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# Restrictive Use of Detergents in the Functional Reconstitution of the Secondary Multidrug Transporter LmrP†

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ABSTRACT: The histidine-tagged secondary multidrug transporter LmrP was overexpressed in *Lactococcus lactis*, using a novel protein expression system for cytotoxic proteins based on the tightly regulated, nisin-inducible *nisA* promoter. LmrP-mediated $H^+$/drug antiport activity in inside-out membrane vesicles was inhibited by detergents, such as Triton X-100, Triton X-114, and Tween 80, at low concentrations that did not affect the magnitude or composition of the proton motive force. The inhibition of the activity of LmrP by detergents restricted the range of compounds that could be used for the solubilization and reconstitution of the protein because low concentrations of detergent are retained in proteoliposomes. Surprisingly, dodecyl maltoside did not modulate the activity of LmrP. Therefore, LmrP was solubilized with dodecyl maltoside, purified by nickel−chelate affinity chromatography, and reconstituted in dodecyl maltoside-destabilized, preformed liposomes prepared from *Escherichia coli* phospholipids and egg phosphatidylcholine. Reconstituted LmrP mediated the transport of multiple drugs in response to an artificially imposed pH gradient, demonstrating that the protein functions as a proton motive force-dependent multidrug transporter, independent of accessory proteins. These observations are relevant for the effective solubilization and reconstitution of multidrug transporters belonging to the major facilitator superfamily, which, in view of their broad drug specificity, may strongly interact with detergents.

The emergence of multidrug resistance (MDR)[1] poses a serious threat to public health. The active extrusion of drugs out of cells is one of the major causes of failure of chemotherapy in the treatment of cancer (*1*). Like cancer cells, microorganisms can develop resistance by the energy-dependent extrusion of toxic compounds from the cell (*2*). These drug efflux processes are mediated by transport proteins, which can be relatively specific for a given substrate or can handle a wide variety of structurally unrelated compounds (*3*−*6*). Although some bacterial transporters utilize the free energy of ATP hydrolysis to extrude cytotoxic compounds (*7*−*9*), most bacterial multidrug efflux systems, known to date, are driven by the proton motive force (*5*). These secondary transporters can be subdivided on the basis of size and similarities in the primary and putative secondary structures. Members of the major facilitator superfamily (MFS) (*10*) and the resistance/nodulation/cell division family (RND) (*11*) are integral membrane proteins with 12 or 14 transmembrane segments and a molecular mass of 45−50 kDa. Members of the small multidrug resistance (SMR) family are functionally similar to MDR transporters from the MFS and RND families, but they only contain four putative transmembrane α-helices (*12*, *13*).

In the Gram-positive bacterium *Lactococcus lactis*, at least four extrusion activities have been detected (*6*, *14*−*16*). Pmf-dependent drug resistance is conferred by the *lmrP* gene, which encodes a hydrophobic polypeptide of 408 amino acid residues with 12 putative membrane-spanning segments (*17*). LmrP-mediated transport of $TPP^+$ in *Escherichia coli* membrane vesicles is driven by the membrane potential ($\Delta\psi$) and the transmembrane proton gradient ($\Delta$pH), which is indicative of an electrogenic $nH^+$/drug ($n \geq 2$) antiport mechanism. Molecular characterization of LmrP-dependent transport of the fluorescent membrane probe TMA-DPH demonstrated a relation between the transport rate and the amount of TMA-DPH associated with the inner leaflet of the lipid bilayer, providing evidence for the active removal of the probe from the cytoplasmic membrane (*18*).

In this paper, we have used the tightly regulated *nisA* promoter to overexpress LmrP in *L. lactis* with the aim of purifying and constituting the protein in proteoliposomes. To our knowledge, the only secondary MDR transporters that have been reconstituted so far are the SMR members EmrE from *E. coli* (*12*) and Smr from *Staphyllococcus aureus* (*19*). Surprisingly, Smr members can be quantitatively extracted with organic solvents such as chloroform/methanol, and are pure and stable after this extraction. For LmrP, the purification and reconstitution of the protein required the use

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[1] Abbreviations: Hoechst 33342, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5-bis(1*H*-benzimidazole); MDR, multidrug resistance; MFS, major facilitator superfamily; RND, resistance/nodulation/cell division; SMR, small multidrug resistance; pmf, proton motive force; $\Delta\psi$, membrane potential; $\Delta$pH, transmembrane proton gradient; $TPP^+$, tetraphenylphosphonium; PCR, polymerase chain reaction; Ni−NTA, $Ni^{2+}$−nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; DDM, *n*-dodecyl β-D-maltoside; C10E8, octaethylene glycol monodecyl ether; C12E8, octaethylene glycol monododecyl ether; NICE, nisin-controlled gene expression.

