The Purified and Functionally Reconstituted Multidrug Transporter LmrA of *Lactococcus lactis* Mediates the Transbilayer Movement of Specific Fluorescent Phospholipids†

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ABSTRACT: *Lactococcus lactis* possesses an ATP-binding cassette transporter, LmrA, which is a homolog of the mammalian multidrug resistance (MDR) P-glycoprotein, and is able to transport a broad range of structurally unrelated amphiphilic drugs. A histidine tag was introduced at the N-terminus of LmrA to facilitate purification by nickel affinity chromatography. The histidine-tagged protein was overexpressed in *L. lactis* using a novel protein expression system for cytotoxic proteins based on the tightly regulated, nisin-inducible *nisA* promoter. This system allowed us to get functional overexpression of LmrA up to a level of 30% of total membrane protein. For reconstitution, LmrA was solubilized with dodecylmaltoside, purified by nickel-chelate affinity chromatography, and reconstituted in dodecylmaltoside-destabilized, preformed liposomes prepared from *L. lactis* phospholipids. The detergent was removed by adsorption onto polystyrene beads. The LmrA protein was reconstituted in a functional form, and mediated the ATP-dependent transport of the fluorescent substrate Hoechst-33342 into the proteoliposomes. Interestingly, reconstituted LmrA also catalyzed the ATP-dependent transport of fluorescent phosphatidylethanolamine, but not of fluorescent phosphatidylcholine. These data demonstrate that LmrA activity is independent of accessory proteins and support the notion that LmrA may be involved in the transport of specific lipids or lipid-linked precursors in *L. lactis*.

Mammalian tumors can develop simultaneous resistance to various structurally unrelated cytotoxic drugs (*1*). This resistance is a serious problem in the treatment of human cancer and is commonly associated with high expression levels of the MDR1-encoded multidrug resistance P-glycoprotein (*2*). P-glycoprotein reduces the cellular accumulation and cytotoxicity of drugs by mediating the ATP-dependent extrusion of these compounds from the cell (*2*−*4*). Like human cells, the extrusion of drugs in bacteria can be mediated by transport proteins which can be dedicated to a single drug or class of drugs (*5*, *6*) or to multiple structurally unrelated amphiphilic compounds (*7*). Such multidrug transport systems resemble P-glycoprotein (*8*−*10*).

Multidrug transporters in pro- and eukaryotic cells can be subdivided in several groups according to structural and bioenergetic criteria (*11*). One of these groups belongs to the ABC superfamily which includes, among others, the mammalian cystic fibrosis transmembrane conductance regulator (CFTR) (*12*), the multidrug resistance protein MRP1 (*13*), P-glycoprotein (*2*), and LmrA from *Lactococcus lactis* (*10, 14*). LmrA is a 590 amino acid polypeptide with 6 transmembrane segments in the N-terminal hydrophobic domain, followed by a hydrophilic ATP-binding cassette (ABC) domain. Interestingly, the sequence conservation between LmrA and P-glycoprotein is relatively high and includes particular regions that have been implicated as determinants of drug recognition and binding in P-glycoprotein (*10*). Furthermore, this protein can even functionally complement P-glycoprotein in human lung fibroblast cells (*14*), suggesting a fundamental biological role for this type of ABC transporter in prokaryotic and eukaryotic cells.

Previously, LmrA function has been studied in whole cells of *L. lactis* and in plasma membrane vesicles derived thereof. In these systems, other cellular components and processes may interfere with a direct characterization of the protein at the molecular level. Here, we report the overexpression, purification, and functional reconstitution of histidine-tagged LmrA into proteoliposomes. We have used the tightly regulated *nisA* promoter to overproduce LmrA in *L. lactis*. After optimization of the expression of the *lmrA* gene, the

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hoechst-33342, 2′-(4-ethoxylphenyl)-5-(4-methyl-1-piperazinyl)-2,5-bis(1*H*-benzimidazole); MDR, multidrug resistance; ABC, ATP-binding cassette; PCR, polymerase chain reaction; Ni-NTA, Ni^{2+} nitrilotriacetic acid; DDM, *n*-dodecyl-*β*-D-maltoside; $C_{10}E_8$, octaethylene glycol monodecyl ether; TX100, Triton X-100; NICE, nisin-controlled gene expression; ATP, adenosine 5′-triphosphate; ATP-*γ*-S, adenosine 5′-*O*-(3-thiotriphosphate); AMP-PNP, 5′-adenylyl imidodiphosphate; PVDF, polyvinylidene difluoride; C_6-NBD-PE, 1-myristoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3 phosphoethanolamine; C_6-NBD-PC, 1-myristoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; N-Rh-PE, L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (egg).

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protein was purified in a single step by nickel-chelate affinity chromatography and reconstituted by the detergent titration technique (*15*) using *n*-dodecyl-*β*-D-maltoside (DDM) as the detergent. The resulting proteoliposomes exhibited ATP-dependent transport of Hoechst-33342 and C_6-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-labeled phosphatidylethanolamine. Interestingly, C_6-NBD-labeled phosphatidylcholine was not transported by LmrA, suggesting that LmrA exhibits specificity for phospholipid headgroups.

MATERIALS AND METHODS

Materials. M-17 medium was obtained from Difco, and synthetic oligonucleotides were from Eurosequence, Groningen. Restriction enzymes, T4 DNA ligase, ATP, AMP-PNP, and ATP-*γ*-S were obtained from Boehringer Mannheim; lysozyme and chloramphenicol were from Merck, and DDM was from Anatrace. Ni^{2+} nitrilotriacetic acid (Ni-NTA) resin was obtained from Qiagen, Inc.; Bio-Beads SM-2 from Bio-Rad; polycarbonate filters from Avestin; recombinant enterokinase from Novagen; and Hoechst-33342 from Molecular Probes Europe BV, Leiden, Holland. All the reagents for immunoblotting (I-block, alkaline phosphatase conjugated goat anti-mouse antibody, Nitroblock, and CSPD) were supplied by Tropix, except the histidine tag monoclonal antibody (DIA 900), which was supplied by Dianova. Verapamil, sodium orthovanadate, and DNase were from Sigma. 1-Myristoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]caproyl]-*sn*-glycero-3-phosphoethanolamine (C_6-NBD-PE), 1-myristoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]caproyl]-*sn*-glycero-3-phosphocholine (C_6-NBD-PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (egg) (N-Rh-PE) were purchased from Avanti Polar Lipids. All other materials were reagent grade and obtained from commercial sources.

