Multidrug and peptide export in Lactococcus lactis
Berg van Saparoea, Hendrik Bart van den

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Chapter 3

Proton motive force-dependent Hoechst 33342-transport by the ABC transporter LmrA of *Lactococcus lactis*

H. Bart van den Berg van Saparoea, Jacek Lubelski, Ronald van Merkerk, Piotr S. Mazurkiewicz, and Arnold J.M. Driessen

Summary

The fluorescent compound Hoechst 33342 is a substrate for many multidrug resistance (MDR) transporters and is widely used to characterize their transport activity. We have constructed mutants of the adenosine triphosphate (ATP) binding cassette (ABC)-type MDR transporter LmrA of *Lactococcus lactis* that are defective in ATP hydrolysis. These mutants and wild-type LmrA showed an atypical behavior in the Hoechst 33342-transport assay. In membrane vesicles, Hoechst 33342-transport was shown to be independent of the ATPase activity of LmrA, and it was not inhibited by orthovanadate but sensitive to uncouplers that collapse the proton gradient and to N,N’-dicyclohexylcarbodiimide, an inhibitor of the F$_0$F$_1$-ATPase. In contrast, transport of Hoechst 33342 by the homologous, heterodimeric MDR transporter LmrCD showed a normal ATP dependence and was insensitive to uncouplers of the proton gradient. With intact cells, expression of LmrA resulted in an increased rate of Hoechst 33342-influx while LmrCD caused a decrease in the rate of Hoechst 33342-influx. Cellular toxicity assays using a triple knockout strain, i.e., *L. lactis* ΔlmrA ΔlmrCD, demonstrate that expression of LmrCD protects cells against the growth inhibitory effects of Hoechst 33342, while in the presence of LmrA, cells are more susceptible to Hoechst 33342. Our data demonstrate that the LmrA-mediated Hoechst 33342-transport in membrane vesicles is influenced by the transmembrane pH gradient due to a pH-dependent partitioning of Hoechst 33342 into the membrane.

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Introduction

The bisbenzimide dye Hoechst 33342 is a cell membrane-permeant DNA stain that exhibits a bright blue fluorescence upon binding to DNA. In Hoechst 33342-stained murine lymphocyte cells, it was observed that the fluorescence intensity is not always related to DNA content (102). This led to the suggestion that membranes may exhibit different permeabilities for Hoechst 33342. Involvement of drug resistance proteins followed from experiments with colchicine-resistant Chinese hamster ovary cell lines (102). Cell lines with an increased resistance to colchicine concomitantly showed a decreased uptake of the dye. At a later stage, Hoechst 33342 was identified as a substrate for P-glycoprotein (P-gp) (103).

Hoechst 33342 is not only fluorescent when bound to DNA but also exhibits an increased fluorescence when it partitions into the hydrophobic environment of the lipid membrane. Since Hoechst 33342 is essentially nonfluorescent in an aqueous environment, (net) transport of the compound from the membrane to the aqueous phase will result in a decrease in fluorescence. This has been the basis of Hoechst 33342-transport assays performed with reconstituted proteoliposomes and membrane vesicles derived from cells. Transport of Hoechst 33342 can be continuously monitored by the changes in fluorescence. The Hoechst 33342-transport assay was first used to show transport activity by reconstituted P-gp (103) and with plasma membrane vesicles derived from highly multidrug resistant cells that express high levels of P-gp (104-106). The assay has been widely used for the analysis of bacterial multidrug transporters, like BmrA from Bacillus subtilis (107), HorA from Lactobacillus brevis (108), MdfA from Escherichia coli (109), NorA from Staphylococcus aureus (110), and LmrP, LmrA and LmrCD from Lactococcus lactis (4;26;32). Likewise, Hoechst 33342 can also be used for binding studies (8).

To study the self-association of the LmrA dimer and the presumed cooperativity between the nucleotide binding domains, LmrA-mutants defective in ATPase activity have been constructed. As expected, these mutations resulted in an inactivation of ATPase activity. Remarkably, the ATPase mutants of LmrA showed Hoechst 33342-transport. Here we show that LmrA mediates transmembrane passage of Hoechst 33342 in a pH-dependent manner.

Experimental procedures

Bacterial strains, plasmids, and growth conditions. Lactococcus lactis NZ9000 (ΔlmrA), an lmrA deletion strain (49), and NZ9000 (ΔlmrA ΔlmrCD) (44) were used as a host for pNZ8048-derived plasmids that permit expression of wild-type and mutant His-tagged LmrA and Strep-tagged LmrCD proteins under control of the nisin system (NICE) (Table 1) (111). Cells were grown in M17 medium (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/mL chloramphenicol.

