The Multidrug Transporter LmrP Protein Mediates Selective Calcium Efflux*

Received for publication, April 19, 2012, and in revised form, June 8, 2012. Published, JBC Papers in Press, June 22, 2012, DOI 10.1074/jbc.M112.372334

Theresia A. Schaedler, Zhen Tong, and Hendrik W. van Veen

From the Department of Pharmacology, University of Cambridge, Cambridge, United Kingdom

Background: The multidrug transporter LmrP confers drug resistance on cells by mediating efflux of structurally dissimilar cytotoxic substrates.

Results: Surprisingly, LmrP catalyzes the selective, high affinity binding and extrusion of Ca\(^{2+}\), which inhibits multidrug transport by LmrP.

Conclusion: LmrP can act as a calcium/proton antiporter.

Significance: Multidrug transporters might fulfill additional physiological roles, which could promote their persistence in the absence of antibiotics.

LmrP is a major facilitator superfamily multidrug transporter from *Lactococcus lactis* that mediates the efflux of cationic amphiphilic substrates from the cell in a proton-motive force-dependent fashion. Interestingly, motif searches and docking studies suggested the presence of a putative Ca\(^{2+}\)-binding site close to the interface between the two halves of inward facing LmrP. Binding experiments with radioactive \(^{45}\)Ca\(^{2+}\) demonstrated the presence of a high affinity Ca\(^{2+}\)-binding site in purified LmrP, with an apparent \(K_d\) of 7.2 \(\mu M\), which is selective for Ca\(^{2+}\) and Ba\(^{2+}\) but not for Mn\(^{2+}\), Mg\(^{2+}\), or Co\(^{2+}\). Consistent with our structure model and analogous to crystal structures of EF hand Ca\(^{2+}\)-binding proteins, two carboxylates (Asp-235 and Glu-327) were found to be critical for \(^{45}\)Ca\(^{2+}\) binding. Using \(^{45}\)Ca\(^{2+}\) and a fluorescent Ca\(^{2+}\)-selective probe, calcium transport measurements in intact cells, inside-out membrane vesicles, and proteoliposomes containing functionally reconstituted purified protein provided strong evidence for active efflux of Ca\(^{2+}\) by LmrP with an apparent \(K_i\) of 8.6 \(\mu M\) via electrogenic exchange with three or more protons. These observations demonstrate for the first time that LmrP mediates selective calcium/proton antiport and raise interesting questions about the functional and physiological links between this reaction and that of multidrug transport.

Multidrug transporters are fascinating proteins that mediate the extrusion of structurally dissimilar chemotherapeutic agents away from their targets in the cell. The expression of multidrug exporters can significantly contribute to the development of drug resistance among (pathogenic) microorganisms. LmrP is a well studied member of the major facilitator superfamily that can transport a wide range of amphiphilic cationic drugs from *Lactococcus lactis* (1). Previous work indicates that LmrP exports monovalent cationic ethidium by electrogenic exchange with protons (2) in a reaction that is dependent on the transmembrane H\(^+\) gradient (\(\Delta pH\)) and membrane potential (\(\Delta \psi\)) components of the proton-motive force (\(\Delta \mu\)).

To further investigate this exchange reaction, an inward facing three-dimensional homology model of LmrP was constructed that was based on the crystal structure of the glycerol-3P/P\(_i\) antiporter GlpT from *Escherichia coli* (3). In this model, LmrP is predicted to contain an internal cavity formed at the interface between the two halves of the transporter. On the surface of this cavity lie two clusters of polar, aromatic, and carboxyl residues with potentially important functions in proton shuttling and substrate interactions (3). Cluster 1 in the C-terminal half contains Asp-235 and Glu-327 in immediate proximity (<3.5 Å) of each other and is located near the apex of the cavity, whereas Cluster 2 in the N-terminal half contains Asp-142. Mutational analyses of these carboxylates suggested that both clusters act as separate proton conduction points (3) by a mechanism in which the carboxylates are protonated in the outward facing conformation and deprotonated in the inward facing conformation.

Recent studies on the energetics of ethidium\(^{2+}\) and propidium\(^{2+}\) transport by LmrP point to a variable proton-substrate stoichiometry, which is thought to be related to substrate-dependent changes in the geometry and distance between Asp-235 and Glu-327 in the inward facing substrate-binding chamber (4). During transport of ethidium, binding of this substrate from the inside surface would decrease the proximity between the side chains of Glu-327 and Asp-235, thus allowing the formation of a carboxyl-carboxylate pair containing Asp-235 as a single proton release site that is stabilized through hydrogen bonding with undissociated Glu-327. In contrast, during the binding of propidium, the side chains of Glu-327 and Asp-235 would not directly interact with each other, and both carboxylates would function as independent proton release sites (4). The observation that in many other secondary active multidrug transporters, catalytic carboxylates are located too far away from each other to directly interact raised the question of why Asp-235 and Glu-327 are localized in close proximity in LmrP. Here, we describe (i) in silico analyses

---

*This work was supported by the Biotechnology and Biological Sciences Research Council.

1 Recipient of awards from Pembroke College, the Cambridge European Trusts, and the Kurt Hahn Trust.

2 To whom correspondence should be addressed: Dept. of Pharmacology, University of Cambridge, Tennis Court Rd., Cambridge CB2 1PD, UK. Tel.: 44-1223-765295; Fax: 44-1223-334100; E-mail: hww20@cam.ac.uk.
suggesting that Asp-235 and Glu-327 are part of a metal ion binding site with selectivity for Ca\(^{2+}\) and (ii) our experimental analyses demonstrating that LmrP mediates the selective binding and proton-coupled efflux of Ca\(^{2+}\).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—** *L. lactis* strain NZ9000 ΔlmrA ΔlmrCD (5), harboring empty expression vector pNZ8048 (6) or derivatives encoding C-terminally His\(_{6}\)-tagged WT LmrP (pHLPS) (2) or His\(_{6}\)-tagged double D235N/E327Q (DE) mutant LmrP (3, 4) downstream of a nisin A inducible promoter, was grown at 30 °C in M17 Broth (Oxoid) supplemented with 0.5% glucose and 5 μg/ml chloramphenicol. Medium was inoculated with a 1:50 dilution of an overnight culture, and cells were grown to an _A_\(_{660}\) of 0.5–0.6. Expression of LmrP proteins was then induced by previously described methods (3, 4) for 1 h at 30 °C in the presence of 0.001% (v/v) of nisin A-containing supernatant of the nisin-producing strain *L. lactis* NZ9700 (6).

