of the major facilitator superfamily of membrane proteins and shows homology to Bmr and NorA, the MDRs of Bacillus subtilis (7) and Staphylococcus aureus (8), respectively, as well as to the transporters involved in tetracycline resistance and bicyclomycin resistance in Escherichia coli (9). Cells expressing LmrP have increased resistance to several clinically important antibiotics from different pharmacological classes (10), which indicates the frightening possibility that its homologues in pathogenic bacteria may reduce the efficacy of important antibiotics in clinical settings.

In this study we report that LmrP is a true secondary transporter and is able to mediate active efflux and, under non-energized conditions, downhill drug influx and efflux. We show that residue Asp128, located in the cytoplasmic loop between transmembrane segments (TMS) II and III, is involved in the energy-coupling mechanism but not in substrate binding, whereas Asp126 in the cytoplasmic loop between TMS IV and V is not participating in the binding of ethidium either, but it might be part of the structural framework responsible for retaining a correct folding of the transporter during the transport cycle. Glu327 appears not to be involved either in the binding of ethidium or the coupling of proton flux to ethidium transport. Energy-uncoupled mutants of LmrP were constructed, which showed increased rates of transmembrane electrical potential (Δψ)-driven influx of cationic drugs. The expression of such uncoupled secondary MDRs is disadvantageous and causes increased drug susceptibility to the host cells. This observation may be useful for developing alternative and more efficient strategies of antibiotic therapies.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—L. lactis NZ9000 (∆lmrA) (11), which lacks the gene encoding ATP-binding cassette-type MDR transporter LmrA (a kind gift from O. Gajic and J. Kok, Department of Genetics, University of Groningen), was used in combination with the NICE system (12, 13) for overexpression of wild type LmrP (WT LmrP) and the mutant proteins (14, 15). L. lactis cells were grown at 30 °C in M17 medium (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol. For the growth experiments, the M17 medium was prepared in 50 mM potassium phosphate (pH 7). Expression of LmrP variants from pNZ8048-derived plasmids was induced by adding 10 ng of nisin A per milliliter at an A600 of 0.6, and cells were harvested 60 min after induction.

Recombinant DNA Techniques—General procedures for cloning and DNA manipulation were performed essentially as described by Sambrook et al. (16). The PCR overlap extension method (17) was used to introduce mutations in the lmrP gene on the pHIL8 expression plasmid (14), which encodes LmrP with a C-terminal His tag. All PCR-amplified DNA fragments were sequenced to verify that only the intended changes were introduced. DNA sequencing was performed at the Bio-

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Fig. 1. Secondary structure model of LmrP. Acidic residues and the native cysteine are indicated. The model is based on the hydropathy profile of the amino acid sequence, the distribution of the arginine and lysine residues according to the “positive inside rule” (6), and the accessibility of cysteine mutants for membrane-impermeant, cysteine-reactive agents (15). Circed letters are single-letter amino acid designations accompanied by their position numbers.

Fig. 2. Influence of pCMBS on transport of Hoechst 33342 in inside-out membrane vesicles prepared from cells expressing the WT and the cysteine-less variant (C270A) of LmrP. Membrane vesicles (20 mg of protein per milliliter) were incubated with or without 0.5 mM pCMBS for 5 min at 30°C. Vesicles were subsequently washed once with 50 mM potassium phosphate, pH 7, and suspended to 0.5 mg protein/ml in the buffer for Hoechst 33342 transport (see “Experimental Procedures”). Hoechst 33342 (1 μM) was added, and its binding to the membrane vesicles was followed in time until a steady state was reached. Hoechst 33342 transport was initiated by the addition of 0.5 mM Mg-ATP. Dotted lines, WT LmrP; dashed lines, mutant C270A; minus sign (−), not treated with pCMBS; and plus sign (+), treated with pCMBS.

RESULTS

Wild Type LmrP Is Sensitive to pCMBS—Wild type LmrP contains one native cysteine residue, Cys270, which is not accessible to the cysteine-reactive agent fluorescein maleimide and is most likely located in the putative TMS VIII (Fig. 1) (15). In a previous report (15), a cysteine-less mutant, C270A, was described that retained significant transport activity and was expressed to similar levels as wild type LmrP. To evaluate the role of Cys270, the effects of several cysteine-reactive agents were tested on LmrP-mediated transport of Hoechst 33342 and ethidium. These compounds are good substrates of LmrP and can be used for transport studies in whole cells and inside-out membrane vesicles (6, 14, 19).