Bacterial Strains and Growth Conditions. The *L. lactis* subsp. *lactis* strain NZ9000 was used as a host for pNZ8048-related plasmids (obtained from Dr. O. Kuipers, NIZO, The Netherlands) used in this work. *L. lactis* strain NZ9700 (*16*) was used as a nisin-producing strain. The cells were grown at 30 °C in M-17 medium supplemented with 0.5% glucose and 5 μg/mL chloramphenicol when appropriate.

Construction of the Expression Vector pNHLmrA. The *lmrA* gene was amplified from pCHLmrA (*14*) by the polymerase chain reaction (PCR) using the oligonucleotide 5′-TCTAGACCACCATGGGGCATCACCATCACCATC-ACGAT GACGATGACAAAGCCGAAAGAGG-3′ to introduce a *Nco*I site upstream of the hexa-histidine tag and to delete the internal *Nco*I site in pCHLmrA at the start of the *lmrA* gene, and the oligonucleotide 5′-GCCGACTCTA-GATTATTATTGACCAAC-3′ to introduce a *Xba*I site at the 3′ end of *lmrA*. The PCR product was purified on an agarose gel, digested with *Nco*I and *Xba*I, and ligated with the similarly treated plasmid pNZ8048, resulting in pNHLmrA. This plasmid was transformed into *L. lactis* NZ9000, and the transformants were screened by restriction analysis of the plasmids. The *Nco*I−*Xba*I fragment containing the *lmrA* gene was sequenced to ensure that only the intended changes had been introduced.

Preparation of Inside-Out Membrane Vesicles. For the isolation of inside-out membrane vesicles of *L. lactis* NZ9000, cells were grown at 30 °C to an OD_{660} of about 0.8. At this point, 0.4% (v/v) of the supernatant of the nisin-producing *L. lactis* strain NZ 9700, containing approximately 10 ng of nisin A/mL, was added to the culture to trigger transcription of the *lmrA* gene from the *nisA* promoter. Subsequently, the cells were incubated for 1 h at 30 °C, harvested at an OD_{660} of approximately 1.5 by centrifugation, and washed with 100 mM potassium phosphate, pH 7.0. The pellet was resuspended in the same buffer, and the cell wall was digested with 10 mg/mL lysozyme for 30 min at 30 °C. Cells were lysed by passage through a French pressure cell (20 000 psi). DNase (100 μg/mL) and 10 mM $MgSO_4$ were added to the cell suspension, which was further incubated for 20 min at 30 °C. Unbroken cells and cell debris were removed by two consecutive centrifugation steps at 13000*g* for 10 min at 4 °C. Inside-out membrane vesicles were collected by centrifugation at 125000*g* for 60 min at 4 °C and resuspended in 50 mM potassium phosphate, pH 7.0, containing 10% glycerol. The membrane vesicles were stored in small aliquots in liquid nitrogen.

Measurement of Expression Levels. Total membrane protein was assayed according to Lowry et al. (*17*) in the presence of 0.5% SDS, using bovine serum albumin as the standard. To determine the ratio between the total amount of membrane protein and the total amount of LmrA, samples of membrane vesicles were solubilized in SDS−PAGE loading buffer and dot-blotted using pure LmrA with known concentration as a standard. The concentration of the LmrA standard was determined from the OD_{280} and the calculated extinction coefficient of the protein ($\epsilon = 45\,090$). The level of LmrA expression in NZ9000/pNHLmrA membrane vesicles was also estimated by densitometry of a SDS−PAGE gel stained with Coomassie-brillant blue.

Solubilization. Inside-out membrane vesicles containing overexpressed LmrA protein (24−28 mg of total membrane protein/mL) were solubilized in 50 mM potassium phosphate, pH 8.0, containing 10% (v/v) glycerol, 100 mM NaCl, and 1% (w/v) DDM. The suspension was mixed, and, after 30 min incubation at 4 °C, the insoluble material was removed by centrifugation (280000*g*, 15 min, 4 °C). The amounts of LmrA in the soluble and insoluble fractions were quantified by Coomassie staining of SDS−PAGE gels and immunoblotting using monoclonal anti-histidine tag antibody as described previously (*18*).

Purification of Histidine-Tagged LmrA. Solubilized membrane proteins were mixed and incubated for 1 h at 4 °C with Ni-NTA agarose (∼8 mg of LmrA/mL of resin) which was preequilibrated in buffer A [50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM] plus 10 mM imidazole. After incubation, the resin was transferred to a Bio-spin column (Bio-Rad) and washed first with 20 column volumes of buffer A containing 10 mM imidazole, and subsequently with 40 column volumes of buffer A (pH 7.0) containing 40 mM imidazole. The protein was eluted with buffer A, pH 7.0, supplemented with 150 mM imidazole and immediately used for reconstitution. All handlings were carried out at 4 °C.