DNA manipulation. General procedures for cloning and DNA manipulation were performed essentially as described by Sambrook et al. (112). For
site-directed mutagenesis to create the nucleotide binding site-mutants G490A and E512Q of LmrA, the following primers were used: 5'-ggagtcaaaattttctggtgcacaaagacaacg-3' (G490A-FWD), 5'-cgtgtgctttgtgcaccagaaattttgactcc-3' (G490A-REV), 5'-ctaatgcttgcatcaagcaacagc-3' (E512Q-FWD), 5'-gctgttgctttgatcaagcattag-3' (E512Q-REV), 5'-gattttgctttacgatgattctgaacaaatattgc-3' [“upstream” primer (EcoRV)] and 5'-ggttttctaattttggttcaaagaaagcttgagc-3' [“downstream” primer (XbaI)]. The PCR overlap extension method ([113]) was used to introduce the G490A (codon “gca” instead of “gga” on the DNA level) and E512Q (codon “caa” instead of “gaa” on the DNA level) in the \textit{lmrA} gene on the expression plasmid pNHLmrA yielding pHLA490A and pHLA512Q, respectively (Table 3.1).

**Table 3.1: \textit{L. lactis} plasmids**

<table>
<thead>
<tr>
<th>plasmid</th>
<th>relevant characteristics</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vector pNZ8048</td>
<td>expression vector carrying the inducible ( P_{\text{nlsA}} ), \text{Cm}^f</td>
<td>111</td>
</tr>
<tr>
<td>pNHLmrA</td>
<td>pNZ8048 harboring the \textit{lmrA} gene with upstream regions encoding an N-terminal six-histidine tag and an enterokinase cleavage site</td>
<td>32</td>
</tr>
<tr>
<td>pHLA490A</td>
<td>pNHLmrA encoding G490A ATPase mutant of LmrA</td>
<td>this study</td>
</tr>
<tr>
<td>pHLA512Q</td>
<td>pNHLmrA encoding E512Q ATPase mutant of LmrA</td>
<td>this study</td>
</tr>
<tr>
<td>pNSGA</td>
<td>pNZ8048 harboring the \textit{LmrCD} gene with a downstream region encoding a Streptag II</td>
<td>4</td>
</tr>
</tbody>
</table>

Preparation of inside-out membrane vesicles. Inside-out membrane vesicles were prepared from \textit{L. lactis} NZ9000 \( \Delta \text{lmrA} \) cells harboring pNZ8048-based expression or control vectors. Cells were grown at 30 °C to an \( \text{OD}_{660} \) of 0.6 - 0.8, whereupon 0.5 ng/mL of Nisin A was added. Growth was continued for a further 90 min. Cells were harvested by centrifugation, washed with 100 mM Hepes-KOH (pH 7.0), and resuspended in the same buffer. Inside-out membrane vesicles were prepared by French pressure cell treatment as described previously ([32]). Membrane vesicles suspended in 100 mM Hepes-KOH (pH 7.0) containing 10% (v/v) glycerol were stored at -80 °C until they were used.

**DCCD treatment of membrane vesicles.** To inactivate the \( \text{F}_0\text{F}_1\)-ATPase, membranes (10 mg/mL total protein) were incubated for 30 min with 10 mM \( N,N'\)-dicyclohexylcarbodiimide (DCCD) on ice. Membrane vesicles were collected by centrifugation, resuspended in 50 mM Hepes-KOH (pH 7.0) containing 10% (v/v) glycerol, and stored at -80 °C.

**Purification of His\_LmrA.** Membranes (10 - 20 mg/mL total protein) bearing overexpressed histidine-tagged wild-type or mutant LmrA were solubilized in 50 mM Hepes-KOH (pH 8.0), containing 0.3 M NaCl, 10% (v/v) glycerol and 1% (w/v) \( n\)-dodecyl-\( \beta\)-D-maltoside (DDM). The suspension was mixed and incubated on ice for 30 min. Insoluble material was removed by centrifugation at 280,000g (20 min at 4 °C). Solubilized membrane proteins were mixed with
Ni\textsuperscript{2+}-NTA agarose (Qiagen, \textasciitilde 25 µL of resin/mg of protein) which was pre-equilibrated in buffer A [50 mM Hepes-KOH (pH 8.0), 0.3 M NaCl, 10% (v/v) glycerol, and 0.05% (w/v) DDM] containing 20 mM imidazole. The suspension was incubated for 1 h at 4 °C, transferred to a Bio-spin column (Bio-Rad), washed with 20 column volumes of buffer A containing 20 mM imidazole and eluted with buffer A (pH 7.0) containing 250 mM imidazole (all at 4 °C). The purified protein was used immediately for reconstitution.