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Table 1: Bacterial Strains and Plasmids

| | relevant properties | reference |
|---|---|---|
| strain | | |
| MG1363 | plasmid-free and prophage-cured derivative of NCDO 712 | *35* |
| NZ9000 | MG1363 derivative, *pepN::nisRK* | O. Kuipers, unpublished |
| NZ9700 | nisin-producing transconjugant containing TN5276 | *36* |
| NZ9800 | NZ9700 derivative, Δ*nisA* | *36* |
| plasmid | | |
| pNZ8048 | $Cm^R$, pSH71 replicon, inducible *nisA* promoter | O. Kuipers, unpublished |
| pSKoppAChis | $pSKII^+$ carrying His-tagged OppA of *L. lactis* | Picon et al., in preparation |
| pNZ8048oppAChis | pNZ8048 carrying His-tagged OppA of *L. lactis* | this work |
| pHLP5 | pNZ8048 carrying His-tagged LmrP of *L. lactis* | this work |

of detergents. Our studies show that low concentrations of detergents Triton X-100, Triton X-114, and Tween 80 already inhibit LmrP-mediated transport, which posed problems in the development of purification and reconstitution procedures. Dodecyl maltoside did not modulate the activity of LmrP at low concentrations, and was used to efficiently solubilize LmrP from *L. lactis* membranes. The C-terminal His-tagged protein was purified by nickel−chelate affinity chromatography and functionally incorporated into dodecyl maltoside-saturated, preformed liposomes. To our knowledge, this work represents the first report on the functional reconstitution of a multidrug transporter of the MFS family.

## MATERIALS AND METHODS

*Bacterial Strains and Growth Conditions*. Bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose (w/v) and 5 μg/mL chloramphenicol when appropriate.

*DNA Manipulations and Plasmid Constructions*. General procedures for cloning and DNA manipulations were performed as described by Sambrook et al. (*20*). The 2128 bp *Nco*I−*Sac*I fragment of plasmid pSKoppAChis was ligated into the *Nco*I−*Sac*I sites of the nisin-inducible vector pNZ8048, yielding the expression vector pNZ8048oppAChis (Table 1).

A *Nco*I restriction site at the ATG start codon of the *lmrP* gene and a *Bam*HI restriction site that overlaps with the stop codon of *lmrP* were introduced by the polymerase chain reaction, using the primers GGGGCCATGGGGAAAGAGTTTTGGAATTTA and GGGGGGATCCATTTAATTTGTTTTC, and chromosomal DNA of *L. lactis* MG1363 as a template. The PCR product was digested with *Nco*I and *Bam*HI, and ligated with the 3.7 kb vector fragment obtained by digestion of pNZ8048oppAChis with *Nco*I and *Bam*HI. The *lmrP* coding sequence in the resulting plasmid pHLP5 was sequenced to ensure that only the intended changes had been introduced.

*Preparation of Membrane Vesicles*. *L. lactis* NZ9000 harboring either pNZ8048 or pHLP5 was grown at 30 °C to an $A_{660}$ of about 0.5, after which a 1:1000 dilution of NZ9700 supernatant, containing approximately 10 ng of nisin A/mL (O. Kuipers, personal communication), was added to trigger transcription from the *nisA* promoter. Following incubation for an additional 1 h at 30 °C, the cells were harvested by centrifugation. For the isolation of inside-out membrane vesicles, cells were lysed by a 2-fold passage through a French pressure cell, after digestion of the cell wall with 10 mg/mL lysozyme (incubation for 20 min at 30 °C). The crude membranes were incubated for 30 min at 30 °C with 10 mM $MgSO_4$ and 100 μg/mL DNase, and unbroken cells and cell debris were removed by centrifugation at 13000*g* for 10 min at 4 °C. Subsequently, the inside-out membrane vesicles were collected from the supernatant by centrifugation at 125000*g* for 1 h at 4 °C. The membrane vesicles were resuspended in 50 mM potassium phosphate (pH 7.0) containing 10% (v/v) glycerol and stored in liquid nitrogen.

For the estimation of LmrP expression levels, peripheral membrane proteins as well as cytosolic contaminants were extracted with 5 M urea and 6% (w/v) sodium cholate as described previously (*21*). Protein was assayed according to the method of Lowry et al. (*22*) in the presence of 0.5% SDS, using bovine serum albumin as a standard.