**Preparation of Inside-out Membrane Vesicles**—The cells were harvested by centrifugation at 13,000 × _g_ for 10 min at 4 °C. The pellet was washed with 50 ml of ice-cold 100 mM K-HEPES (pH 7.0). The cells were resuspended in 25 ml of 100 mM K-HEPES (pH 7.0) containing 2 mg/ml of lysozyme and one tablet of Complete protease inhibitor (Roche Applied Science) and incubated at 30 °C for 30 min. The cells were disrupted by passing them twice through a Basic Z 0.75-kilowatt Benchtop Cell Disruptor (Constant Systems, Northants, UK) at 20,000 p.s.i. Disrupted cells were incubated for 30 min in the presence of 10 μg/ml DNase, 2 μg/ml RNase, and 10 mM MgSO\(_4\). K-EDTA was added to a final concentration of 15 mM. Cell debris and undisrupted cells were removed by centrifugation at 13,000 × _g_ for 15 min at 4 °C. Inside-out membrane vesicles were harvested by centrifugation of the supernatant at 125,000 × _g_ for 30 min, resuspended in 100 mM K-HEPES (pH 7.0) containing 10% glycerol, and stored in liquid nitrogen. Protein concentration was determined using the Bio-Rad DC assay kit, and expression of the His\(_{6}\)-tagged proteins was confirmed on Western blot probed with anti-His\(_{6}\) tag antibody (Sigma-Aldrich).

**Protein Purification**—Inside-out membrane vesicles (40–60 mg of total protein) were solubilized in 7.5 ml of solubilization buffer (50 mM K-HEPES buffer, pH 8.0, containing 100 mM NaCl, 10% (v/v) glycerol, and 1.5% β-D-decyl maltoside (DDM))\(^3\) (Melford). The solubilization mix was incubated on a rotating wheel at 4 °C. Unsolubilized particles were removed by centrifugation at 125,000 × _g_ for 30 min at 4 °C, and the supernatant was immediately used in subsequent steps. For purification of the protein by nickel-affinity chromatography 400 μl of nickel-nitrilotriacetic acid (Ni-NTA)-agarose resin suspension (50% (w/w) in 30% (v/v) ethanol) (Sigma-Aldrich) was used. The resin was first equilibrated by washing three times with 5 ml of Milli-Q water and twice with 5 ml of Buffer A (50 mM K-HEPES, pH 8.0, containing 100 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, and 20 mM imidazole). The solubilized protein was added to the resin, after which the suspension was incubated on a rotating wheel at 4 °C overnight. The resin was washed five times with 5 ml of Buffer A and then six times with 5 ml of Buffer B (50 mM K-HEPES, pH 7.0, containing 100 mM NaCl, 10% (v/v) glycerol, 0.05% (w/v) DDM, and 20 mM imidazole). The buffer was discarded, and the resin was transferred to a Bio-spin column (Bio-Rad) and eluted with 500 μl elution buffer (Buffer B supplemented with 200 mM imidazole and 5% glycerol). As a control protein in drug binding studies, the His\(_{6}\)-tagged galactose-H\(^{+}\) symporter GalP from _E. coli_ (7) was solubilized using the solubilization buffer as described above containing 300 mM NaCl instead of 100 mM (4, 8). The protein was purified on Ni-NTA resin by the method as described for LmrP using salt-free elution buffer. Protein concentrations were determined using the Micro-BCA assay kit (Pierce). The purity of protein samples was checked on Coomassie-stained SDS-PAGE and quantified by densitometric analysis of individual lanes using ImageJ software, version 1.43 (National Institutes of Health).

**Substrate Binding to Purified Protein**—WT LmrP, DE mutant LmrP, and GalP were purified as described under “Protein Purification” and diluted to a final concentration of 400 μg/ml. For Ca\(^{2+}\) binding measurements, samples contained 10 μg of purified protein each and were prepared in 50 mM Tris-Cl at the pH indicated in the legend to Fig. 3, in a total volume of 500 μl. SDS-PAGE was run to confirm that the amount of protein in each samples was identical. 45CaCl\(_2\) (1.66 or 1.04 GBq/mg; PerkinElmer Life Sciences) was added with 0.57 TBq/mmol for the single concentrations as indicated in legend to Fig. 3 and 0.12–0.57 TBq/ml for the concentration range in the kinetic experiment in Fig. 3B, after which the samples were incubated at room temperature for 30 min under mild shaking. The samples were subsequently filtered over nitrocellulose filters (Whatman; 0.2-μm diameter) using a vacuum pump to enable rapid filtration, during which the protein strongly binds to the filter and is separated from buffer containing free 45Ca\(^{2+}\). Filters were immediately washed twice with 3 ml of ice-cold 50 mM Tris-Cl of the same pH as the assay buffer used for the experiment. The filters were transferred to tubes containing scintillation Ultima Gold XR (PerkinElmer Life Sciences) and subjected to liquid scintillation counting. Binding data were corrected for binding of 45Ca\(^{2+}\) to the filters.