Reconstitution of LmrA into liposomes. E. coli total lipid extract (Avanti Polar Lipids) was washed with an acetone/ether mixture (114), dried, and resuspended in 50 mM Hepes-KOH (pH 7.2) at a concentration of 20 mg/mL. The suspension was frozen in liquid nitrogen, slowly thawed at room temperature, and sonicated on ice using a tip sonicator at an intensity of 4 µm (peak to peak) for four cycles of sonication for 15 s and resting for 45 s. Aliquots of 2 mL were frozen in liquid nitrogen and slowly thawed at room temperature. This freeze-thaw step was repeated once, and aliquots of the liposome suspension were frozen in liquid nitrogen and stored at -80 °C. To obtain unilamellar liposomes with a relatively homogeneous size, the frozen liposome suspension was slowly thawed at room temperature and extruded 11 times through a 400 nm polycarbonate filter. After dilution to 4 mg of lipid/mL, liposomes were saturated with DDM and solubilization was monitored at OD\textsubscript{450} as described by Paternostre et al. (115). Purified LmrA was mixed with DDM-saturated liposomes (1 µmol of DDM/mg of lipid) at a 1/20 ratio (w/w) and incubated for 30 min at room temperature under gentle agitation. The detergent was removed by two successive extractions with polystyrene beads (Bio-Beads SM-2, Bio-Rad, extensively washed with methanol, ethanol and water), first at a wet weight of 80 mg/mL of liposome suspension for 2 h at room temperature and then at a wet weight of 160 mg/mL of liposome suspension overnight at 4 °C, under gentle agitation. The proteoliposomes were harvested by centrifugation (280,000 g for 30 min at 4 °C), resuspended in 50 mM Hepes-KOH (pH 7.4) at a final concentration of 1 mg/mL, frozen in liquid nitrogen, and stored at -80 °C.

ATPase assay. The ATPase activity of reconstituted LmrA was determined using the colorimetric assay of Lanzetta et al. (116). Proteoliposomes were incubated at 30 °C in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NH\textsubscript{4}Cl, 5 mM MgSO\textsubscript{4}, 15 mM MgCl\textsubscript{2}, and 1 mM ATP. At regular time intervals, samples of 30 µL were transferred to a 96-well microplate and 150 µL of malachite green molybdate reagent was added. After 5 min, 34% citric acid was added. Absorbance at 600 nm was measured after incubation for 50 min at room temperature and compared with that of a phosphorus standard.

Hoechst 33342-transport assays. The transport activity of LmrA and LmrCD was assayed by means of the drug Hoechst 33342 [2’-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5’-bi-1H-benzimidazole, Molecular Probes] (4;32). Membrane vesicles: Inside-out membrane vesicles (0.5 mg of protein/mL) were suspended in 50 mM Hepes-KOH (pH 7.4) containing 2 mM MgSO\textsubscript{4}, 8.5 mM NaCl, 5 mM phosphocreatine, and 0.1 mg/mL creatine kinase. After incubation for 1 min at
30 °C, 0.5 μM of Hoechst 33342 was added. Once the fluorescence signal was stable, 2 mM of Mg\textsuperscript{2+}-ATP was added and the Hoechst 33342 fluorescence was recorded over time.

Cells: L. lactis NZ9000 ΔlmrA cells harboring pNZ8048-based expression or control vectors were grown at 30 °C to an OD\textsubscript{660} of 0.6-0.8, whereupon 0.5 ng/mL of nisin A was added. Growth was continued for a further 90 min. The cells were harvested and resuspended in 50 mM potassium phosphate (pH 7.0) containing 5 mM MgSO\textsubscript{4}. Dinitrophenol was added to 0.5 mM, and the cells were incubated for 30 min at 30 °C. The cells were washed four times with 50 mM potassium phosphate (pH 7.0) containing 5 mM MgSO\textsubscript{4}, resuspended in the same buffer to an OD\textsubscript{660} of 5, and kept on ice. For the actual experiment, the cells were diluted to an OD\textsubscript{660} of 0.5 in the same buffer at 30 °C. Hoechst 33342 was added to a concentration of 0.5 μM. All measurements were performed with a PerkinElmer Life Sciences model 50B fluorometer with a magnetic stirred holder at 30 °C using emission and excitation wavelengths of 355 and 457 nm, respectively.

*pH dependence of Hoechst 33342 fluorescence.* Extruded liposomes (20 μL) in 50 mM Hepes-KOH (pH 7.2) at a concentration of 20 mg/mL were diluted in 2 mL of a buffer with an appropriate pH (50 mM Bis-Tris for pH 5.2, 6.0 and 6.5, and 50 mM Hepes-KOH for pH 7.0, 7.5 and 7.8), and then 1 μM nigericin and 1 μM Hoechst 33342 were added. For comparison, Hoechst 33342 fluorescence measurements were performed in the absence of liposomes. All data were acquired with a PerkinElmer Life Sciences model 50B fluorometer with a magnetic stirred holder at 20 °C using emission and excitation wavelengths of 355 and 457 nm, respectively.