*Measurement of the Δ*p*H in Membrane Vesicles*. The ΔpH in inside-out membrane vesicles was monitored by fluorescence quenching of Acridine orange (*23*). Membrane vesicles (0.5 mg of protein/mL) were resuspended in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. Acridine orange was added at a final concentration of 1.25 μM. Upon the addition of 0.5 mM $Mg^{2+}$-ATP, a pmf (interior positive and acid) was generated by the $F_0F_1$ $H^+$-ATPase. The ionophores valinomycin and nigericin were added to a final concentration of 1 μM each, to dissipate the $\Delta\psi$ and ΔpH, respectively. Acridine orange fluorescence was monitored at 30 °C, in a Perkin-Elmer LS-50B fluorimeter, using excitation and emission wavelengths of 494 and 530 nm, respectively, with slit widths of 3 nm each.

*Preparation of Liposomes*. The *E. coli* total lipid extract (Avanti Polar Lipids) was acetone/ether washed as described by Viitanen et al. (*24*), dissolved in chloroform, and stored at −20 °C under $N_2$. For the preparation of liposomes, the *E. coli* lipids and egg yolk phosphatidylcholine (Avanti Polar Lipids) in a 3:1 ratio (w/w) were evaporated to dryness under vacuum using a rotary evaporator. The lipids were resuspended in 50 mM potassium phosphate (pH 7.0) at a concentration of 20 mg/mL, using a syringe equipped with a 0.55 mm × 250 mm hypodermic needle. The suspension was sonicated under $N_2$, on ice, using a tip sonicator at an intensity of 4 μm (peak to peak) for four cycles with intervals of 15 s of sonication and 45 s of rest. Samples of 1 mL were frozen in liquid $N_2$ and slowly thawed at room temperature. This freeze−thaw step was repeated once, and the liposomes were stored in liquid $N_2$.

*Solubilization*. Membrane vesicles (1−4 mg of membrane protein) from *L. lactis* NZ9000 harboring pHLP5 were solubilized in 50 mM potassium phosphate (pH 8.0) containing 100 mM NaCl, 10% (v/v) glycerol, and 1% (w/v)

detergent. The suspensions were mixed and incubated on ice for 30 min. The insoluble material was pelleted by centrifugation (280000*g* for 20 min at 4 °C). The amount of His-tagged LmrP in the soluble and insoluble fractions was quantified by immunoblotting as described previously (*25*) using monoclonal anti His-tag antibody DIA 900 (Dianova).

*Purification and Reconstitution of LmrP*. His-tagged LmrP was purified by affinity chromatography using $Ni^{2+}$−nitrilotriacetic acid (Ni−NTA)−agarose (Qiagen Inc.). The solubilized membrane proteins were mixed with Ni−NTA resin (∼25 $\mu$L of resin/mg of protein), which was equilibrated with 50 mM potassium phosphate (pH 8.0) supplemented with 100 mM NaCl, 10% (v/v) glycerol, 0.2 mg/mL *E. coli* lipids, 0.05% detergent (buffer A), and 10 mM imidazole, and gently shaken for 60 min at 4 °C. The resin was transferred to a Bio-spin column (Bio-Rad) and subsequently washed with 20 column volumes of buffer A and 10 mM imidazole and 10 column volumes of buffer A and 20 mM imidazole. The protein was eluted with buffer A (pH 7.0) containing 250 mM imidazole, and fractions of 500 $\mu$L were collected. The fractions were analyzed by SDS−PAGE (*26*).

For membrane reconstitution of LmrP, the liposomes were slowly thawed and extruded (11 times) through a 400 nm polycarbonate filter to obtain unilamellar liposomes with a relatively homogeneous size. Subsequently, the liposomes were diluted to 4 mg of lipid/mL and saturated with detergent, which was followed by measuring the $A_{540}$, as described by Paternostre et al. (*27*). The purified LmrP protein was mixed with the detergent-saturated liposomes (1 $\mu$mol of dodecyl maltoside/mg of lipid) at a protein:lipid ratio of 1:150 (w/w) and incubated for 30 min at room temperature under gentle agitation. The detergent was removed by absorption to polystyrene beads as described previously (*25*). Finally, the proteoliposomes were collected by centrifugation (280000*g* for 20 min at 4 °C), resuspended in 20 mM potassium phosphate (pH 7.0) supplemented with 100 mM KAc and 2 mM $MgSO_4$, and stored in liquid nitrogen.

### *Transport Assays*

*Intact Cells.* For the transport of ethidium bromide (*17*), cells were washed and resuspended in 50 mM potassium phosphate (pH 7.0) containing 5 mM $MgSO_4$. Glucose (25 mM) was added to supply the cells with metabolic energy.