In Hoescht 33342 binding assays, 25 μg of purified protein was added to 2 ml of 100 mM K-HEPES (pH 7.0) containing 5 mM MgSO\(_4\) and CaCl\(_2\) at concentrations as indicated in Fig. 7, in a cuvette under mild stirring in a LS 55B fluorescence spectrometer (PerkinElmer). Hoescht 33342 was added in a stepwise manner (2 × 0.125 μM followed by 1 × 0.25 μM and 15 × 0.5 μM) every 20 s. Fluorescence was monitored over time until a plateau phase was reached. Hoescht 33342 fluorescence was monitored at excitation and emission wavelengths of 355 and 457 nm, respectively, with a slit width of 10 nm for emission and 5 nm for excitation. Elution buffer was used as a control for background binding to components of the buffer, and purified GalP served as a control for nonspecific binding.

**Ca\(^{2+}\) Transport in Intact Cells—** *L. lactis* cells were grown to an _A_\(_{660}\) of 0.4–0.6 at 30 °C, after which LmrP proteins were
expressed as described under “Bacterial Strains, Plasmids, and Growth Conditions.” The cells were subsequently harvested by centrifugation at 6,500 × g for 8 min at 4 °C, washed three times with ice-cold 50 mM K-HEPES (pH 7.0), and then resuspended in the same buffer to an A₆₆₀ of 5.0. The cells were preloaded with 0.5 μM Fura-2-AM (Invitrogen) and 250 μM CaCl₂ (when indicated) for 1.5 h at 30 °C under shaking. The cells were subsequently washed three times with ice-cold 50 mM K-HEPES (pH 7.0) in the same volume that was used for resuspension to remove any remaining extracellular Fura-2-AM. The cells were diluted 40-fold into a cuvette containing 2 ml of 50 mM K-HEPES (pH 7.0) containing 1.8 mM probenecid to inhibit endogenous anion efflux activity (9–12) and block background export of Fura-2 from L. lactis cells. Upon the addition of 25 mM glucose, Fura-2 fluorescence was monitored at an excitation wavelength of 340 nm and emission wavelength of 510 nm with slit widths of 5 or 10 nm, respectively.

Substrate Transport in Inside-out Membrane Vesicles—Inside-out membrane vesicles containing WT LmrP, DE mutant LmrP, or no LmrP protein (control) were prepared as described under “Preparation of Inside-out Membrane Vesicles.” The samples contained 1 mg/ml membrane vesicles, 2.5 mM ATP, 0.1 mg/ml creatine kinase, 5 mM phosphocreatine, and, where indicated, ionophores nigericin and/or valinomycin to a final concentration of 0.5 μM each in a buffer composed of 10 mM Tris-Cl, 10 mM KPi, and 150 mM KCl (pH 7.5) in a total volume of 500 μl. The samples were prepared in sulfuric acid-washed glass tubes and incubated for 3 min before start of the measurements. ⁴⁵CaCl₂ (0.57 TBq/mol) was then added at concentrations as given in Fig. 5 and its legend. The samples were filtered over nitrocellulose filters (Whatman; 0.45 μm) to allow binding of the membrane vesicles to the filters, after which the filters were washed with 3 ml of ice-cold 100 mM LiCl to eliminate any remaining extracellular Fura-2-AM. The cells were subsequently harvested by centrifugation at 3,000 × g for 10 min. The supernatant was completely evaporated in a rotation evaporator at 25 °C. The lipids were then dissolved in chloroform and transferred into a preweighed glass tube to allow accurate dilution of the lipid in chloroform to a concentration of 100 mg/ml. The lipid solution was kept at −20 °C in the dark under a N₂ atmosphere.

Transport Measurements in Proteoliposomes—WT LmrP and DE mutant LmrP were purified as described under “Protein Purification.” Reconstitution was performed using a combination of previously published methods (13, 14). Phospholipids (1.5 mg) were dried by evaporation under a stream of N₂ gas. Liposomes were prepared by sonication for 8 min in 2.7 ml of 10 mM Tris-Cl (pH 7.4), 0.5 mM K-EDTA, 1 mM β-mercaptoethanol, and 75 mM NaCl. Detergent DDM was added to the lipid suspension in a 1:2 w/w lipid/detergent ratio. Purified LmrP proteins in elution buffer, or equal volume of elution buffer as a control, were added in a 1:10 protein/lipid ratio (w/w). The samples were incubated at 30 min at room temperature under gentle agitation. Subsequently, the detergent DDM was removed by two incubations with 80 mg of biobeads for 2 h at room temperature under mild stirring. The third incubation with 80 mg of biobeads was performed overnight at 4 °C. Proteoliposomes were collected by centrifugation at 18,000 × g for 30 min and resuspended in 3 ml of buffer I (10 mM Tris-Cl, 10 mM KPi, 150 mM KCl and 150 mM NH₄Cl, pH 6.8). The samples were frozen in liquid nitrogen and thawed at room temperature. Subsequently, the proteoliposomes were collected by centrifugation at 18,000 × g for 30 min, resuspended to a final concentration of 2 mg/ml in buffer I, and immediately used in an experiment.

For measurement of Hoechst 33342 transport in (proteo)liposomes in the presence of an imposed ΔpH (inside acidic) (Fig. 6A), proteoliposomes or empty liposomes (control) were diluted 200-fold in Buffer II (10 mM Tris-Cl, 10 mM KPi, 150 mM KCl, and 150 mM NaCl, pH 7.5) in a total reaction volume of 2 ml. After 40 s, Hoechst 33342 was added to a final concentration of 0.25 μM, and changes in fluorescence were recorded in a PerkinElmer LS 55B fluorimeter for 800 s at excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 10 and 5 nm, respectively.