Hoechst 33342-resistance assay. To biologically monitor the activity of the MDR transporters, the growth rates of L. lactis NZ9000 ΔlmrA ΔlmrCD cells overexpressing LmrA, LmrCD, or the empty vector control in the presence of various concentrations of Hoechst 33342 were recorded. Overnight cultures were diluted 20 times into fresh medium and grown for 1 h. Expression was induced by addition of 0.1 ng/mL nisin A, and cells were grown for an additional hour. The induced cells were diluted to an OD\textsubscript{660} of 0.05, and 150 μL aliquots were transferred to a 96-well microplate containing 50 μL of various concentrations of Hoechst 33342 in growth medium containing 0.1 ng/mL Nisin A. Silicon oil (50 μL) was placed on top of the samples to prevent evaporation. Cells were grown at 30 °C, and the cell density was monitored by measuring the OD\textsubscript{660} every 6 min for 10 h in a microplate reader (Molecular Devices, Thermomax).

**Results**

Construction, expression and ATPase activity of LmrA mutants. To study the self-association of the LmrA dimer and the presumed cooperativity between the nucleotide binding domains (NBD), two conserved amino acids that are critical for ATP hydrolysis were replaced by site-directed mutagenesis. This concerned substitution of the glycine at position 490 in the ABC signature motif with an
alanine, and replacement of the glutamic acid at position 512, the putative catalytic base in the ATPase reaction, with glutamine. These residues have been shown to be critical in other ABC transporters, and their mutagenesis normally results in an abolishment of both the ATPase and transport activity (117-119). The individual mutants (G490A and E512Q LmrA) were overexpressed with an N-terminal His-tag, purified to homogeneity, and reconstituted into proteoliposomes composed of \textit{E. coli} lipids. The ATPase activity of LmrA and the two mutants was determined by monitoring the release of inorganic phosphate. The ATPase activity of both mutants was less than 3% of that observed for wild-type LmrA (Figure 3.1), demonstrating that the mutations in the conserved NBD motifs inactivate the LmrA ATPase activity.

\textit{Hoechst 33342}-transport activity of LmrA ATPase mutants. To determine if the inactivation of the ATPase activity of LmrA also interferes with the ATP-dependent expulsion of drugs, the transport of Hoechst 33342 was examined. Hoechst 33342 is a substrate for LmrA (31;32;37) and many other MDR transporters (107;110). Transport of this compound in inside-out membrane vesicles can be readily monitored by its fluorescence properties. In aqueous solution, Hoechst 33342 is essentially nonfluorescent, whereas partitioning into the membrane results in a significant increase in the fluorescence (103). Addition of Hoechst 33342 to \textit{L. lactis} membrane vesicles indeed results in a marked increase in fluorescence (Figure 3.2). Upon addition of ATP, membrane vesicles harboring elevated levels of wild-type LmrA showed a high Hoechst 33342-transport activity as evidenced by a rapid fluorescence quenching (Figure 3.2A). In contrast, \textit{ΔlmrA} membranes show only a very small level of fluorescence quenching. Surprisingly, membranes bearing the inactive G490A and E512Q ATPase mutants of LmrA showed a remarkably high level of ATP-dependent quenching of Hoechst 33342 fluorescence (Figure 3.2A). Whereas the transport activity of the G490A LmrA mutant was indistinguishable from the wild-type LmrA, the activity of the E512Q LmrA mutant was partially impaired.
Figure 3.2. Hoechst 33342-transport in inside-out membrane vesicles prepared from control-, LmrA-, and LmrCD-expressing cells. Hoechst 33342 was added (H) to inside-out membrane vesicles, and transport was initiated by the addition of 2 mM Mg2+-ATP (ATP). (A) Inside-out membrane vesicles prepared from cells expressing LmrA (4), G490A LmrA (5), and E512Q LmrA (3), or control cells (2). Experiments were performed in the presence of 1 µM valinomycin. As indicated, at the end of the experiment, 2 µM nigericin was added. When nigericin was included in the assay before transport was initiated by the addition of ATP, only a low residual Hoechst 33342-transport activity was observed (1). For clarity, only the trace for inside-out membrane vesicles prepared from cells expressing E512Q mutant LmrA is shown. (B) Inside-out membrane vesicles prepared from cells expressing LmrA (2) or from control cells (1). As indicated, 25 mM methylammonium chloride was added. (C) Inside-out membrane vesicles prepared from LmrCD-expressing (2-5) or control (1) cells. Transport was assayed under standard conditions (1 and 5), and in the presence of 1 µM valinomycin (4), 1 µM nigericin (3), or 0.1 mM orthovanadate (2). (D) Inside-out membrane vesicles prepared from cells expressing wild-type LmrCD (3), double ATPase mutants of LmrC and LmrD, D495N and E587Q, respectively (2) and control cells (1).