*Membrane Vesicles*. To study transport of Hoechst 33342 (Molecular Probes Inc.) by LmrP, inside-out membrane vesicles (0.5 mg of protein/mL) were resuspended in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. After incubation for 30 s at 30 °C, Hoechst 33342 was added at 1 $\mu$M (final concentration). LmrP was activated, following the generation of a pmf by the $F_0F_1$ $H^+$-ATPase, upon the addition of 0.5 mM $Mg^{2+}$-ATP. The amount of membrane-associated Hoechst 33342 was measured fluorimetrically (Perkin-Elmer LS 50B fluorometer), using excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 5 nm.

*Proteoliposomes.* Hoechst 33342 transport in proteoliposomes was driven by an artificially imposed proton gradient (alkaline inside). The frozen proteoliposomes, in 20 mM potassium phosphate (pH 7.0), 100 mM KAc, and 2 mM $MgSO_4$ (buffer B), were thawed undisturbed at room temperature, extruded (11 times) through a 400 nm polycarbonate filter, collected by centrifugation (280000*g* for 20 min at 4 °C), and resuspended to a final concentration of 0.4 mg of protein/mL in buffer B. A 5 $\mu$L sample was diluted 100-fold into 20 mM potassium phosphate (pH 7.0) containing 50 mM $K_2SO_4$ and 2 mM $MgSO_4$. After 20 s, Hoechst 33342 in water was added at a final concentration of 1 $\mu$M; other additions were made as described in the figure legends.

## RESULTS

*Overexpression of Functional Histidine-Tagged LmrP in L. lactis*. In a previous study, LmrP was heterologously expressed in *E. coli* strain DH5α, using the IPTG-inducible *trc* promoter (*18*). It was observed that the *E. coli* cells stopped growing upon induction of LmrP expression, even though the expression levels were maximally only 0.1% of the total amount of the membrane protein. We therefore switched to a homologous expression system that takes advantage of the nisin-inducible, tightly regulated *nisA* promoter (*28*). The *lmrP* gene was inserted into pNZ8048, yielding pHLP5. To facilitate the purification of LmrP, a factor Xa cleavage site and a six-histidine tag were introduced at the C-terminus of the protein.

Energized *L. lactis* NZ9000/pHLP5 cells, overexpressing LmrP, showed strongly reduced uptake of ethidium (Figure 1A). This observation indicates that the histidine-tagged LmrP protein is functional. In addition, Hoechst 33342 transport studies were performed using inside-out membrane vesicles. Hoechst 33342, a substrate of various MDR transporters, including LmrP (*29*, *30*), is strongly fluorescent in a hydrophobic environment, and essentially nonfluorescent in an aqueous environment. Therefore, a decrease in fluorescence upon activation of LmrP corresponds with a net movement of Hoechst 33342 from the membrane to the aqueous phase. Figure 1B demonstrates that LmrP efficiently expels Hoechst 33342 LmrP from the NZ9000/pHLP5 membranes upon the generation of a pmf. The residual Hoechst 33342 efflux activity, which is observed in NZ9000/pNZ8048 membrane vesicles (Figure 1B, dotted line), is most likely mediated by endogenous, chromosomally encoded LmrA, LmrP, and/or other drug efflux activities. When both the membrane potential ($\Delta\psi$) and the transmembrane proton gradient ($\Delta$pH) were dissipated by the addition of the ionophores valinomycin and nigericin, LmrP-mediated drug transport was inhibited and Hoechst 33342 fluorescence increased to the level observed with control membrane vesicles. To quantitate the level of expression of LmrP, inside-out membrane vesicles of *L. lactis* NZ9000/pHLP5, extracted with 5 M urea/6% (w/v) sodium cholate, were analyzed by SDS−PAGE and stained with Coomassie Brilliant Blue (Figure 1C, lane 2). The level of LmrP was estimated to be 5% of the total amount of integral membrane protein.

*Effect of Detergents on LmrP Activity*. Bolhuis et al. (*18*) provided evidence for transport of TMA-DPH by LmrP from the inner leaflet of the cytoplasmic membrane. Other amphiphilic compounds that partition in the membrane may be recognized by LmrP in a similar fashion. Thus, the detergents used in the purification and reconstitution of membrane proteins are potential substrates of LmrP. The