Purification of E. coli Lipids for Reconstitution—Lipids from E. coli total lipid extract (Avanti Polar lipids) containing approximately 67% phosphatidylethanolamine, 23% phosphatidylglycerol, and 10% cardiolipin were purified before use. 200 mg of the lipid was weighed out and dissolved in 3 ml of chloroform. The lipid solution was added dropwise into ice-cold acetone perfused with liquid N₂ and containing 2 μl/ml β-mercaptoethanol. The mixture was stirred gently at 4 °C overnight, during which contaminants dissolved in the acetone phase. The insoluble lipids were collected by centrifugation (3,000 × g rpm, 20 min, 4 °C). The pellet was solubilized with 40 ml of diethyl ether containing 2 μl/ml β-mercaptoethanol and perfused with N₂. The tube was vortexed until the pellet was dissolved. Subsequently, the soluble lipid fraction was separated from insoluble contaminants by centrifugation at 3,000 × g for 10 min. The supernatant was completely evaporated in a rotation evaporator at 25 °C. The lipids were then dissolved in chloroform and transferred into a preweighed glass tube to allow accurate dilution of the lipid in chloroform to a concentration of 100 mg/ml. The lipid solution was kept at −20 °C in the dark under a N₂ atmosphere.
The C-terminal half of LmrP (Fig. 1). Membrane helix 8), which are all located in close proximity in oxygen from Ile-264 (transmembrane helix 8) and a cation-pi (transmembrane helix 9) in addition to the main chain carbonyl predicted distance and geometry that is analogous to that of two key carboxylates in the Ca2+ binding site of many EF hand Ca2+-binding proteins. Example of co-crystallized Ca2+-troponin C complex (Protein Data Bank 1A2X) at 2.3 Å resolution (45), in which, for clarity of presentation, only the positions of the two carboxyl residues (Asp-143 and Glu-150) are shown. The images were generated in MacPyMOL v1.3.

as described under “Substrate Transport in Inverse-out Membrane Vesicles” and washed two times with 3 ml of ice-cold 10 mM KPi, 10 mM Tris-Cl, 150 mM KCl (pH 7.5). Filters were transferred into scintillation vials, and the radioactivity was determined.

RESULTS

In Silico Prediction of a Ca2+-binding Site in LmrP—LmrP contains two acidic residues Asp-235 and Glu-327 that are predicted to be located in close proximity of each other in our inward facing structure model (3, 4). A motif search was performed in the data base SPASM (Spatial Arrangements of Side chains and Main chains; Uppsala Software Factory) to test the occurrence of this arrangement of carboxyl residues in other proteins. Surprisingly, when only the coordinates of Asp-235 and Glu-327 were used as input, the search revealed that the Asp-235/Glu-327 arrangement is very similar to that of two key carboxyl residues in certain EF hand Ca2+-binding proteins such as calmodulin, troponin C, and calsequestrin (Fig. 1). To confirm and extend these predictions, we used the maximum entropy-based docking web server MEDock for efficient prediction of whether and how Ca2+ would bind in our LmrP model. MEDock predicted the binding of Ca2+, but not of Mg2+, in a fashion that involves coordination by the carboxyl oxygens of Asp-235 (transmembrane helix 7) and Glu-327 (transmembrane helix 9) in addition to the main chain carboxyl oxygen from Ile-264 (transmembrane helix 8) and a cation-pi electron interaction on the aromatic ring of Tyr-265 (transmembrane helix 8), which are all located in close proximity in the C-terminal half of LmrP (Fig. 1).

High Affinity Binding of Ca2+ to Purified LmrP—Following the in silico predictions of Ca2+ binding by MEDock, binding experiments with radioactive 45Ca2+ were performed using purified LmrP in detergent solution. In these measurements, the major facilitator superfamily galactose/H+ symporter GalP from E. coli (7), with a similar overall architecture as LmrP and a dedicated role in the transport of monosaccharides, was used as a control for nonspecific substrate binding (4, 8). Using the attached His tag, WT LmrP, DE mutant LmrP, and GalP were purified from detergent-solubilized total membrane proteins in inside-out membrane vesicles by Ni-NTA affinity chromatography to purities above 95% (Fig. 2). The purified proteins were incubated for 30 min in the presence of 45Ca2+ at a concentration of 10.3 μM and subsequently filtered over 0.2-μm nitrocellulose filters. The radioactivity retained on the filters was determined. Fig. 3A shows that 45Ca2+ binding to WT LmrP was substantially elevated compared with the GalP control. Post-hoc analysis using Tukey’s test suggested a significant difference between the means for LmrP and GalP (p < 0.03). Analysis by analysis of variance confirmed that the difference between the data sets was significant (p < 0.01). These Ca2+ binding experiments with WT LmrP and GalP were repeated using a range of Ca2+ concentrations. Subtraction of the nonspecific binding obtained for GalP from the total binding obtained for LmrP allowed determination of specific binding to LmrP as a function of the Ca2+ concentration (Fig. 3B). Fitting of the specific binding data to a hyperbola showed Ca2+ binding to LmrP with an apparent dissociation constant (Kd) of 7.2 ± 4.3 μM Ca2+ and a maximum binding (Bmax) of 3.7 ± 0.9 nmol/mg protein.

Importance of Asp-235 and Glu-327 in Ca2+ Binding—To test the predicted role of Asp-235 and Glu-327 in Ca2+ coordination, 45Ca2+ binding to purified double mutant D235N/E327Q (DE) LmrP was determined. 45Ca2+ binding to the DE mutant was strongly reduced compared with WT LmrP and was close to the GalP control (Fig. 3A). Analysis of the data both by Tukey’s test and by analysis of variance indicated a significant difference between the binding to WT LmrP and DE LmrP (p < 0.02 and 0.01, respectively) but not between DE LmrP and GalP. As carboxyl moieties coordinate Ca2+ in their dissociated
In control experiments, no significant changes in the Fura-2 fluorescence were observed in control cells or cells expressing WT LmrP or DE mutant LmrP in the absence of the preloading with Ca\(^{2+}\) (Fig. 4B). Furthermore, Fura-2 remained undetectable in the external buffer during these experiments, indicating that LmrP did not mediate Fura-2 efflux at a significant level (data not shown). Because WT LmrP and the DE mutant are equally well expressed in the lactococcal cells under the experimental conditions (4), our findings demonstrate the Ca\(^{2+}\) extrusion activity of LmrP in intact cells and the importance of Asp-235 and Glu-327 for this activity.