Reconstitution of Histidine-Tagged LmrA in Proteoliposomes. The phospholipids of *L. lactis* NZ9000 were extracted and purified from the cell mass as described (*19*). Cells (500 g wet weight) were resuspended in 510 mL of 20 mM potassium phosphate, pH 7.0, and incubated at 37 °C for 30

min with 4.2 mg/mL lysozyme and 100 μg/mL DNase. All the following steps were performed in a N_2 atmosphere to minimize oxidation. One liter of chloroform and 2 L of methanol were added, and the suspension was stirred overnight at 4 °C. Undissolved material was removed by centrifugation in glass tubes at 900*g* for 10 min. To the supernatant were added 0.95 L of chloroform and 0.95 L of water, and the mixture was stirred for 3 h at room temperature. The phases were separated for 12 h at 4 °C, and the lower phase (chloroform) was evaporated to dryness in a rotary evaporator at 30 °C, yielding approximately 9 mg of lipids/g of cells. The lipids were resuspended in 50 mM potassium phosphate, pH 7.0, at a final concentration of 20 mg/mL and stored in liquid nitrogen. Unilamellar liposomes with relatively homogeneous size were prepared by freezing in liquid nitrogen, by slow-thawing at room temperature, and by extruding the liposomes 12 times through a 400 nm polycarbonate filter. Subsequently, the liposomes were diluted to 4 mg of phospholipids/mL, and the detergent destabilization of the liposomes was followed at an optical density of 540 nm (*20*) by the addition of increasing amounts of DDM. For the reconstitution of solubilized and purified LmrA, protein was mixed with DDM-destabilized liposomes (1 μmol of DDM/mg of lipids) in a 1/100 ratio (w/w), and incubated for 30 min at room temperature under gentle agitation. The detergent was removed by three successive extractions with polystyrene beads (Bio-Beads SM-2, Bio-Rad, extensively washed with methanol and water) at a wet weight of 80 mg/mL of liposome suspension. The first extraction was done at room temperature for 2 h, and the second and third at 4 °C for 2 and 16 h, respectively. Finally, the liposomes were harvested by centrifugation (280000*g*, 15 min, 20 °C), resuspended in 50 mM potassium phosphate, pH 7.0, at a final protein concentration of 1 mg/mL, and stored in liquid nitrogen. All transport, orientation, and incorporation experiments were performed after a freeze/thaw step followed by extrusion of the proteoliposomes through a 400 nm polycarbonate filter.

Incorporation of LmrA into Liposomes. Discontinuous sucrose gradients were performed in Beckman ultracentrifugation tubes on top of a 40% (w/v) sucrose cushion. The discontinuous gradients were prepared from the following sucrose concentrations in 50 mM potassium Hepes, pH 7.4: 32% (1 mL), 26% (1 mL), 21% (1 mL), 16% (1 mL), 11% (1 mL), 7% (1 mL), 3% (1 mL), 0% (1 mL). Proteoliposomes were washed twice and resuspended to a final protein concentration of 1 mg/mL in 50 mM potassium Hepes, pH 7.4. Then 100 μL of proteoliposome suspension was layered on top of the gradient and centrifuged for 16 h at 150000*g* in an SW41 swing-out rotor (Beckman) at 20 °C. Subsequently, fractions of 0.7 mL were collected from the bottom. A sample of 500 μL of each fraction was dialyzed overnight in 1 L of 50 mM potassium Hepes, pH 7.4, to remove the sucrose. The amount of phospholipids in the fractions was calculated from the inorganic phosphate liberated by the addition of 300 μL of perchloric acid as described (*21*). The presence of LmrA in the fractions was estimated by Western blotting as described (*18*).

Orientation of the Reconstituted LmrA Protein. Proteoliposomes were washed twice and resuspended to a final protein concentration of 1 mg/mL in 50 mM potassium Hepes, pH 7.4. An enterokinase cleavage site localized between the histidine tag and the N-terminus of the protein was used to determine the orientation of the reconstituted LmrA. To make the cleavage site accessible for the enterokinase enzyme from both the outside and the inside of the membrane, proteoliposomes (10 μL) were sonicated 3 times for 5 s on ice in the presence of 2 units of enterokinase. A nonsonicated sample, in which only the cleavage sites located on the outside of the membrane were accessible for the proteinase, and a sample without enterokinase treatment were used as controls. The reaction mixtures (final volume 50 μL) were incubated for 16 h at 21 °C. The samples were electrophoresed, transferred to a PVDF membrane, and analyzed by Western blotting using anti-histidine tag antibody. The detection limit of the immunoblotting assay was less then 100 pg.

Transport Assays. (A) Ethidium Transport in Whole Cells. The accumulation of ethidium bromide in cells of *L. lactis* was measured by fluorometry as described (*10, 22*). Briefly, LmrA expression in *L. lactis* was induced by the addition of nisin A as described above, after which the cells were collected by centrifugation, washed with 50 mM potassium phosphate, pH 7.0, plus 5 mM $MgSO_4$, and resuspended to an OD_{660} of 0.5 in this buffer. Cells were preenergized for 3 min in the presence of 25 mM glucose. Subsequently, ethidium bromide was added to a final concentration of 2 μM, and the fluorescence was followed using excitation and emission wavelengths of 500 and 580 nm, respectively. The excitation and emission bandwidths were 5 and 10 nm, respectively.

(B) Hoechst-33342 Transport in Membrane Vesicles. Inside-out membrane vesicles (1 mg of total membrane protein) were diluted in 2 mL of 50 mM potassium Hepes, pH 7.4, containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine in a 3 mL cuvette. After 1 min incubation at 30 °C, 4 μL of 1 mM Hoechst-33342 in water was added, after which Hoechst fluorescence was followed until steady state. Mg-ATP or Mg-ATP-γ-S was added to a final concentration of 2 mM, and the fluorescence intensity was followed for 15 min with a Perkin-Elmer LS 50B fluorometer, using excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 3 nm each.

(C) Hoechst-33342 Transport in Proteoliposomes. Proteoliposomes in 50 mM potassium phosphate, pH 7.0, were thawed slowly at room temperature and extruded through to a 400 nm polycarbonate filter. Subsequently, the proteoliposomes were washed twice in 50 mM potassium Hepes, pH 7.4, by ultracentrifugation (280000*g* for 15 min at 4 °C), and resuspended in the same buffer to a final concentration of 1 mg of protein/mL. Samples of 10 μL were added to 2 mL of 50 mM potassium Hepes, pH 7.4, containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine in a 3 mL cuvette. After 60 s of data acquisition, 1.25 μM Hoechst-33342 was added. Mg-ATP or Mg-ATP-γ-S (1.25 mM) was added after 120 s of data acquisition. The difference between the Hoechst-33342 fluorescence in liposomes and in proteoliposomes containing LmrA before the addition of nucleotides was less than 5% in all the experiments.