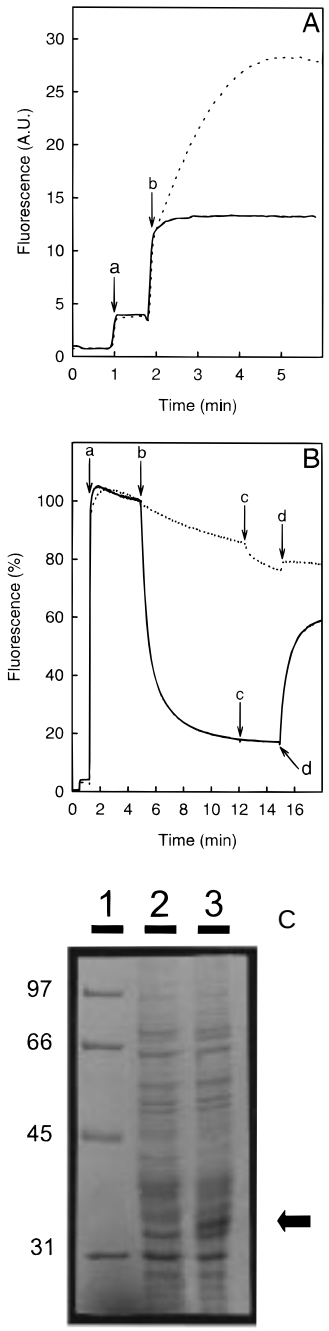


FIGURE 1: Overexpression of His-tagged LmrP in *L. lactis*. (A) Ethidium accumulation in *L. lactis* NZ9000/pNZ8048 (control cells, dotted line) and NZ9000/pHLP5 (LmrP-expressing cells, solid line). Cells (a) were incubated for 1 min in 50 mM potassium phosphate (pH 7.0) supplemented with 5 mM $MgSO_4$ and 25 mM glucose. The assay was started upon addition of 10 $\mu$M ethidium bromide (b) to the cell suspension. (B) Inside-out membrane vesicles prepared from control cells (dotted line) and LmrP-expressing cells (solid line) were diluted to a concentration of 0.5 mg of protein/mL in 50 mM potassium Hepes (pH 7.0) containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. After incubation for 30 s, 1 $\mu$M Hoechst 33342 was added (a). Transport was initiated upon addition of 0.5 mM $Mg^{2+}$-ATP (b). Valinomycin (c) and nigericin (d) were added to a final concentration of 1 $\mu$M each. (C) Coomassie Brilliant Blue-stained SDS−PAGE gel (10%) with samples of urea/cholate extracted inside-out membrane vesicles prepared from *L. lactis* NZ9000/pNZ8048 (control cells, lane 2) or NZ9000/pHLP5 (LmrP-expressing cells, lane 3). Molecular weight markers are shown in lane 1. The arrow indicates the position of the His-tagged LmrP protein.

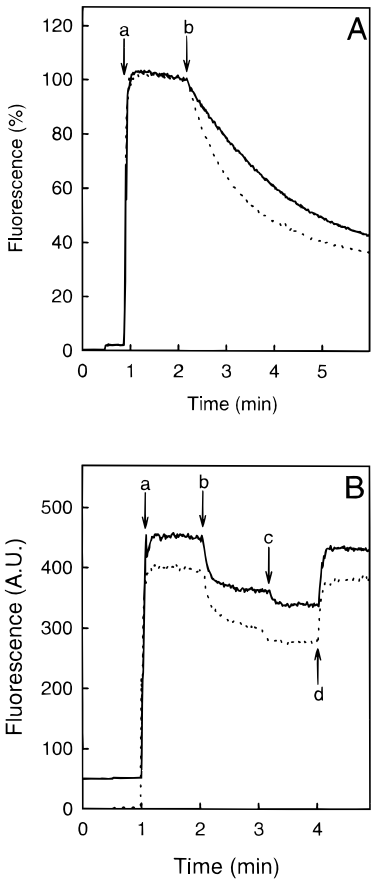


FIGURE 2: Effect of Triton X-100 on Hoechst 33342 transport and proton motive force generation in inside-out membrane vesicles. (A) Inhibition of Hoechst 33342 transport in membrane vesicles by Triton X-100. The rate of Hoechst 33342 transport (as described in the legend of Figure 1) was measured in the presence (solid line) or absence (dotted line) of 0.005% (w/v) Triton X-100. The addition of 1 $\mu$M Hoechst 33342 (a) and 0.5 mM $Mg^{2+}$-ATP (b) are indicated. (B) Measurement of the $\Delta$pH (interior acid) in membrane vesicles, in the presence (solid line) or absence (dotted line) of 0.005% (w/v) Triton X-100. For clarity of presentation, the solid trace is offset 50 units from the dotted trace. Acridine orange was added to a final concentration of 1.25 $\mu$M (a). A proton motive force (interior acid and positive) was generated by the $F_1F_0$ $H^+$-ATPase upon the addition of 0.5 mM $Mg^{2+}$-ATP (b). Valinomycin (c) and nigericin (d) were added to a final concentration of 1 $\mu$M each, to interconvert $\Delta\psi$ into $\Delta$pH, and to dissipate the $\Delta$pH, respectively.