**Transport Studies in Inside-out Membrane Vesicles**—To further test the ability of LmrP to mediate Ca\(^{2+}\) transport, \(^{45}\)Ca\(^{2+}\) uptake was measured in inside-out membrane vesicles. For this purpose, inside-out membrane vesicles were diluted to a final protein concentration of 2 mg/ml into 10 mM Tris-Cl, 10 mM KCl, 150 mM KCl (pH 7.0) containing an ATP-regenerating system and 2.5 mM ATP, which is the source of metabolic energy for the formation of the \(\Delta\psi - Z\Delta\phi\) in which \(Z = 58.1\) mV at 20 °C, interior positive and acidic) through proton pumping by the F\(_{1}\)F\(_{0}\) ATPase. The transport reaction was started with the addition of \(^{45}\)Ca\(^{2+}\) to a final concentration of 10.3 \(\mu\)M, after which samples were taken over time, and the amount of \(^{45}\)Ca\(^{2+}\) accumulated in the inside-out membrane vesicles was determined by rapid filtration. In these experiments, the magnitude and composition of the \(\Delta\psi\) was manipulated using the ionophores nigericin and valinomycin. Nigeri-
cin mediates the antipoint of H\(^+\) and K\(^+\) down their concentration gradients, thereby selectively dissipating the \(\Delta\phi\) in an electroneutral manner. Valinomycin mediates electronegenic uniport of K\(^+\), allowing the electrophoretic uptake of K\(^+\) in cells with dissipation of the \(\Delta\phi\). In the presence of a \(\Delta\phi\) only (through the addition of valinomycin), the uptake of 45Ca\(^{2+}\) was reduced slightly compared with \(\Delta\phi\)-dependent uptake, pointing to the \(\Delta\phi\) dependence of Ca\(^{2+}\) transport (Fig. 5A). In the presence of a \(\Delta\phi\) only (through the addition of nigericin), 45Ca\(^{2+}\) uptake was reduced more strongly but remained significantly above control uptake in the absence of the \(\Delta\phi\) (in the presence of both ionophores). This result points to the \(\Delta\phi\) dependence of the transport reaction (Fig. 5A).

The absence of robust 45Ca\(^{2+}\) uptake in control inside-out membrane vesicles lacking LmrP (Fig. 5B) and in inside-out membranes vesicles containing DE mutant LmrP (see below) under these conditions indicates that the observed Ca\(^{2+}\) transport reaction is LmrP-dependent. Taken together, these data suggest that LmrP mediates calcium transport by a \(\Delta\phi\)-dependent, electronegenic Ca\(^{2+}\)/H\(^+\) exchange reaction in which \(n \approx 3\). To determine the affinity of LmrP for Ca\(^{2+}\) in the transport reaction in inside-out membrane vesicles, the rate of uptake of 45Ca\(^{2+}\) in LmrP-containing inside-out membrane vesicles was followed at 45Ca\(^{2+}\) concentrations ranging from 0.1 to 100.3 \(\mu M\). The initial rates of linear uptake were determined between 20 and 90 s following 45Ca\(^{2+}\) addition and were plotted against the 45Ca\(^{2+}\) concentration (Fig. 5C). The data were fitted to a hyperbola from which an apparent affinity constant (\(K_a\)) of 8.6 \(\pm\) 2.3 \(\mu M\) and a maximum rate \(\left(V_{\text{max}}\right)\) of 2.5 \(\pm\) 0.3 pmol Ca\(^{2+}\)/mg of protein/s was estimated. This \(K_a\) value corresponds well to the apparent \(K_a\) of 7.2 \(\mu M\) derived in the Ca\(^{2+}\)-binding experiments (Fig. 3B). Compared with the GalP control, DE mutant LmrP did not mediate a significant transport of 45Ca\(^{2+}\) in the kinetic experiments (Fig. 5C). In control experiments, the functionality of the LmrP proteins was tested in a Hoechst 33342 transport assay, in which the fluorescence quenching of the dye was detected following its transport from the membrane into the acidic, aqueous lumen of the inside-out membrane vesicles (Fig. 5D). Both WT LmrP and the DE mutant exhibited a substantial activity in this assay.

**Substrate Transport by Purified LmrP in Proteoliposomes**—To further exclude a significant role of endogenous Ca\(^{2+}\) transporters in the observed Ca\(^{2+}\) transport activity in LmrP-containing inside-out membrane vesicles, LmrP was purified and functionally reconstituted in proteoliposomes. To impose a reversed \(\Delta\phi\) (interior acidic) across the liposomal membrane that could drive LmrP-mediated 45Ca\(^{2+}\) accumulation, proteoliposomes were prepared in NH\(_4\)Cl-containing buffer (pH 6.8) (see “Experimental Procedures”) and diluted in NH\(_4\)Cl-free buffer (pH 7.5) to allow the transmembrane diffusion of NH\(_3\) from the (proteo)liposomes with concomitant acidification of the lumen. To test transport in the absence of the \(\Delta\phi\), (proteo)liposomes were diluted in the buffer in which they were prepared, thus preventing the outward diffusion of NH\(_3\). The functionality of the reconstituted LmrP was first confirmed in a Hoechst 33342 transport assay, as described previously (15), in which the (proteo)liposomes were diluted in buffers containing 0.25 \(\mu M\) Hoechst 33342. The transport-associated quenching of the dye relative to empty liposomes (control) was only obtained for WT LmrP and the DE mutant upon imposition of the \(\Delta\phi\) (Fig. 6A), but not in the absence of the \(\Delta\phi\) (Fig. 6B).

When the external buffer was supplemented with 1.1 \(\mu M\) 45Ca\(^{2+}\) instead of Hoechst 33342, a significant \(\Delta\phi\)-dependent accumulation of the radioactive ion was obtained in proteoliposomes containing WT LmrP, whereas the accumulation for the DE mutant was at the background level in empty liposomes (Fig. 6C). In absence of the \(\Delta\phi\), no 45Ca\(^{2+}\) accumulation was detected in any of the samples (Fig. 6D). These experiments provide strong evidence for the ability of LmrP itself to mediate proton-coupled Ca\(^{2+}\) transport.