(D) C_6-NBD-Phospholipid Transport Assay. Donor liposomes containing 3 mol % C_6-NBD-phospholipid, 6 mol % N-Rh-PE, and 91 mol % POPC were prepared essentially as described by Kremer et al. (*23*). The lipid mixture was

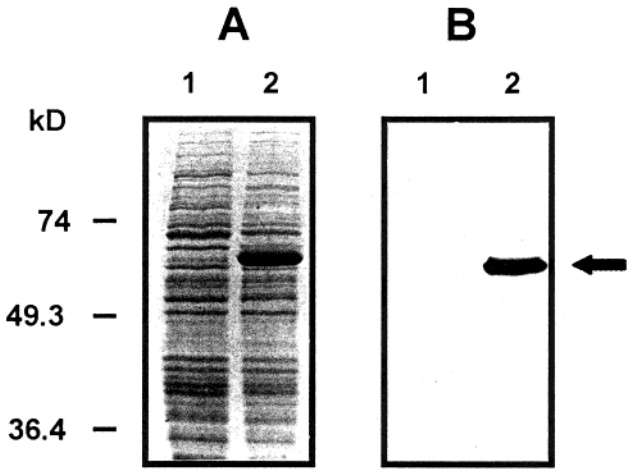

FIGURE 1: Overexpression of LmrA in *Lactococcus lactis* NZ9000. (A) Coomassie Brilliant Blue-stained SDS−PAGE gel (10%) with inside-out membrane vesicles (50 μg of protein/lane) prepared from *L. lactis* NZ9000/pNZ8048 control cells (lane 1) and LmrA expressing *L. lactis* NZ9000/pNHLmrA cells (lane 2). (B) Western blot of the SDS−PAGE gel shown in (A) using monoclonal antibodies directed against the histidine tag. The arrow indicates the position of the histidine-tagged LmrA protein.

dried under a nitrogen atmosphere and resuspended in ethanol at a total lipid concentration of about 15 mg/mL. Subsequently, the ethanol solution was slowly injected into 20 volumes of 50 mM potassium Hepes, pH 7.4, at room temperature under continuous vortexing. The resulting liposomes were dialyzed overnight at 4 °C against 100 volumes of 50 mM potassium Hepes, pH 7.4, and used within the next 48 h. NBD-phospholipid transport was measured in a cuvette containing 1.9 mL of 50 mM potassium Hepes, pH 7.4, 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, 5 mM phosphocreatine, and 50 μg of donor liposomes/mL. Mg-ATP, Mg-AMP-PNP, or Mg-ATP plus vinblastine were added to the buffer when appropriate (see the legend of Figure 6 for additional details). Transport was initiated after 1 min by the addition of proteoliposomes to the cuvette at a final protein concentration of 0.1 μg of membrane protein/mL and a donor liposome/proteoliposome ratio of 5. NBD fluorescence [excitation 475 nm (15 nm slit width), emission 530 nm (4 nm slit width)] was monitored at 0.1 s intervals under continuous stirring, at a constant temperature of 30 °C.

RESULTS

Overexpression and Quantification of the Expression Levels of Histidine-Tagged LmrA. The *lmrA* gene and the upstream coding regions for an N-terminal six-histidine tag and an enterokinase cleavage site were amplified from pCHLmrA by PCR (*14*). *Nco*I and *Xba*I sites were introduced at the 5′ and 3′ ends of the DNA fragment to allow cloning downstream of the nisin-inducible *nisA* promoter in pNZ8048 (*24*), yielding pNHLmrA. The addition of nisin A to *L. lactis* NZ9000 harboring pNHLmrA resulted in the synthesis of histidine-tagged LmrA with an expected molecular mass of 66 kDa (Figure 1). Control cells, harboring the empty vector, did not show a signal on the Western blot.

To determine the expression levels of LmrA in inside-out membrane vesicles prepared of LmrA-overexpressing cells, two methods were used. The amount of LmrA was determined by quantitative immunoblotting. Different dilutions of total membrane protein and pure LmrA protein were made and dotted on a PVDF membrane. After immunochemical staining with anti-histidine tag antibody, the blots were scanned, and a calibration curve was made using the pure protein. From five independent experiments, the level of LmrA was estimated to be 30 ± 5% of total membrane protein. In the second method, the amount of LmrA in membrane vesicles was determined by densitometry of a Coomassie-brilliant blue-stained SDS−PAGE gel of total membrane protein of *L. lactis* NZ9000/pNHLmrA. In this case, the amount of LmrA was estimated to be about 35% of total membrane protein (data not shown).

Ethidium Bromide Accumulation in L. lactis Cells. Ethidium accumulation was measured indirectly by monitoring the fluorescence of the intracellular ethidium−polynucleotide complex (Figure 2A). Glucose-energized *L. lactis* NZ9000 cells overexpressing LmrA showed a lower accumulation of ethidium, and, hence, a higher rate of ethidium efflux than the control strain (NZ9000/pNZ8048). In the presence of the LmrA inhibitor reserpine (*10*), the ethidium fluorescence in LmrA-expressing cells quickly increased to the same level as observed in control cells (Figure 2A), demonstrating the involvement of LmrA in the ethidium efflux process.

Hoechst-33342 Transport in Membrane Vesicles. The amphiphilic drug Hoechst-33342 is transported by several MDR proteins (*14*, *25*, *26*). The probe is only fluorescent when it is present in the lipid environment of biomembranes, and essentially nonfluorescent in the aqueous phase (*25*). Transport experiments performed in inside-out membrane vesicles of *L. lactis* NZ9000 cells showed enhanced transport of Hoechst-33342 in membrane vesicles harboring LmrA in the presence of ATP but not in the presence of ATP-γ-S (Figure 2B). ATP-dependent transport of Hoechst-33342 was not observed in control membrane vesicles (data not shown). The transport of Hoechst-33342 was inhibited by the addition of verapamil, a compound which was shown to inhibit P-glycoprotein and LmrA (*14*). These observations indicate that the histidine-tagged LmrA protein is functional in whole cells of *L. lactis* NZ9000 and in isolated inside-out membrane vesicles derived thereof.