ability of octyl glucoside, C10E8, C12E8, decyl maltoside, dodecyl maltoside, Tween 20, Tween 80, Triton X-100, Triton X-114, and SDS to compete with Hoechst 33342 for LmrP-mediated transport in inside-out membrane vesicles was tested. Figure 2A shows that energy-dependent Hoechst 33342 transport in inside-out membrane vesicles was significantly inhibited by Triton X-100. Similar results were obtained for Triton X-114 and Tween 80. A concentration of 0.005% (w/v) Triton X-100, Triton X-114, or Tween 80 resulted in 46, 36, and 24% inhibition of the initial rate of Hoechst 33342 transport, respectively. The fluorescent probe Acridine orange was used to study the effect of the detergents on the pmf (Figure 2B). Generation of a $\Delta$pH (inside acid) causes quenching of Acridine orange fluorescence. Upon addition of valinomycin, the fluorescence further decreases, due to the interconversion of $\Delta\psi$ into $\Delta$pH. The subsequent dissipation of the pH gradient, by the addition of nigericin, leads to the release of Acridine orange from the vesicles,

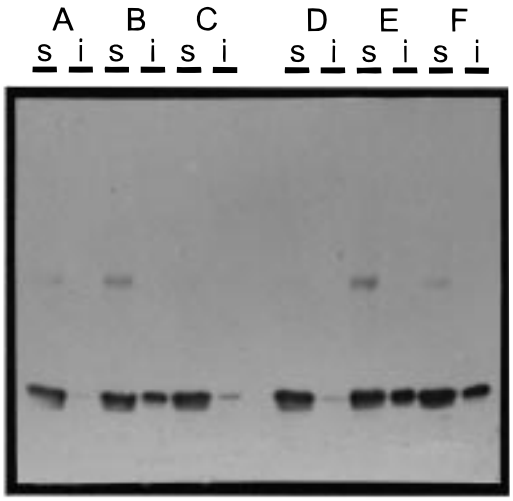


FIGURE 3: Solubilization of LmrP from inside-out membrane vesicles of *L. lactis* NZ9000/pHLP5. Membranes (1 mg of protein) were solubilized in 50 mM potassium phosphate (pH 8.0) supplemented with 100 mM NaCl, 10% (v/v) glycerol, and 1% (w/v) detergent: (A) Triton X-100, (B) octyl glucoside, (C) decyl maltoside, (D) dodecyl maltoside, (E) C10E8, and (F) C12E8. The suspensions were mixed and incubated on ice for 30 min. The insoluble material was pelleted by centrifugation (280000*g* for 20 min at 4 °C), and the soluble (s) and insoluble (i) fractions were analyzed by SDS−PAGE and immunoblotting using monoclonal antibodies directed against the histidine tag.

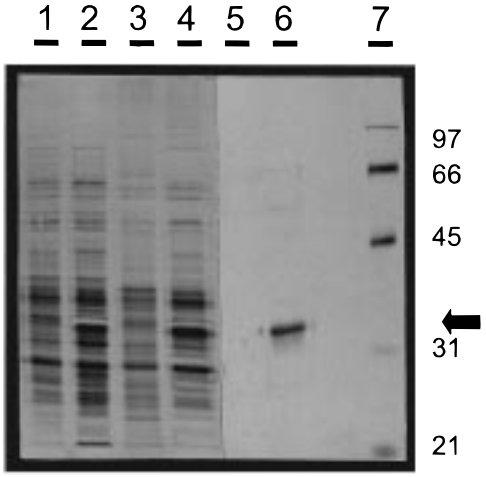


FIGURE 4: Purification of the histidine-tagged LmrP protein. Silver-stained SDS−PAGE gel (10%) with samples from NZ9000/pNZ8048 (lanes 1, 3, and 5) and NZ9000/pHLP5 (lanes 2, 4, and 6): total membranes (lanes 1 and 2), supernatant (20 min at 280000*g*) after solubilization with 1% (w/v) dodecyl maltoside (lanes 3 and 4), and Ni−NTA eluates at 250 mM imidazole (lanes 5 and 6). The molecular weight markers are shown in lane 7. The arrow indicates the position of the His-tagged LmrP protein.

with a concomitant increase in fluorescence (*23*). This increase is indicative for the magnitude of the pmf that was generated. The Acridine orange measurements revealed that, at the concentrations used, the detergents did not affect the magnitude and composition of the pmf. These results imply that Triton X-100, Triton X-114, and Tween 80 affect LmrP directly, either by competing with the substrate for LmrP-mediated transport or by modulating the LmrP activity otherwise. In view of the retention of detergents in procedures aimed at the incorporation of proteins into artificial membranes (*31*; J. Knol et al., in preparation), Triton X-100,