Hoechst 33342 is highly fluorescent when present in the lipid environment of membranes but essentially non-fluorescent in the aqueous phase. Transport of Hoechst 33342 from the membrane into the aqueous phase can therefore be followed in membrane vesicles by monitoring the Hoechst 33342 fluorescence over time. The addition of ATP to inside-out membrane vesicles of L. lactis results in the generation of a pmf through proton pumping by the F$_{1}$F$_{0}$-ATPase. This pmf drives the transport of Hoechst 33342 by LmrP out of the membrane as indicated by the decrease in Hoechst 33342 fluorescence (Fig. 2). Wild type LmrP-mediated Hoechst 33342 transport was not affected by 2-[trimethylammonium]ethyl]methanethiosulfon-
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Fig. 3. Fluxes of ethidium in energized and non-energized cells of *L. lactis*. Binding of ethidium to the nucleic acids was followed fluorometrically. *A*, accumulation of ethidium under energized conditions. Cells were pre-energized for 2 min in the presence of 25 mM glucose, after which 10 μM ethidium was added. *B*, influx of ethidium into non-energized cells. *C*, efflux of ethidium from non-energized cells. The cells were pre-loaded with ethidium (25 μM) for 30 min at 30 °C, spun down, suspended in 100 μl of buffer, and immediately transferred to the measurement cuvette. *D*, effect of pCMBS on influx of ethidium into non-energized cells. Cells were challenged with 0.5 mM pCMBS for 5 min at 30 °C, washed twice with 50 mM potassium phosphate, pH 7, and then used for measurements of ethidium transport. *E*, the influence of pCMBS on the efflux of ethidium from non-energized cells. After loading with ethidium, 0.5 mM pCMBS was added to the cell suspension followed by 5 min of incubation at 30 °C and two washings with 50 mM potassium phosphate, pH 7, and then used for measurements of ethidium transport. *F*, inhibition of efflux of ethidium from preloaded cells by verapamil. After loading with ethidium, 1 μM verapamil was added to the cell suspension followed by 5 min of incubation at 30 °C. The cells were washed twice with 50 mM potassium phosphate, pH 7, containing 1 μM verapamil and then used for measurements of ethidium efflux in the presence of 1 μM verapamil. Solid lines, cells harboring empty vector; dotted lines, cells expressing WT LmrP; dashed lines, cells expressing mutant C270A; minus sign (−), not treated with pCMBS; plus sign (+), treated with pCMBS; and A.U., arbitrary units.

Fig. 4. Effect of expression of WT and mutants of LmrP on ethidium accumulation in energized *L. lactis* cells. The cells were pre-energized for 2 min in the presence of 25 mM glucose, after which 10 μM ethidium was added. Solid line, cells harboring empty vector; dotted line, cells expressing WT LmrP; dash-dot line, cells expressing mutant D68/C/C270A; long dashed line, cells expressing mutant D128/C/C270A; and dash-double dot line, cells expressing E327/C/C270A mutant. A.U., arbitrary units.
cells than from ethidium-preloaded WT LmrP and LmrP C270A mutant-expressing cells (Fig. 3C). The rates of the ethidium fluxes were proportional to the level of expression of LmrP (data not shown). These observations demonstrate that LmrP facilitates the flux of ethidium across the membrane in the absence of a pmf. Additional evidence that the ethidium flux is, to a large extent, LmrP-mediated was obtained by treatment of cells with pCMBS. pCMBS did not affect ethidium fluxes in control cells not expressing LmrP or in cells expressing C270A mutant LmrP, whereas it decreased drastically the fluxes of ethidium in cells expressing the WT LmrP (Figs. 2 and 3, D and E). These ethidium fluxes were inhibited by tetraphenylphosphonium, a well-known substrate of LmrP (22), and the inhibitor verapamil (23), providing further support for the proposal that LmrP mediates facilitated fluxes of ethidium in non-energized cells (Fig. 3F and data not shown).