Solubilization and Purification of LmrA. The detergents octyl glucoside, DDM, $C_{10}E_8$, and TX100 were examined for their ability to solubilize LmrA from *L. lactis* NZ9000/pNHLmrA membranes: 1% (w/v) dodecylmaltoside (DDM) and 1% (v/v) Triton X-100 solubilized 91% and 87% of LmrA, respectively, whereas 1% (w/v) octyl glucoside and $C_{10}E_8$ solubilized only 72% and 58%, respectively (data not shown). In view of the inhibition of the transport activity of the lactococcal multidrug transporter LmrP (*26*) and other multidrug transporters (*27*) by Triton X-100, DDM was selected for the purification of LmrA.

The purification was carried out in a single affinity chromatography step. DDM-solubilized membranes were mixed with Ni-NTA resin and incubated for 1 h at 4 °C under continuous shaking. The resin was subsequently washed with buffers containing 10 and 40 mM imidazole, to remove impurities. The protein was eluted by increasing the imidazole concentration to 150 mM. Samples of solubilized membranes, fractions collected at different steps of the purification procedure, and purified protein were analyzed by SDS−PAGE (Figure 3). Analysis of SDS−PAGE gels

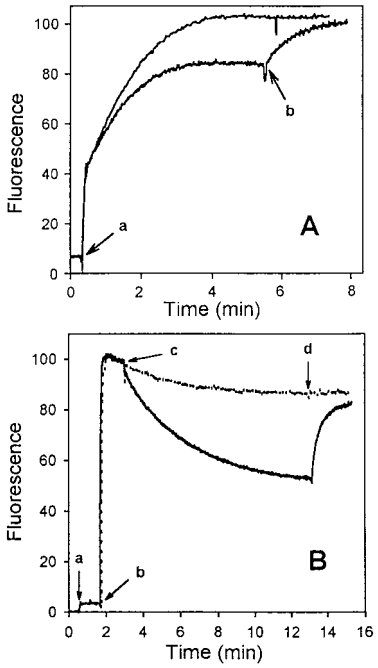

FIGURE 2: Overexpressed LmrA is functional in *Lactococcus lactis* NZ9000. LmrA-mediated drug transport was measured by fluorometry. Panel A: Accumulation of ethidium bromide in cells. Ethidium accumulation in *L. lactis* NZ9000 harboring pNHLmrA (lower line) or pNZ8048 (upper line). The cells were preenergized by 25 mM glucose. The following additions were made to cells expressing LmrA and to control cells: (a) ethidium bromide to a final concentration of 2 μM; (b) reserpine to a final concentration of 50 μM, to inhibit LmrA activity. Panel B: Hoechst-33342 transport in inside-out membrane vesicles prepared from LmrA-expressing cells. The experiments were measured by fluorometry. The experiments were performed in 50 mM potassium Hepes, pH 7.4, containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. Protein (final concentration of 0.5 mg/mL) was added after 30 s of incubation (a). Hoechst-33342 (2 μM) was added after 1 min (b). Mg-ATP (c, solid line) and Mg-ATP-γ-S (c, dotted line) were added after 2 min to a final concentration of 2 mM. Verapamil (d) was added to LmrA-containing membrane vesicles to a final concentration of 50 μM in order to inhibit the Hoechst-33342 transport mediated by LmrA. Hoechst-33342 transport in inside-out membrane vesicles prepared from control cells without overexpressed LmrA was not observed under the same assay conditions (data not shown).

and immunoblots by densitometry showed that full-length LmrA can be isolated at levels of approximately 97% of purity (data not shown). The eluted fraction containing histidine-tagged LmrA was typically at a concentration of 1.2 mg/mL, and the procedure yielded about 1 mg of LmrA from 15 mg of total membrane protein.

Reconstitution of LmrA into Liposomes. Purified LmrA was immediately reconstituted in liposomes by mixing the protein with DDM-destabilized liposomes containing *L. lactis* NZ9000 phospholipids. Subsequently, DDM was removed by adsorption on polystyrene beads. The incorporation of the protein into the liposomal membranes was assessed by sucrose gradient centrifugation. Proteoliposomes were applied on the top of the gradient, and, after centrifugation, the position of the membranes in the sucrose density gradient was determined by measurements of the phosphate content of the collected fractions. The position of the histidine-tagged LmrA protein in the sucrose density gradient was determined by Western blotting. Reconstitution of LmrA in the liposomes is evident from the co-centrifugation of LmrA and the lipid

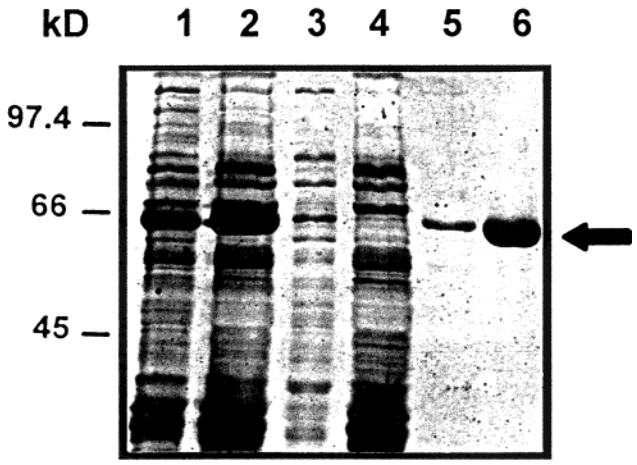

FIGURE 3: Purification of histidine-tagged LmrA. Silver-stained SDS−PAGE gel (10%) showing the intermediate steps in the solubilization/purification procedure of histidine-tagged LmrA. Lane 1, total membrane protein of *L. lactis* NZ9000/pNHLmrA; lane 2, supernatant containing DDM-solubilized protein; lane 3, DDM-insoluble membrane proteins; lanes 4 and 5, protein eluted from the Ni-NTA resin during the washing with 10 mM imidazole (lane 4) and 40 mM imidazole (lane 5); lane 6, purified histidine-tagged LmrA eluted from a Ni-NTA column with 150 mM imidazole. The arrow indicates the position of LmrA.

fraction (data not shown). It is interesting to note that no free protein was detected at the bottom of the gradient, indicating that all LmrA molecules were inserted into the liposomal membranes during reconstitution.