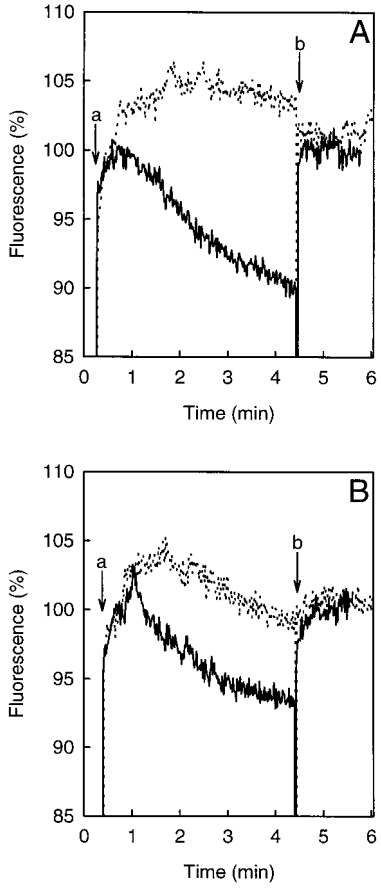


FIGURE 5: Hoechst 33342 transport in proteoliposomes. Proteoliposomes were loaded with 20 mM potassium phosphate (pH 7.0) containing 100 mM potassium acetate and 2 mM $MgSO_4$. Hoechst 33342 (a) and nigericin (b) were added to final concentrations of 1 $\mu$M each. The fluorescence intensity after addition of nigericin was normalized to 100%. (A) Hoechst 33342 fluorescence for liposomes containing LmrP (solid line) and control liposomes (dotted line), in which an artificial $\Delta$pH (interior alkaline) was imposed by a 100-fold dilution of the proteoliposomes into 20 mM potassium phosphate (pH 7.0) containing 50 mM $K_2SO_4$ and 2 mM $MgSO_4$. (B) $\Delta$pH (interior alkaline)-driven, LmrP-mediated Hoechst 33342 transport in proteoliposomes in the absense (solid line) and presence of 20 $\mu$M erythromycin (dotted line).

Triton X-114, and Tween 80 are not suitable for reconstitution of LmrP.

*Solubilization of LmrP from Inside-Out Membrane Vesicles*. Various detergents were evaluated for their efficiency to solubilize LmrP from inside-out membrane vesicles. Figure 3 shows that octyl glucoside, C10E8, and C12E8 solubilized only 70% of LmrP from NZ9000/pHLP5 membranes. Better results were obtained with the detergents Triton X-100, decyl maltoside, and dodecyl maltoside, which solubilized more than 90% of the LmrP. Analysis of the protein samples on SDS−PAGE showed that solubilization with Triton X-100, octyl glucoside, C10E8, and C12E8 resulted in the formation of relatively large amounts of di- and trimeric structures (Figure 3, lanes As, Bs, Es, and Fs). Therefore, dodecyl maltoside was chosen for the purification of LmrP.

*Purification*. The solubilized membrane proteins from *L. lactis* NZ9000 harboring pNZ8048 or pHLP5 were applied to a nickel chelate affinity resin, and contaminating proteins were removed by washing the column with 10 and 20 mM imidazole. LmrP was eluted by lowering the pH of the buffer to 7.0 and increasing the imidazole concentration to 250 mM.

LmrP was purified in a single step to a high degree of purity (>90%). Besides a major band at 36 kDa, minor bands were found at 62 and 102 kDa (Figure 4, lane 6). Western blot analysis, using monoclonal anti His-tag antibodies, showed that these bands represent di- and trimeric species of LmrP (data not shown). The absorption spectrum of the purified protein showed very little absorbance in the 320−340 nm range, indicating that higher-order aggregates of LmrP are not formed in the solubilization or purification steps (data not shown). Approximately 1 mg of purified LmrP was obtained from 1 L of *L. lactis* NZ9000/pHLP5 grown to an $A_{660}$ of 0.7−0.9.

*Reconstitution*. LmrP was reconstituted by mixing the purified protein with preformed dodecyl maltoside-destabilized liposomes, composed of *E. coli* phospholipids and egg phosphatidylcholine in a 3:1 ratio, followed by the slow removal of detergent. Treatment of liposomes with detergent was followed by measuring the $A_{540}$ (*25*, *27*). The onset of solubilization (liposomes saturated with detergent) was reached at a concentration of 1 $\mu$mol of dodecyl maltoside/mg of lipid (data not shown), and this detergent concentration was used to reconstitute purified LmrP (100 $\mu$g) at a protein:lipid ratio of 1:150 (w/w). After removal of the detergent by absorption to polystyrene beads (Biobeads SM-2) and a freeze−thaw step, the sample was extruded through a 400 nm polycarbonate filter to obtain unilamellar proteoliposomes with a relatively homogeneous size.