Energy-uncoupled Mutants of LmrP Also Facilitate Drug Influx in Energized Cells—Recently, the interesting observation was made that energized cells of L. lactis expressing variants of LmrP C270A with a single cysteine substitution 2 (D68C/C270A, D128C/C270A, or E327C/C270A) accumulated ethidium rapidly (Fig. 4) (15), whereas WT LmrP extrudes ethidium under these conditions. The ethidium influx rates into these cells were comparable with the influx rates observed in non-energized cells expressing WT LmrP (Fig. 3B). These data indicate that the cysteine mutants D68C/C270A, D128C/C270A, and E327C/C270A are energy-uncoupled and capable of catalyzing only a facilitated diffusion of substrates.

It is noteworthy that the influx rates into cells expressing these mutant proteins are slightly lower under non-energized conditions than under energized conditions (compare Figs. 4 and 5), which is consistent with the notion that the Δψ (inside negative) accelerates the facilitated diffusion process of this positively charged substrate.

Sensitivity of Single Cysteine Mutants to pCMBS—The effects of pCMBS on ethidium fluxes in L. lactis cells expressing the single cysteine mutants of LmrP were also investigated (Fig. 5). pCMBS (0.5 mM) strongly inhibited the influx and efflux of ethidium in cells expressing the D68C/C270A mutant or WT LmrP (Fig. 5, A and D). In contrast, pCMBS did not inhibit either influx or efflux of ethidium in cells expressing D128C/C270A (Fig. 5, B and E) or E327C/C270A (Fig. 5, C and F) mutants. Labeling of residues D128C/C270A and E327C/C270A by fluorescein maleimide could be prevented by preincubation of protein with pCMBS, demonstrating that both residues are accessible to this mercurial (data not shown).

Phenotype of D68C, D128C, and E327C Mutants of LmrP—To investigate whether the observed uncoupled phenotype of the D68C/C270A, D128C/C270A, and E327C/C270A mutants was caused by changing two residues in LmrP, the mutations giving an uncoupled phenotype in the cysteine-less background of the mutant C270A were also introduced into WT LmrP possessing Cys 270. All three mutants were expressed to levels similar to that of WT LmrP (data not shown). The D68C mutant retained the uncoupled phenotype of the D68C/C270A mutant, whereas the D128C mutant catalyzed facilitated diffusion of ethidium into energized cells, but to a lower extent than the D128C/C270A mutant did. Unexpectedly, the E327C mutant exhibited a high transport activity of ethidium (Fig. 6), indicating that both mutations are responsible for the uncoupled phenotype of the E327C/C270A mutant.

Cells Expressing the Energy-uncoupled Mutants of LmrP Are More Susceptible to Ethidium—Because the LmrP mutants D68C/C270A, D128C/C270A, and E327C/C270A facilitate influx of ethidium, cells expressing these mutants should be more sensitive to drugs than nonexpressing control cells. To test this hypothesis, we followed the effects of ethidium on the growth of L. lactis NZ9000 (∆lmrA) harboring the empty vector pNZ8048 or its derivatives encoding WT LmrP or mutants D270A, D68C/C270A, D128C/C270A, and E327C/C270A (Fig. 7). Concentrations of ethidium that resulted in a 50% reduction of the growth rates (IC 50) are given in Table I. For cells harboring the empty

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2 It should be noted that the annotation of the cysteine mutants used in this manuscript differs from the annotation used in Ref. 15 (D68C/C270A was D68C, D128C/C270A was D128C, and E327C/C270A was E327C).
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Fig. 6. Ethidium accumulation in energized L. lactis cells expressing WT and mutants of LmrP containing the endogenous Cys270. The cells were pre-energized for 2 min in the presence of 25 mM glucose, after which 10 μM ethidium was added. Solid line, cells harboring empty vector; dotted line, cells expressing WT LmrP; dash-dot line, cells expressing mutant D68C; long dashed line, cells expressing mutant D128C; dash-double dot line, cells expressing E327C mutant; and A.U., arbitrary units.