Effect of ATPase inhibitors on the Hoechst 33342-transport activity of LmrA. Orthovanadate (VO$_4^{3-}$) is an inhibitor of several ATPase enzymes, including P-gp (120) and other ABC transporters (121;122). The ATPase activity of LmrA is also inhibited by orthovanadate (Figure 3.1). At 0.1 mM orthovanadate, less than 3% of the ATPase activity remained. Next, the effect of orthovanadate on
LmrA-mediated Hoechst 33342-transport was assayed in membrane vesicles. At a concentration of orthovanadate that nearly completely abolished the ATPase activity of LmrA, essentially no effect was observed on the transport of Hoechst 33342 when ATP was added to the membrane vesicles (data not shown).

**Effect of ionophores on the Hoechst 33342-transport activity of LmrA.** In the inside-out membrane system, ATP drives not only the LmrA-dependent transport of drugs but also proton influx by the H+-translocating $F_0 F_1$-ATPase, resulting in the generating a proton motive force (pmf), inside acidic and positive. Since LmrA is an ATP-dependent transporter, its activity is not expected to be influenced by dissipation of the pmf by ionophores. Nigericin is an ionophore that mediates the electroneutral exchange of K⁺ for H⁺, thereby collapsing the transmembrane pH gradient in these membrane vesicles. Addition of this ionophore completely reversed the LmrA-dependent Hoechst 33342-transport activity (Figure 3.2A). When nigericin was included in the assay before transport was initiated by the addition of ATP, only a low residual Hoechst 33342-transport activity was observed (Figure 3.2A). Addition of the ionophore valinomycin, which mediates electrogenic K⁺ movements, thereby collapsing the transmembrane electrical potential, had no effect. Transport of Hoechst 33342 by the two LmrA ATPase mutants was also completely inhibited by nigericin. An alternative explanation for the nigericin sensitivity of the Hoechst 33342-transport activity of LmrA is that this ionophore is a substrate of the transporter. It may compete with Hoechst 33342 for binding and transport and thereby block the transport. This hypothesis, however, then assumes that LmrA discriminates between nigericin and valinomycin, another peptide ionophore that has no effect on Hoechst 33342-transport. For that reason we also used an alternative means to dissipate the ΔpH, employing the weak base methylammonium. Addition of 25 mM methylammonium completely reversed the LmrA-dependent Hoechst 33342-transport activity (Figure 3.2B). Taken together, these data suggest that LmrA-dependent Hoechst 33342-transport in membrane vesicles is dependent on the transmembrane pH gradient.

**Figure 3.3. Effect of DCCD on LmrA-mediated Hoechst 33342-transport in membrane vesicles.** Hoechst 33342 was added (H) to inside-out membrane vesicles prepared from control cells (2 and 3) or LmrA-expressing cells (1 and 4) that were treated with DCCD (1 and 2) or mock-treated (3 and 4). The system was energized by the addition of 2 mM Mg²⁺-ATP (ATP).
Hoechst 33342-transport activity of LmrCD. We have recently described another MDR transporter of L. lactis termed LmrC and LmrD (4). These are ABC half-transporters that constitute a functional heterodimeric ABC-type MDR transporter. In membrane vesicles, LmrCD shows a high ATP-dependent transport activity of Hoechst 33342 (4) (Figure 3.2C). In contrast to the LmrA-dependent activity, transport of Hoechst 33342 by LmrCD was only marginally affected by the ionophore nigericin and strongly inhibited by orthovanadate (Figure 3.2C). Likewise, we generated ATPase mutants of LmrC and LmrD, D495N and E587Q, respectively (44). When the double mutant LmrCD proteins were expressed, no Hoechst 33342-transport activity was observed (Figure 3.2D). This shows that LmrCD functions as a typical ATP-dependent MDR transporter, whereas the Hoechst 33342-transport activity observed with LmrA is apparently dependent on the pH gradient.

Effect of \( F_0F_1 \)-ATPase inhibition on the LmrA-dependent Hoechst 33342-transport in membrane vesicles. Since proton motive force generation in membrane vesicles in the presence of ATP requires the activity of the \( F_0F_1 \)-ATPase, we next tested the effect of \( N,N' \)-dicyclohexylcarbodiimide (DCCD), an effective inhibitor of the \( H^+ \) translocation activity of the \( F_0 \) domain (123). The ATPase activity of purified LmrA was not affected by 10 mM DCCD (Figure 3.1). However, treatment of the membrane vesicles with 10 mM DCCD completely abolished the ATP-dependent Hoechst 33342-transport activity of LmrA (Figure 3.3). Taken together, these data strongly suggest that LmrA-mediated transport of Hoechst 33342 is not dependent on the LmrA ATPase activity, but that it is mediated through the transmembrane pH gradient which in the membrane vesicles is generated by the \( F_0F_1 \)-ATPase.