**Ca\(^{2+}\) Inhibits the Interaction of LmrP with Hoechst 33342**—Because LmrP can transport both Hoechst 33342 and Ca\(^{2+}\), we tested the potential interaction between these substrates on LmrP. Ca\(^{2+}\) at a 1 mm concentration inhibited the \(\Delta\phi\)-dependent transport of Hoechst 33342 by WT LmrP in inside-out membrane vesicles, whereas an equal concentration of Mn\(^{2+}\) had no inhibitory effect (Fig. 7A). Under identical conditions, neither of these cations influenced Hoechst 33342 fluorescence.
LmrP-mediated Calcium/Proton Antiport

in control membrane vesicles lacking LmrP (Fig. 7B). The mechanism of inhibition by Ca2+ was further examined in a Hoechst 33432 binding assay (16). In this assay, the binding of Hoechst 33432 (0.7–6.0 μM) to purified WT LmrP and the DE mutant LmrP (0.28 μM) as a control was determined from the enhancement of fluorescence upon transfer of the probe from the aqueous buffer to nonpolar binding site(s) on the protein. Lineweaver-Burk plots for the data yielded an apparent Km of 1.1 μM for the DE mutant LmrP (0.28 μM) in the absence or presence of 1 mM Ca2+. The buffer contained a standard amount of 5 mM Mg2+ that did not significantly inhibit transport. The binding of Hoechst 33432 to 0.28 μM purified WT LmrP (C) or DE mutant LmrP (D) was measured over a range of Hoechst 33432 concentrations (0.5–6.0 μM) in the absence (C) or presence of a fixed concentration of 1 mM Ca2+ (●) or 2 mM Ca2+ (●). Kinetic parameters in the main text represent the means ± S.E. of three independent experiments.

**DISCUSSION**

Similar to eukaryotic cells, eubacterial cells are equipped with transporters to efflux Ca2+ and maintain Ca2+ homeostasis (17). The cytosolic Ca2+ concentration is usually significantly lower than in the extracellular environment, roughly between 0.1 and 1 μM in *E. coli* (18, 19). Early studies by Rosen and McClees (20) and Harold and co-workers (21) first identified a Ca2+/H+ antiporter in *E. coli* and an ATP-dependent P-type Ca2+ efflux pump in *Streptococcus faecalis*, and these types of systems were later shown to also exist in other bacteria (22, 23) including *L. lactis* (24, 25). However, Ca2+ transport has not been studied extensively in bacteria, and in addition to the bacterial transport systems listed above, others might exist.

Our data suggest that LmrP mediates the selective binding and transport of Ca2+ in cells, inside-out membrane vesicles, and proteoliposomes containing purified protein. No significant Ca2+ binding or transport activities were obtained in any of the experimental systems lacking LmrP (Figs. 2–4). Similar to published observations for Ca2+ channels, for which Ba2+ can pass through the channel apart from Ca2+ (26, 27), Ca2+ can be replaced by Ba2+ but not by Mg2+, Mn2+, or Co2+ in the binding experiments with LmrP (Fig. 3D). The determined affinities of LmrP for Ca2+ in our binding assay (apparent Kd of 7.2 ± 4.3 μM) (Fig. 3B) and transport assay (apparent K0.5 of 8.6 ± 2.3 μM) (Fig. 5C) are consistent with each other. For the designated calcium/proton antiporter from *Bacillus subtilis*, an
apparent $K_i$ of 40 $\mu$M Ca$^{2+}$ was found (28), whereas for the Ca$^{2+}$/H$^+$ antiporter YfkE in this organism, an apparent $K_i$ was determined of $\sim$12.5 $\mu$M (29). The apparent $K_d$ of mammalian calmodulin for Ca$^{2+}$ covers a range from 14 $\mu$M down to 0.5 $\mu$M in the presence of the activatable target enzyme (30, 31). Hence, the apparent affinity of LmrP for Ca$^{2+}$ lies in a similar range as observed for proteins for which the interaction with Ca$^{2+}$ is established. The affinity of LmrP for Ca$^{2+}$ is also in a similar range as the affinity for a typical multidrug substrate such as Hoechst 33342, which is bound by LmrP with an apparent $K_d$ of 2.6 $\pm$ 0.3 $\mu$M (Fig. 7C).

In many Ca$^{2+}$-binding proteins, the Ca$^{2+}$-binding pocket exhibits a pentagonal bipyramidal geometry (32), through positioning of side chains in a characteristic helix-loop-helix structural motif termed the EF hand (33). The end of the first helix and the start of the second helix often contain a carboxyl residue that is crucial for coordinating Ca$^{2+}$ (see Fig. 1B for example). It is this pair of carboxyl residues that led us to Ca$^{2+}$-binding proteins during a motif search in the SPASM database with the coordinates of the Asp-235/Glu-327 side chains of LmrP as the bait. Mutations of acidic residues are known to significantly change (mainly decrease) the affinity of calcium binding to EF hand proteins (30, 34). The importance of Asp-235 and Glu-327 in Ca$^{2+}$ binding was predicted in our docking studies (Fig. 1A) and was demonstrated in binding and transport assays using DE mutant LmrP (Figs. 3–6). In a study on the pH dependence of Ca$^{2+}$ binding, a pH optimum was reached between pH 6.0 and 7.0 for WT LmrP but not for the DE mutant (Fig. 3C), suggesting that deprotonation of Asp-235 and Glu-327 in the binding pocket is crucial for Ca$^{2+}$ binding. Very recently, a crystal structure of the liganded Na$^+$/Ca$^{2+}$ exchanger NCX from Methanococcus jannaschii was resolved at 1.9 Å resolution, showing the direct role of Glu-54 and Glu-213 in the coordination of Ca$^{2+}$ in its binding pocket (35). It is important to note that although the DE mutant is inhibited in Ca$^{2+}$ binding and transport (Figs. 3–7) and propidium$^+$ transport (4), electroneutral ethidium$^+$-H$^+$ antiport (4) and Hoechst 33342 transport (Figs. 5D and 6A) are retained. Hence, the DE mutant is strongly inhibited in Ca$^{2+}$ transport activity but can still function as a multidrug transporter with a different selectivity compared with the wild-type protein.