Fig. 7. Resistance of L. lactis NZ9000 (ΔlmrA) to ethidium. Cells carrying empty vector pNZ8048 (filled circle), plasmids coding for WT LmrP (inverted triangle), and the mutants C270A (square), D68C/C270A (diamond), D128C/C270A (triangle), and E327C/C270A (pentagon) were grown in M17 medium in the presence of different concentrations of ethidium. The relative growth rate is plotted as a function of the drug concentration. The data are from two independent experiments.

Table I

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<th>Drug Influx via a Multidrug Transporter</th>
<th>Ethidium susceptibilities of L. lactis NZ9000 (ΔlmrA) harboring vector pNZ8048 and its derivatives encoding WT LmrP and the mutants C270A, D68C/C270A, D128C/C270A, and E327C/C270A</th>
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<td>Data are ± S.E. and represent the average of two independent experiments.</td>
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<td><strong>IC</strong>&lt;sub&gt;50&lt;/sub&gt;</td>
<td><strong>μM</strong></td>
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<tr>
<td>Empty vector</td>
<td>6.5 ± 0.2</td>
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<td>WT LmrP</td>
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<td>D68C/C270A</td>
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<td>E327C/C270A</td>
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Ethidium influx of non-energized cells expressing the cysteine-less mutant C270A. The rate of ethidium influx of non-energized cells (Fig. 3) and the efflux of ethidium out of cells expressing mutant D68C, D128C/C270A, and E327C/C270A were significantly more sensitive to ethidium than the control cells not expressing LmrP and displayed IC<sub>50</sub> values of ~1.9, 2, and 3.2 μM, respectively.

DISCUSSION

In this study the sensitivity of WT LmrP to pCMBS was used to demonstrate that LmrP plays a major role in the high fluxes of ethidium across the membranes under non-energized conditions. Ethidium accumulation was followed by measuring fluorescence of the cellular ethidium-poly nucleotide complex. The influx of ethidium into (Fig. 3D) and the efflux of ethidium out of non-energized cells (Fig. 3E) were blocked upon the incubation of cells with pCMBS in cells expressing WT LmrP but not in cells expressing the cysteine-less mutant C270A. The rate of facilitated diffusion of ethidium under non-energized conditions increased with the expression levels of LmrP, and the ethidium fluxes are inhibited by other substrates of LmrP. Furthermore, we have generated LmrP mutants that are energy-uncoupled. These mutants facilitate rapid entry and exit of drugs into and out of the cell but are unable to secrete the drugs against their concentration gradient. These mutants resemble previously described energy-uncoupled mutants of the lactose permease (24, 25) and demonstrate that LmrP behaves as a true secondary transporter capable of mediating facilitated diffusion of substrates in both directions, depending on the direction of the substrate gradient.