pH-dependent fluorescence of Hoechst 33342 in lipid vesicles. Acidification of the lumen of the membrane vesicles by the activity of \( F_0F_1 \)-ATPase may influence the Hoechst 33342 fluorescence in the transport assay. To determine the influence of the pH on the fluorescence of Hoechst 33342, we have analyzed Hoechst 33342 fluorescence in buffers of different pH values, both in the absence and in the presence of liposomes. Hoechst 33342 has a complex, pH-dependent spectrum, and when the dye is dissolved in a buffer, the fluorescence quantum yield is much higher at pH 5.0 than at pH 8.0 (Figure 3.4, triangles) (124). When liposomes are added to the Hoechst 33342 solution, the fluorescence increases by at least 2 orders of magnitude. However, now an inverted pH-dependence of the fluorescence is observed (Figure 3.4, circles). The pH-dependence of the dye fluorescence in aqueous solution suggests that the compound undergoes protonation and/or deprotonation of the nitrogen atoms in the piperazinyl and benzimidazole rings. Although our experiments do not directly measure partitioning, the increase of Hoechst 33342 fluorescence with an increase in pH in the presence of membranes is most likely caused by a more efficient partitioning of deprotonated and less charged Hoechst 33342 into the lipid membrane.

Transport of Hoechst 33342 in intact cells. The Hoechst 33342-transport assay in membrane vesicles essentially monitors repartitioning of Hoechst 33342 from...
the membrane to the aqueous phase. This assay does not reveal the direction of transport per se; i.e., the Hoechst 33342 may either be transported into the lumen of the vesicles or be expelled into the suspending medium. In cells, Hoechst 33342 becomes highly fluorescent when it intercalates with the DNA. This feature can be used to monitor Hoechst 33342-transport in intact cells as excretion should result in a shift of the equilibrium from the DNA to the free, soluble state of Hoechst 33342. In energy-depleted cells of *L. lactis* bearing overexpressed levels of LmrA, a rapid influx of Hoechst 33342 was observed (Figure 3.5) that was barely distinguishable from the influx of Hoechst 33342 into control cells. When cells were energized with glucose, we reproducibly observed that the Hoechst 33342-influx into cells overexpressing LmrA occurred with a faster rate than in control cells. These data unequivocally show that LmrA does not expel Hoechst

**Figure 3.4. pH-dependence of the Hoechst 33342 fluorescence.**
The fluorescence of free Hoechst 33342 (1 µM) in buffer (triangles, scale on the right axis) and in the presence of 0.2 mg/mL liposomes and 1 µM nigericin (circles, scale on the left axis). a.u. means arbitrary units. The data points represent the average of 30 s readings recorded after stabilization of the fluorescence signal of Hoechst 33342.

**Figure 3.5. Influx of Hoechst 33342 into cells overexpressing LmrA.**
Influx of Hoechst 33342 into washed, energy-deprived cells expressing LmrA (1) or control cells (2) or cells re-energized with 25 mM glucose for 3 min (3 and 4 respectively) was assayed in time. Hoechst 33342 (H) was added to a final concentration of 0.5 µM.
Hoechst 33342-resistance of cells overexpressing LmrA or LmrCD. Hoechst 33342-transport in intact cells was further studied in a cytotoxicity assay. For this assay, we have used a strain that lacks both LmrA and LmrCD, i.e., *L. lactis* NZ9000 Δ*mra* Δ*mrcd*. This strain is like the individual Δ*mrcd* strain that is hypersensitive to Hoechst 33342 and other drugs (14). Unlike the parental strain, *L. lactis* NZ9000 Δ*mra* Δ*mrcd* is readily killed by low concentrations of Hoechst 33342. LmrA or LmrCD was expressed, and the Hoechst 33342-resistance of the cells was compared with that of control cells that were transformed with the empty vector. Expression of LmrA and LmrCD was confirmed by western blotting (data not shown). Figure 3.6 shows the growth rate as a function of the Hoechst 33342 concentration. The growth rates of cells expressing LmrCD are similar to those of the parental *L. lactis* NZ9000 cells harboring the empty vector, showing that expression of LmrCD in the (triple) deletion mutant completely rescues the hypersensitive phenotype of the cells towards Hoechst 33342. No protection was observed when LmrA was expressed. Rather, we reproducibly found cells to be more sensitive to the drug in the presence of LmrA. Moreover, the individual *L. lactis* NZ9000 Δ*mra* strain (49) exhibited a Hoechst 33342-resistance similar to that of the parental strain (data not shown). Taken together, these data demonstrate that unlike LmrCD, LmrA does not protect cells against the cytotoxic effects of Hoechst 33342.
Hoechst 33342 is an organic cation that is used to stain chromosomal DNA in live cells. It has also been used extensively to characterize the multidrug transporters in mammalian cells (103-106) and bacteria (4;26;32;107-110). Hoechst 33342 is strongly fluorescent in the hydrophobic environment of the membrane, or when intercalated with DNA. However, it is virtually nonfluorescent in aqueous solution. Because of this property, Hoechst 33342 is a very useful compound in studying the transport activity of MDR transporters which extrude substrates from the cellular membrane into the external medium. We have compared the Hoechst 33342-transport activity of two bacterial ABC-type MDR transporters, i.e., LmrA of *L. lactis* which is both structurally and functionally a homolog of P-gp (5;31) and LmrCD of *L. lactis*, a recently identified heterodimeric ABC transporter that is highly active in drug excretion (4). Both systems are ABC transporters, and their activity is therefore expected to be dependent on hydrolysis of ATP. Both have been shown to transport Hoechst 33342 in a membrane vesicle system in an ATP-dependent manner. However, here we report that the Hoechst 33342-transport associated with LmrA in membrane vesicles does not require the ATPase activity of LmrA itself. Rather, it involves ATP hydrolysis by the F$_0$F$_1$-ATPase which generates a transmembrane pH gradient which in turn affects the membrane partitioning and/or the fluorescence properties of Hoechst 33342 that enters the membrane vesicles via LmrA. Hoechst 33342 transport is, however, LmrA-dependent as it is not observed with control membrane vesicles. Moreover, an extensive study to obtain structural information about LmrA using a cysteine-scanning approach (37) shows that transmembrane segment 6 (TMS 6) of LmrA is particularly sensitive to mutations that interfere with Hoechst 33342 transport. Verapamil, a calcium-channel blocker, is also known as an inhibitor of transport of Hoechst 33342-transport by LmrA (31;32). In four of the mutants, the extent of inhibition of Hoechst 33342-transport by verapamil is also reduced, indicating that these residues are involved in both Hoechst 33342-transport and binding of verapamil. Finally, a study using a set of substrate photoaffinity ligands to identify the substrate-binding domain of LmrA confirms that TMS 6 is involved in substrate binding (38). Taken together these data indicate that Hoechst 33342 moves via the substrate binding domain of LmrA.