Orientation of Reconstituted LmrA Protein. The orientation of LmrA protein incorporated into the proteoliposomes was determined by monitoring the accessibility to enterokinase of the enterokinase cleavage site located behind the histidine tag in His_6-LmrA. The histidine tag which remained attached to LmrA after enterokinase cleavage was detected by Western blotting using an anti-histidine tag antibody. When proteoliposomes were sonicated in the presence of enterokinase, to allow the exposure to enterokinase of both the external and luminal sides of the proteoliposomes, less than 10% of the histidine tag remained attached to LmrA compared to the signal obtained for control incubations lacking enterokinase. Without sonication of the proteoliposomes, only the external side of the proteoliposomes was accessible for enterokinase. Under these conditions, 45% of the histidine tags remained present after enterokinase treatment (Figure 4). These data suggest that about half of the enterokinase cleavage sites were present at the internal surface of the proteoliposomes, and half of the enterokinase cleavage sites were present on the external surface of the proteoliposomes. Thus, histidine-tagged LmrA protein adopts a 50% in, 50% out orientation in the proteoliposomes.

Hoechst-33342 Transport in Proteoliposomes. To check the functionality of the reconstituted LmrA protein, the fluorescent probe Hoechst-33342 was used (Figure 5). In the presence of ATP, the Hoechst-33342 fluorescence in LmrA-containing proteoliposomes decreased more rapidly than in the presence of the slowly hydrolyzable ATP analogue ATP-γ-S. ATP-dependent changes in Hoechst-33342 fluorescence were not observed in liposomes lacking LmrA. Because Hoechst-33342 is only fluorescent in the membrane, a decrease in the fluorescence intensity of Hoecht-33342 represents a decrease in the amount of membrane-associated

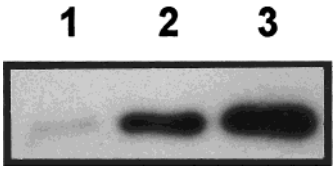

FIGURE 4: Orientation of LmrA in proteoliposomes. The orientation of reconstituted LmrA in proteoliposomes was determined by cleavage of the histidine tag, using the enterokinase cleavage site located between the tag and LmrA, and the detection of residual histidine-tagged LmrA by Western blotting using anti-histidine-tag antibody. Lane 1, sample of proteoliposomes sonicated 3 times for 5 s and subsequently incubated at 21 °C for 16 h in the presence of 2 units of enterokinase; lane 2, nonsonicated proteoliposomes incubated at 21 °C for 16 h in the presence of 2 units of enterokinase; lane 3, nonsonicated proteoliposomes without enterokinase treatment.

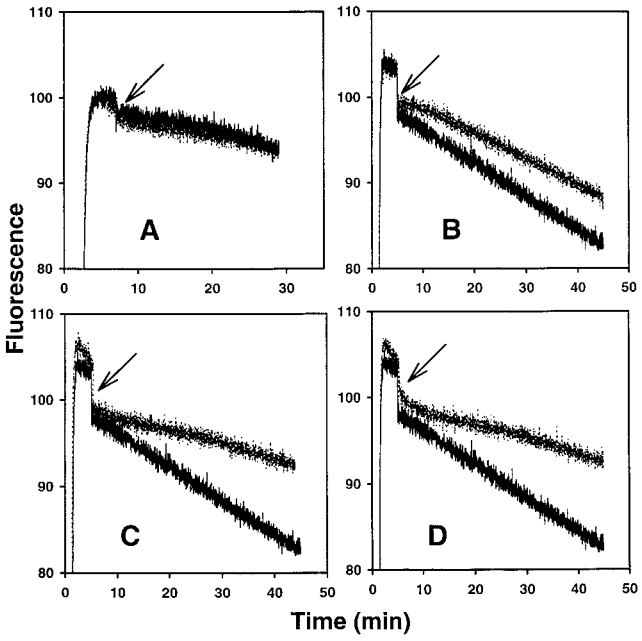

FIGURE 5: Hoechst-33342 transport in proteoliposomes. Proteoliposomes were diluted to a final protein concentration of 5 μg/mL in 2 mL of 50 mM potassium Hepes, pH 7.4, containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. Hoechst-33342 and Mg-ATP or Mg-ATP-γ-S were added to a final concentration of 1.25 μM, 1.25 mM, and 1.25 mM, respectively. Panel A: Hoechst-33342 fluorescence for control liposomes, without LmrA, after addition of Mg-ATP (solid line) or Mg-ATP-γ-S (dotted lines). Panel B: Hoechst-33342 fluorescence for proteoliposomes containing LmrA, after addition of Mg-ATP (solid line) or Mg-ATP-γ-S (dotted lines). Panel C: Hoechst-33342 fluorescence for proteoliposomes containing LmrA after addition of Mg-ATP in the absence (solid line) or presence (dotted line) of 100 μM sodium orthovanadate. Panel D: Hoechst-33342 fluorescence for proteoliposomes containing LmrA after addition of Mg-ATP in the absence (solid line) or presence (dotted line) of 50 μM verapamil. The arrow indicates the addition of Mg-ATP or Mg-ATP-γ-S.

Hoecht-33342. The calculation of the initial concentration of membrane-associated Hoechst-33342 in the proteoliposomes, from the total amount of Hoechst-33342 and liposomes added, and the previously determined membrane/water partition coefficient of 5100 (*28*), allowed the conversion of fluorescence intensity units into a concentration of membrane-associated Hoechst-33342. By this method, a transport rate of 0.5 nmol (mg of membrane protein)$^{-1}$ min^{-1} was obtained for ATP-dependent, LmrA-mediated transport under the experimental conditions.