The Hoechst 33342 transport assay was used to assess the transport activity of membrane-reconstituted LmrP. When an artificial proton gradient was imposed, the Hoechst 33342 fluorescence of LmrP-containing liposomes decreased in time (Figure 5A). Following the dissipation of $\Delta$pH by the addition of nigericin, Hoechst 33342 fluorescence of the LmrP-containing liposomes increased to the initial level, demonstrating that the LmrP-mediated Hoechst 33342 transport was $\Delta$pH-dependent. Hoechst 33342 transport was not observed in liposomes lacking LmrP or in LmrP-containing proteoliposomes in the absence of a $\Delta$pH (data not shown). Furthermore, addition of the LmrP substrate erythromycin resulted in inhibition of the LmrP-mediated Hoechst 33342 transport in proteoliposomes (Figure 5B).

## DISCUSSION

The nisin-controlled gene expression system (NICE), based on the *L. lactis nisA* promoter (*28*), was used for amplified expression of LmrP. Nisin induces transcription from the *nisA* promoter via a two-component regulatory system that is composed of the nisin sensor NisK and the transcription factor NisR (*32*). The *nisA* promoter is more tightly regulated than other promoters, which offers the advantage of overexpressing toxic proteins in *L. lactis* (*28*). In the absence of nisin, we did not observe detectable amounts of LmrP in *L. lactis*. Upon induction, the level of LmrP expression in *L. lactis* was approximately 5% of the total amount of integral membrane protein. Ethidium uptake was significantly reduced in *L. lactis* cells overexpressing LmrP, demonstrating that the protein is functional.

Since Hoechst 33342 is highly fluorescent in a hydrophobic environment and not in a hydrophilic environment, the probe proved to be very suitable for studying its transport from the lipid to the aqueous phase. Hoechst 33342 has also successfully been used in studies of reconstituted P-glycoprotein (*29*). Our study showed that Hoechst 33342 is transported by LmrP, in both inside-out membrane vesicles and proteoliposomes. The artificially imposed proton gradient was large and stable enough to drive Hoechst 33342 transport by the reconstituted LmrP. The observed LmrP-mediated Hoechst 33342 transport in proteoliposomes is much slower than that observed in inside-out membrane vesicles. Similar observations were made by Shapiro et al. (*29*, *30*) for reconstituted P-glycoprotein. It was speculated that the high lipid:protein ratio in proteoliposomes allows a higher rate of passive rebinding of transported Hoechst 33342 to the membrane, as compared to native membranes, which results in a lower net rate of drug transport. In addition, the rate of drug extrusion by purified MDR transport proteins may be affected by the significant retention of detergent during the reconstitution procedures (approximately 1 mol of dodecyl maltoside per 15−20 mol of phospholipid; J. Knol et al., in preparation). The detergent may affect the passive flip-flop of the drug from one leaflet to the other and thereby interfere with net drug transport indirectly. Due to their amphiphilic character, detergents are potential substrates of drug transporters. This aspect is particularly relevant for those transporters that are able to accept their substrates from the membrane phase, as was shown for TMA-DPH extrusion by LmrP (*18*). For LmrP, it was observed that Triton X-100, Triton X-114, and Tween 80 inhibit Hoechst 33342 transport in inside-out membrane vesicles at low concentrations that do not affect the magnitude or composition of the proton motive force, whereas decyl maltoside, dodecyl maltoside, octyl glucoside, C10E8, C12E8, and Tween 20 do not inhibit LmrP activity. Thus, the choice of detergent for solubilization and reconstitution of MDR transporters may be crucial for activity measurements of the purified proteins in proteoliposomes. In previous work, Van Helvoort et al. (*33*) and Bosch et al. (*34*) demonstrated that phosphatidylcholine and phosphatidylethanolamine are potential substrates for the human multidrug transporter P-glycoprotein. Therefore, similar considerations may have to be made for the choice of lipids used for the reconstitution of secondary multidrug transporters.

Taken together, the results obtained in this work with purified LmrP reconstituted into proteoliposomes demonstrate that LmrP by itself is capable of mediating drug transport without accessory proteins or cytosolic components. The modulation of the activity of LmrP by detergents restricts the range of compounds that can be used for solubilization and reconstitution protocols.

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