Several lines of evidence indicate that ATP hydrolysis by LmrA is not needed for LmrA-mediated Hoechst 33342-transport in membrane vesicles. First, mutagenesis of the conserved and functionally critical residues of the nucleotide binding domain of LmrA that abolishes its ATPase activity has no or little effect on Hoechst 33342-transport in membrane vesicles. Second, orthovanadate, a potent inhibitor of the LmrA ATPase activity, has no effect on Hoechst 33342-transport in membranes. In a previous study (32) orthovanadate-sensitive Hoechst 33342-transport by LmrA was seen in proteoliposomes. However, the observed transport activity in proteoliposomes was extremely low compared to that in membrane vesicles. We have not been able to detect any significant difference in LmrA proteoliposomes with the Hoechst 33342-transport assay when comparing addition of ATP with that...
of the nonhydrolyzable ATP analogue AMP-PNP (unpublished results). Third, LmrA-mediated Hoechst 33342-transport in membrane vesicles is completely inhibited by nigericin, an ionophore that acts as a H⁺/K⁺ antiporter, and by the weak base methylammonium. Both of these compounds collapse the transmembrane pH gradient in membrane vesicles. In contrast, Hoechst 33342-transport was not inhibited by another peptide ionophore valinomycin, suggesting that it is mostly, if not exclusively, dependent on the transmembrane pH gradient. The alternative explanation that nigericin is a substrate of LmrA and thus competes with Hoechst 33342 for binding and transport by LmrA cannot be ruled out, but this possibility seems unlikely as the same inhibitory effect is observed with methylammonium.

Fourth, LmrA-mediated Hoechst 33342-transport is blocked by DCCD, whereas its ATPase activity is unaffected. DCCD is an efficient inhibitor of the F$_{0}$F$_{1}$-ATPase. The latter enzyme is solely responsible for the generation of a proton motive force in membrane vesicles of L. lactis. For comparison, similar experiments were conducted with membrane vesicles bearing elevated levels of the ABC-type MDR transporter LmrCD. Like LmrA, LmrCD mediates Hoechst 33342-transport in membrane vesicles. However, this transport reaction is strictly dependent on ATP hydrolysis by LmrCD as it is inhibited by nucleotide binding site mutations that block the ATPase activity. Transport is strongly inhibited by orthovanadate, but barely affected by nigericin. Taken together, these data strongly suggest that ΔpH-dependent Hoechst 33342-transport is a reaction that is specific for LmrA as Hoechst 33342-transport via the analogous LmrCD system is not influenced by the proton motive force.