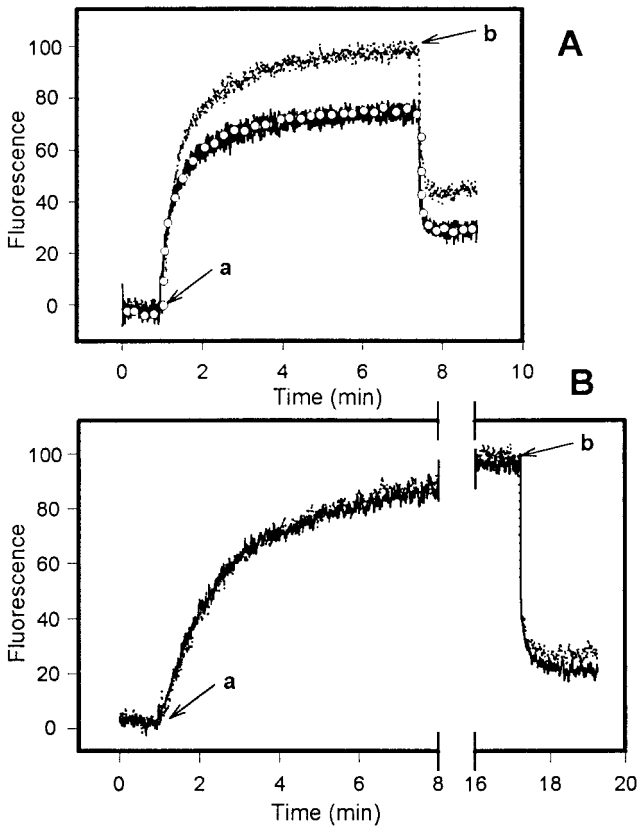

FIGURE 6: Transport of fluorescent phospholipids from donor liposomes to LmrA-containing proteoliposomes. Donor vesicles containing trace amounts of the NBD fluorescence quencher N-Rh-PE and the fluorescent lipid analogues C_6-NBD-PE (panel A) or C_6-NBD-PC (panel B) were diluted at the onset of the experiment in 2 mL of 50 mM potassium Hepes, pH 7.4, containing 2 mM $MgSO_4$, 8.5 mM NaCl, 0.1 mg/mL creatine kinase, and 5 mM phosphocreatine. At arrow a, proteoliposomes were added at a final concentration of 0.1 μg of membrane protein/ mL. Panel A: NBD-PE fluorescence in the presence of 1.25 mM Mg-ATP (dotted line), 1.25 mM Mg-AMP−PNP (black line), or 1.25 mM Mg-ATP plus 10 μM vinblastine (open circles). Panel B: NBD-PC fluorescence in the presence of 1.25 mM Mg-ATP (dotted line) or 1.25 mM Mg-AMP−PNP (black line). At arrow b, 2 mM $CoCl_2$ was added in order to quench the fluorescence of lipid present in the outer leaflet of the proteoliposomes.

The transport activity of LmrA in proteoliposomes was completely inhibited by 100 μM sodium orthovanadate, an inhibitor of LmrA (*10*) and of other ABC transporters and P-type ATPases. In addition, LmrA-mediated Hoechst-33342 transport in proteoliposomes was completely inhibited in the presence of 50 μM of the modulator verapamil. Taken together, these data suggest that reconstituted LmrA protein is able to pump the fluorescence probe Hoechst-33342 out of the proteoliposomal membrane in an ATP-dependent manner.

Transbilayer Movement of Fluorescent Phospholipids in Proteoliposomes. The ability of reconstituted LmrA to mediate the transbilayer movement of fluorescent C_6-NBD-PE or C_6-NBD-PC was investigated (Figure 6). The NBD-labeled lipids were introduced into LmrA-containing proteoliposomes from synthetic donor liposomes containing the NBD fluorescence quencher N-Rh-PE, which itself is unable to transfer from the donor liposomes to proteoliposomes. The addition of proteoliposomes to a suspension of donor liposomes resulted in a biphasic development of the NBD-PE fluorescence: an initial phase in which the NBD

fluorescence increased rapidly was followed by a second phase in which the NBD fluorescence increased at a slower rate (Figure 6A). As was established previously (*29*), the first phase reflects the rapid transfer of NBD-labeled phospholipid from the outer leaflet of the donor liposomes to the outer leaflet of proteoliposomes, whereas the second phase corresponds to the transbilayer movement of the fluorescent phospholipids from the outer leaflet to the inner leaflet of the proteoliposomes, and further transfer of NBD-labeled phospholipid from donor liposomes to proteoliposomes. After the distribution of fluorescent C_6-NBD-PE between donor liposomes and proteoliposomes had reached an equilibrium, the addition of 2 mM $CoCl_2$, a collisional quencher of NBD fluorescence, resulted in a quenching of the NBD fluorescence of about 50% (Figure 6A). The presence of higher concentrations of $CoCl_2$, up to 20 mM, did not further reduce the NBD fluorescence (data not shown). The cobalt quenching experiment demonstrates the existence of two pools of C_6-NBD-PE in the proteoliposomes of roughly the same size, one pool in the inner leaflet being inaccessible to cobalt ions and one pool in the outer leaflet being accessible to cobalt ions.

In the presence of ATP, C_6-NBD-PE was accumulated to a significantly higher level in the LmrA-containing proteoliposomes than in the presence of the nonhydrolyzable ATP analogue AMP-PNP (Figure 6A). This difference in C_6-NBD-PE accumulation was not observed when 10 μM vinblastine, a high-affinity substrate of LmrA (*14*), was included in the assay mixture. The addition of 2 mM $CoCl_2$ to incubations containing ATP again resulted in the quenching of NBD fluorescence by about 50%, suggesting that the concentration of C_6-NBD-PE was increased in both membrane leaflets of the proteoliposomes (Figure 6A). Taken together, these data strongly suggest that C_6-NBD-PE is a substrate for LmrA.