Studies on the energetics of LmrP-mediated $^{45}$Ca$^{2+}$ transport in inside-out membrane vesicles demonstrated that this reaction is based on electrogenic Ca$^{2+}$/nH$^+$ antiport ($n \geq 3$) (Fig. 5A), similar to published observations for the calcium/proton antiporter in B. subtilis (28). The transport of Ca$^{2+}$ by LmrP might therefore be analogous to the transport of divalent cationic propidium, which is based on electrogenic propidium$^+$/3H$^+$ exchange (4). However, compared with Ca$^{2+}$, propidium$^+$ has a much larger structure in which the two positive charges are separated by a C$_4$ alkyl chain. Although Asp-235 and Glu-327 are crucial for binding of each of these substrates (this work and Ref. 4), our docking studies on Ca$^{2+}$ binding predict that the charge neutralization of Ca$^{2+}$ is in part based on direct electrostatic interactions with these carboxylates, whereas for propidium$^+$, the charge neutralization of at least one positive charge is most likely achieved through interactions with residues located further away, e.g., aromatic and polar residues as observed for the binding of berberine to the crystallized multidrug-binding transcriptional regulator QacR (36). The binding sites in LmrP for Ca$^{2+}$ and propidium$^+$ might therefore partially overlap. On the other hand, our findings of non-competitive inhibition of Hoechst 33342 binding by Ca$^{2+}$ (Fig. 7C) point to the binding of Ca$^{2+}$ to unliganded LmrP and the Hoechst 33342-LmrP complex, and hence, to separate, communicating sites for these substrates. Inhibitory effects by Ca$^{2+}$ on drug interactions in LmrP were also observed for ethidium$^+$ as a substrate (data not shown). The mechanism of this inhibition was not further investigated.

In conclusion, we present compelling evidence that the multidrug transporter LmrP can mediate the selective binding and transport of Ca$^{2+}$ and that this activity can inhibit multidrug transport. Ca$^{2+}$ is implicated in a number of important bacterial functions including heat shock, pathogenicity, chemotaxis, differentiation, and cell cycle (17, 22). Our observations relate to previous findings of Na$^+$(K+)/H$^+$ antiport by the secondary active multidrug transporter MdfA (37) and tetracycline transporter Tet(L) (38, 39) in E. coli and of Na$^+$/Cl$^-$–H$^+$ symport by the multidrug ATP-binding cassette transporter LmrA in L. lactis (40–42). These findings raise the paradox that multidrug transporters with a broad specificity for amphiphilic organic cations can, at the same time, be highly selective for certain inorganic ions. They suggest that these multidrug transporters fulfill additional physiological roles to that of multidrug transport and that such roles might promote the persistence of multidrug transporter even in the absence of exposure to antibiotics (43, 44). Indeed, for MdfA a role in alkal tolerance was demonstrated (37). Many molecular details of the “dual specificity” of LmrP and other systems are still unknown, and a systematic comparison with transporters with an established, dedicated role in inorganic ion translocation will be an important challenge. Clearly, there is a lot to be learned about substrate recognition and transport by multidrug transporters, which will be crucial for the generation of selective multidrug resistance inhibitors with a low general toxicity and for our understanding of the factors that can trigger transporter expression.

Acknowledgments—We thank Peter Henderson (Institute of Membrane and Systems Biology, University of Leeds, Leeds, UK) for the gift of membrane vesicles containing GalP and Lucy Crouch for critical reading of the manuscript.

REFERENCES

1. Putman, M., van Veen, H. W., Degener, J. E., and Konings, W. N. (2001) The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines. Microbiology 147, 2873–2880
2. Bolhuis, H., van Veen, H. W., Brands, J. R., Putman, M., Poolman, R., Driessen, A. J., and Konings, W. N. (1996) Energetics and mechanism of drug transport mediated by the lactococcal multidrug transporter LmrP. J. Biol. Chem. 271, 24123–24128
3. Bapna, A., Federici, L., Venter, H., Velamakanni, S., Luisi, B., Fan, T. P., and van Veen, H. W. (2007) Two proton translocation pathways in a secondary active multidrug transporter. J. Mol. Microbiol. Biotechnol. 12, 197–209
4. Schaedler, T. A., and van Veen, H. W. (2010) A flexible cation binding site in the multidrug major facilitator superfamily transporter LmrP is associated with variable proton coupling. FASEB J. 24, 3653–3661
LmrP-mediated Calcium/Proton Antiport

5. Venter, H., Velamakanni, S., Balakrishnan, L., and van Veen, H. W. (2008) On the energy-dependence of Hoechst 33342 transport by the ABC transporter LmrA. Biochem. Pharmacol. 75, 866–874

6. de Ruiter, P. G., Kuipers, O. P., and de Vos, W. M. (1996) Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. Appl. Environ. Microbiol. 62, 3662–3667

7. Ward, A., Sanderson, N. M., O'Reilly, J., Rutherford, N. G., Poolman, B., and Henderson, P. J. (2000) in Membrane Transport: A Practical Approach (Baldwin, S. A., ed) pp. 141–164, Oxford University, Oxford, UK

8. Woebeking, B., Velamakanni, S., Federici, L., Seeger, M. A., Murakami, S., and van Veen, H. W. (2008) Functional role of transmembrane helix 6 in drug binding and transport by the ABC transporter MsbA. Biochemistry 47, 10904–10914

9. McDonough, P. M., and Button, D. C. (1989) Measurement of cytoplasmic calcium concentration in cell suspensions. Correction for extracellular Fura-2 through use of Mn²⁺ and probenecid. Cell Calcium 10, 171–180