Cells expressing a single cysteine variant of C270A LmrP (D68C/C270A, D128C/C270A, or E327C/C270A) rapidly accumulate ethidium under both non-energized and energized conditions (Figs. 4 and 5, A–C) and clearly exhibit an energy-uncoupled phenotype. However, the influx of ethidium is slower under non-energized conditions than under energized conditions, suggesting that the Δϕ (inside negative) pulls cationic drugs into the cells (20, 21). These data also demonstrate that the negatively charged residues Asp<sup>68</sup>, Asp<sup>128</sup>, and Glu<sup>327</sup> are not essential for ethidium binding. pCMBS inhibited the facilitated diffusion of ethidium in the D68C/C270A mutant, but not in the D128C/C270A and E327C/C270A mutants (Fig. 5). Because these residues are all accessible to pCMBS (data not shown), it appears that Asp<sup>68</sup> and Glu<sup>327</sup> are not in close proximity to either the binding site for ethidium or the substrate translocation pathway. To determine whether the uncoupled behavior of the single cysteine variants D68C/C270A, D128C/C270A, and E327C/C270A of LmrP is due to introduction of two mutations, i.e. an acidic amino acid to cysteine and the native Cys<sup>270</sup> to alanine, an additional set of mutants was constructed, namely D68C, D128C, and E327C using WT LmrP as the template. One of these mutants, D68C, catalyzed the facilitated diffusion of ethidium into energized cells (Fig. 6), confirming a role of the Asp<sup>68</sup> in the energy-coupling mechanism. Asp<sup>68</sup> is part of the conserved motif, GXXXD/E(R/K)XGRK, located in the cytoplasmic loop between TMS II and TMS III of the major facilitator superfamily of secondary transporters that are involved in the transport of a variety of compounds (26). The function of this motif has been extensively studied in other transporters. It is of interest to note that, for the tetracycline transporter TetA(B), it has been demonstrated that Asp<sup>68</sup> (homologous to Asp<sup>68</sup> in LmrP) does not directly participate in substrate binding but is part of the gating mechanism of TetA(B) (27). Moreover, N-ethylmaleimide labeling of a cysteine residue introduced next to the Asp<sup>68</sup> in TetA(B) inhibited tetracycline transport, further suggesting that this region is essential for coupling transport to the pmf. In the E. coli lactose permease (LacY), substitutions of the conserved Asp<sup>68</sup> almost completely abolish transport activity of lactose permease (28, 29). However, these mutants have not been reported to be uncoupled and display very low rates of downhill transport of lactose (28, 29). Conservation of this motif among members of the major facilitator superfamily suggests that this region is not directly involved in substrate binding but, instead, may be critical in facilitating conformational changes following...
substrate binding (30). The motif is connected with TMS II, which undergoes a conformational change upon interaction of lactose permease with ligands (25, 31) and faces the substrate binding cavity (32). We hypothesize that mutation D68C alters the structure of the loop connecting TMS II and TMS III and further disturbs the orientation of these two TMSs, thereby interfering with the coupling between the $H^+$ flux and substrate translocation. The D128C mutant appeared to be less efficient in facilitating influx of ethidium into energized cells than the D128C/C270A mutant (compare Figs. 4 and 6). At this stage, the function of Asp128 remains obscure. For the TetA(B) transporter, it has been proposed that Asp128 (corresponding to Asp128 in LmrP) forms an ion pair with an arginine residue present in a loop region between TMS II and TMS III, thereby ensuring a correct structure of the protein (33, 34). The third mutant, E327C, extrudes ethidium efficiently (Fig. 6), ruling out a role of Glu327 in proton translocation or ethidium binding. However, the presence of a negative charge at this location is critical for the interaction of LmrP with the divalent cation Hoechst 33342 (15), suggesting that Glu327 is part of the substrate binding pocket of LmrP. The two mutations in the E327C/C270A mutant thus seem to affect the conformation in this binding region in such a way that pmf-driven transport of ethidium and Hoechst 33342 is no longer possible. In summary, the uncoupled phenotype of mutants D68C and D128C is most likely the result of structural perturbations within LmrP, and the uncoupled phenotype of mutants D68C and D128C is most likely membrane-embedded. In this report we tested several other cysteine-reactive compounds to probe the role of Cys270, but only pCMBS was found to inhibit the transport activity of WT LmrP. Because pCMBS had no effect on the activity of the cysteine-less mutant C270A, we conclude that this mercurial targets Cys270. This residue is likely located in a transmembrane region of the protein that is poorly accessible to the water phase, because the maleimide probe CMBS could be due to the location of Cys270 close to a substrate binding site or to the translocation pathways of substrates and/or protons. A location in the substrate binding site seems unlikely, because the affinity of the C270A mutant for Hoechst 33342 is unchanged upon reaction with pCMBS. Alternatively, pCMBS-labeling of Cys270 may interfere with conformational changes of LmrP during the transport cycle. It is noteworthy that the significant Hoechst 33342 and ethidium transport activity of the C270A mutant excludes a direct role of Cys270 in proton translocation. One conclusion of our work is that the presence of a secondary WT MDR, such as LmrP, causes the rapid influx of drugs under non-energized conditions. This observation demonstrates that LmrP is a proper secondary transporter and mediates active transport and, in the absence of pmf, downhill fluxes of substrates. These modes of transport are typical for secondary transporters and have been described previously (37, 38). Notably, we report here a new important finding that expression of an uncoupled MDR increases the sensitivity of cells to ethidium even under energized conditions. Clearly, expression of such an energy-uncoupled secondary MDR allows a membrane potential-driven accumulation of a toxic cationic 3 P. Mazurkiewicz, unpublished results.