Interestingly, a different result was obtained for C_6-NBD-PC. Cobalt quenching experiments suggested that this lipid analogue distributed asymmetrically between the outer leaflet and inner leaflet of the proteoliposomes, with about 75% outside−25% inside (Figure 6B). Furthermore, no difference was observed between the NBD fluorescence development in the presence of ATP or AMP-PNP, suggesting that C_6-NBD-PC is not a substrate of LmrA.

DISCUSSION

Multidrug transporters mediate the transmembrane movement of drugs with very different chemical structures and cellular targets, and, hence, are able to overcome cell cytotoxicity by lowering the cytoplasmic drug concentration (*7*). To study the structure, function, and mechanism of multidrug transporters at the molecular level, it is invaluable to have the proteins in a pure and active form. In this work, we have established an efficient method for the overexpression, purification, and reconstitution of histidine-tagged LmrA. The nisin-controlled gene expression system (NICE), based on the *L. lactis nisA* promoter (*24, 26*), was successfully used to express LmrA up to levels of about 30−35% of total membrane protein. This overexpressed protein is functional. The accumulation of ethidium was significantly reduced in LmrA-expressing *L. lactis* cells, and the fluorescent probe Hoechst-33342 was transported in an ATP-dependent manner by LmrA in inside-out membrane vesicles derived from these cells.

The solubilization of membrane proteins requires that the proteins are taken from their natural environment and that the phospholipid bilayer is replaced by an artificial environment of the detergent micelle (*30, 31*). The choice of the detergent used for the solubilization (and purification) of multidrug transport proteins is crucial (*18, 32*). Due to their amphiphilic character, detergents are potential substrates of multidrug transporters. Recently, it was demonstrated that the transport activity of the secondary lactococcal MDR LmrP, which has similar substrate specificity as LmrA (*33*), is inhibited by low concentrations of Triton X-100, Triton X-114, and Tween 80 (*26*). In view of the lack of inhibition of LmrA-mediated drug transport by low concentrations of DDM, and the excellent solubilization of the protein by DDM, this detergent was used in further work. Although the addition of phospholipids, osmolites (often glycerol), or substrates has been shown to stabilize membrane proteins in a detergent-solubilized state (*34*−*36*), none of these additions was found to be essential for the activity of reconstituted LmrA. The purification of LmrA to levels of approximately 97% of purity was achieved by applying the histidine peptide fusion technique (*37*) and the use of a single nickel-chelate affinity chromatography step.

The pure LmrA protein was reconstituted into DDM-destabilized proteoliposomes prepared from *L. lactis* lipids. The insertion of a membrane protein into detergent-destabilized liposomes has been shown to result in proteoliposomes with a unidirectional protein insertion while in proteoliposomes formed by coalescence of mixed micelles containing lipid, protein, and detergent a scrambled orientation of the protein is prevalent (*38, 39*). A possible explanation is that proteins with a large hydrophilic domain, such as the H^+-ATPase and Ca^{2+}-ATPase, are inserted into the membrane with their hydrophobic domains first (*15, 40*), and that the hydrophilic domain of these proteins acts like an anchor that prevents random insertion. However, site-specific proteolysis of the N-terminal histidine tag site in LmrA suggest a 50% in, 50% out orientation of purified LmrA in the proteoliposomes. Our results are consistent with previous studies on the reconstitution of the lactose permease LacS from *Streptococcus thermophilus*, in which DDM-solubilized LacS protein inserted in a random orientation in DDM-destabilized liposomes (*18*). Analysis of the structure of DDM-destabilized liposomes revealed that both the inner and outer membrane surfaces are accessible to solubilized membrane proteins (*32*).

The fluorescent substrate Hoechst-33342 was used to assess the transport activity of the reconstituted LmrA protein. This compound has been used in activity measurements of reconstituted drug transporters such as P-glycoprotein (*25*) and LmrP (*26*). ATP-dependent transport of Hoechst-33342 was observed in proteoliposomes containing reconstituted LmrA. Similar observations were made by Shapiro et al. (*25, 41*) for reconstituted P-glycoprotein and by Putman et al. (*26*) for reconstituted LmrP. The LmrA-mediated transport was inhibited by orthovanadate and the chemosensitizer verapamil.

It is known that certain ABC transporters are able to translocate lipids. The mouse Mdr2 (*42*) and mammalian MDR3 (*43*) both function as a phosphatidylcholine translo-

cator. The mammalian ABC transporter ABC1 mediates the transbilayer movement of phosphatidylserine (*44*). Furthermore, the LmrA homologue MsbA mediates the translocation of core-lipid A in *E. coli* (*45*). We have tested the specificity of reconstituted LmrA for C_6-NBD-labeled PE and PC. To our surprise, LmrA was able to mediate the ATP-dependent transport of C_6-NBD-labeled PE, but not of C_6-NBD-labeled PC. Although the fluorescent NBD group may play a role in the recognition of these lipid analogues by LmrA, our results do suggest a specificity of LmrA for the phospholipid headgroup. Our observations support the notion that LmrA may be involved in the transport of specific lipids or lipid-linked precursors in *L. lactis.*

To date, several ABC-type MDR transporters have been overexpressed in homologous and heterologous systems. P-glycoprotein has been overexpressed in yeast (*46*) and in murine lymphoid leukemia P388/ADR25 cells (*47*) up to levels of 7% and 30% of total membrane protein, respectively. MRP1 has been overexpressed at high levels in baby hamster kidney (BHK-21) cells (*48*). However, to our knowledge, the expression of a membrane protein in a prokaryotic expression system to a level as high as 30−35% of total membrane protein, as obtained in our study, has not been reported previously.

This study provides an efficient procedure to obtain highly purified LmrA, functionally reconstituted into artificial phospholipid bilayers prepared from *L. lactis* lipids. The availability of straightforward methods for overexpression and purification of bacterial LmrA, the short generation time, and the ease of mutant selection and DNA manipulation associated with *L. lactis* make LmrA an attractive ABC transporter for future studies on structure/function relationships in P-glycoprotein-like MDR systems. In addition, the methods described in this work may be useful for the overexpression in *L. lactis* of other pro- and eukaryotic ABC transporters.

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