10. Di Virgilio, F., Steinberg, T. H., and Silverstein, S. C. (1990) Inhibition of ATP-dependent calcium transport in cells and membrane vesicles of Staphylococcus aureus. J. Biol. Chem. 265, 686–690

11. Molenaar, D., Bolhuis, H., Abee, T., Poolman, B., and Konings, W. N. (1999) The efflux of a fluorescent probe is catalyzed by an ATP-driven extrusion system in Lactococcus lactis. J. Bacteriol. 174, 3118–3124

12. Norris, V., Grant, S., Freestone, P., Canvin, J., Sheikh, F. N., Toth, I., Trinei, M., Modha, K., and Norman, R. I. (1996) Calcium signalling in bacteria. J. Bacteriol. 178, 3677–3682

13. Ambudkar, S. V., Lynn, A. R., Maloney, P. C., and Rosen, B. P. (1984) Calcium efflux from Escherichia coli. Evidence for two systems. J. Biol. Chem. 259, 6142–6146

14. Ambudkar, S. V., Lynn, A. R., Maloney, P. C., and Rosen, B. P. (1986) Reconstitution of ATP-dependent calcium transport from streptococci. J. Biol. Chem. 261, 15596–15600

15. Griessen, A. J., and Konings, W. N. (1986) Calcium transport in membrane vesicles of Streptococcus cremoris. Eur. J. Biochem. 159, 149–155

16. Hagiwara, S., Fukushima, I., and Eaton, D. C. (1974) Membrane currents carried by Ca, Sr, and Ba in barnacle muscle fiber during voltage clamp. J. Gen. Physiol. 63, 564–578

17. Sather, W. A., and McCleskey, E. W. (2003) Permeation and selectivity in calcium channels. Annu. Rev. Physiol. 65, 133–159

18. Matsushita, T., Ueda, T., and Kubo, A. (1986) Purification and characterization of Ca²⁺ /H⁺ antiporter from Bacillus subtilis. Eur. J. Biochem. 156, 95–100

19. Fujisawa, M., Wada, Y., Tsuchiya, T., and Ito, M. (2009) Characterization of Bacillus subtilis YK1 (ChaA). A calcium-specific Ca²⁺ /H⁺ antiporter of the CaCA family. Arch. Microbiol. 191, 649–657

20. Yang, J. J., Gawthrop, A., and Ye, Y. (2003) Obtaining site-specific calcium-binding affinities of calmodulin. Protein Pept. Lett. 10, 331–345

21. Ye, Y., Lee, H. W., Hellings, H., and Yang, J. J. (2002) Structural analysis, identification, and design of calcium-binding sites in proteins. Proteins 47, 344–356

22. Strnadka, N. C., and James, M. N. (1989) Crystal structures of the helix-loop-helix calcium-binding proteins. Annu. Rev. Biochem. 58, 951–998

23. Protsyshyn, R. M., and Reid, R. E. (1994) A structure/activity study of calcium affinity and selectivity using a synthetic peptide model of the helix-loop-helix calcium-binding motif. J. Biol. Chem. 269, 1641–1647

24. Liese, K. M., Schuman, J. T., Skurray, R. A., Brown, M. H., Brennan, R. G., and Schumacher, M. A. (2008) QacR-cation recognition is mediated by a reducibility of residues capable of charge neutralization. Biochemistry 47, 8122–8129

25. Peters, K. M., Schuman, J. T., Skurray, R. A., Brown, M. H., Brennan, R. G., and Schumacher, M. A. (2008) QacR-cation recognition is mediated by a reducibility of residues capable of charge neutralization. Biochemistry 47, 8122–8129

26. Lewinson, O., Padan, E., and Bibi, E. (2004) Alkalitolerance. A biological function for a multidrug transporter in pH homeostasis. Proc. Natl. Acad. Sci. U.S.A. 101, 14073–14078

27. Cheng, J., Guffanti, A. A., and Krulwich, T. A. (1994) The chromosomal tetracycline resistance locus of Bacillus subtilis encodes a Na+/H⁺ antiporter that is physiologically important at elevated pH. J. Biol. Chem. 269, 27365–27371

28. Guffanti, A. A., Cheng, J., and Krulwich, T. A. (1998) Electrogenic antiport activities of the Gram-positive Tet proteins include a Na⁺ (K⁺)/K⁺ mode that mediates net K⁺ uptake. J. Biol. Chem. 273, 26447–26454

29. Venter, H., Shilling, R. A., Velamakanni, S., Balakrishnan, L., and Van Veen, H. W. (2003) An ABC transporter with a secondary-active multidrug translocator domain. Nature 426, 866–870

30. Shilling, R., Federici, L., Walas, F., Venter, H., Velamakanni, S., Woebeking, B., Balakrishnan, L., and Van Veen, H. W. (2005) A critical role of a carboxylate in proton conduction by the ATP-binding cassette multidrug transporter LmrA. FASEB J. 19, 1698–1700

31. Velamakanni, S., Lau, C. H., Gutmans, D. A., Venter, H., Barrera, N. P., Seeger, M. A., Woebeking, B., Matak-Vinkovic, D., Balakrishnan, L., Yao, Y., U, E. C., Shilling, R. A., Robinson, C. V., Thorn, P., and Van Veen, H. W. (2009) A multidrug ABC transporter with a taste for salt. PLoS One 4, e6137

32. Krulwich, T. A., Lewinson, O., Padan, E., Bibi, E. (2005) Do physiological roles foster persistence of drug/multidrug-efflux transporters? A case study. Nat. Rev. Microbiol. 3, 566–572

33. Piddock, L. J. (2006) Multidrug-resistance efflux pumps. Not just for resistance. Nat. Rev. Microbiol. 4, 629–636

34. Vassylev, D. G., Takeda, S., Nakatsukasa, S., Maeda, K., and Maéda, Y. (1998) Crystal structure of tronemin C complex with tronomin I fragment at 2.3-A resolution. Proc. Natl. Acad. Sci. U.S.A. 95, 4847–4852