It is generally believed that the Hoechst 33342-transport assay in membrane vesicles records the MDR transporter-dependent repartitioning of Hoechst 33342 from the membrane phase into the aqueous solution. It is important to stress that these experiments do not reveal whether Hoechst 33342 is released inside of the lumen of the inside-out vesicles or into the external solution, although transport into the vesicles would be the expected physiological direction of transport. To determine the physiological direction of transport, experiments were carried out with intact cells. In this case, the Hoechst 33342 fluorescence mostly originates from the intercalation of Hoechst 33342 with DNA inside the cell. MDR transport activity in intact cells that is associated with drug extrusion will prevent entry of Hoechst 33342 into the cell. Indeed, this is the case with glucose-energized cells bearing elevated levels of LmrCD (4). In contrast, glucose-energized cells bearing elevated levels of LmrA were unable to prevent the entry of Hoechst 33342. This experiment demonstrates that LmrA does not mediate Hoechst 33342-extrusion; rather, LmrA highly reproducibly permits a slightly higher rate of influx of Hoechst 33342 into the cells as compared to the control. Also in the cytotoxicity tests, LmrA provided no protection against Hoechst 33342 and even makes cells more susceptible to Hoechst 33342. In contrast, expression of LmrCD rendered cells less sensitive to Hoechst 33342. Taken together, these data indicate that LmrA does not seem to function as a true multiple-drug transporter for Hoechst 33342. This appears to be different with other substrates. For instance, N-ethylmaleimide (NEM) labeling of a cysteine mutant of the glycine at position 386 in the Walker A region of the NBD of LmrA completely inhibited the LmrA-dependent fluorescent
phospholipid transbilayer movement in proteoliposomes (36). It is interesting to note that Hoechst 33342-transport in inside-out membrane vesicles bearing the same mutant of LmrA is not sensitive to NEM (G.J. Poelarends, unpublished data). It is, however, unclear how different substrates, i.e., Hoechst 33342 and fluorescent phospholipids, can be transported by different mechanisms by LmrA. As discussed above, our data strongly suggest that LmrA mediates cellular entry of Hoechst 33342. One major caveat of this kind of analysis is that the levels of LmrA upon overexpression can be as high as 30% of the total membrane protein (32). When the expression levels of LmrA are reduced, the Hoechst 33342-transport activity in vesicles can no longer be detected. At this stage, we cannot exclude the possibility that the Hoechst 33342-transport activity of LmrA is due to the large amounts of this membrane protein, but it should be stressed that all drug extrusion tests reported to date were performed under overexpressing conditions and that LmrA mutants have been described that exhibit a reduced Hoechst 33342-transport activity (37). One may argue that LmrA forms a kind of channel in the membrane that allows facilitated influx of Hoechst 33342. Our experiments with the pH dependence of the Hoechst 33342 fluorescence in the presence of liposomes indicate that the transport activity in membrane vesicles is due to a pH-dependent partitioning of the Hoechst 33342 between the membrane and water phase or, alternatively, a pH dependence of the fluorescence of the membrane-inserted Hoechst 33342. We consider the pH-dependent partitioning model, however, more likely. In solution, Hoechst 33342 exhibits a low fluorescence that was even further reduced with an increase in pH. In the presence of liposomes, a marked increase in fluorescence is observed but the pH dependence of the fluorescence now shows a reversed pH profile, i.e., the fluorescence increases with pH. This phenomenon is likely due to a more efficient partitioning of the deprotonated and less charged Hoechst 33342 into lipids. In membrane vesicles, the generation of a transmembrane pH gradient by the F$_{0}$F$_{1}$-ATPase results in an acidic pH inside. This low pH will favor a repartitioning of Hoechst 33342 from the membrane phase into the acidic aqueous phase of the lumen of the vesicles, thus causing a lowering of the dye fluorescence. Since this activity is dependent on LmrA, it seems that LmrA accelerates the movement of Hoechst 33342 from the membrane phase to the aqueous phase. Importantly, with intact cells, LmrA is unable to excrete Hoechst 33342 from the cells, and its presence even increases the cytotoxicity of Hoechst 33342 in L. lactis cells. This strongly suggests that LmrA facilitates the transmembrane movement of Hoechst 33342. It is unclear if this activity relates to the peculiar H$^{+}$/drug symport reaction that was suggested recently for the separately expressed transmembrane domain of LmrA (LmrA-MD) (33), as our study involved only the full-length LmrA. In contrast to the growth studies reported by Venter et al. (33), who used a wild-type L. lactis strain, our study employs a triple knockout strain which lacks LmrA, LmrC and LmrD. Therefore, the results of the growth inhibition studies cannot be directly compared as the triple deletion strain is much more susceptible to Hoechst 33342. It is difficult to envisage how the proton-linked activity of LmrA would contribute to its proposed physiological function as a drug efflux system.
This study raises questions about the physiological significance of LmrA as a multiple-drug transporter. Moreover, a knockout of the \textit{lmrA} gene in \textit{L. lactis} has no effect on the Hoechst 33342 (this chapter) and ethidium (4) sensitivity of the cells, while inactivation of the \textit{lmrCD} genes makes cells highly susceptible to these compounds. Therefore, it will be important to study the exact role of LmrA in the native host \textit{L. lactis}. 