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Energetics of wild-type and mutant multidrug resistance secondary transporter LmrP of *Lactococcus lactis*

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Abstract

LmrP, a proton/multidrug antiporter of *Lactococcus lactis*, transports a variety of cationic substrates. Previously, two membrane-embedded acidic residues, Asp^{142} and Glu^{327}, have been reported to be important for multidrug transport activity of LmrP. Here we show that neither Glu^{327} nor Asp^{142} is essential for ethidium binding but that Glu^{327} is a critical residue for the high affinity binding of Hoechst 33342. Substitution of these two residues, however, negatively influences the transport activity. The energetics of transport was studied of two closely related cationic substrates ethidium and propidium that carry one and two positive charges, respectively. Extrusion of monovalent ethidium is dependent on both the electrical membrane potential ($\Delta\psi$) and transmembrane proton gradient (ΔpH), while extrusion of propidium predominantly depends on the ΔpH only. The LmrP mutants D142C and E327C, however, mediate electroneutral ethidium extrusion, but are unable to mediate ΔpH-dependent extrusion of propidium. These data indicate that Asp^{142} and Glu^{327} are involved in proton translocation.

Keywords: Energetics; LmrP; *Lactococcus lactis*

1. Introduction

Microorganisms are, in their natural environments, continuously exposed to a variety of toxic compounds from natural and/or industrial sources. To survive the poisonous effects of these cytotoxic compounds, several defense mechanisms have been developed. One of these defense mechanisms is the active extrusion of cytotoxic compounds from the cell by drug efflux transporters present in the cytoplasmic membrane. Besides the specific drug efflux systems (SDRs), multidrug resistance (MDR) transporters are found, which can extrude a wide variety of structurally unrelated compounds. The energy for the efflux process by MDR transporters can be supplied directly by either (i) a proton or sodium motive force (pmf or smf) in secondary MDRs or (ii) ATP in ATP-binding cassette (ABC)-MDR transporters [1].

One of the MDR extrusion systems of *Lactococcus lactis* is the proton motive force-driven secondary transporter LmrP. It is a 408-amino-acid protein with 12 putative transmembrane helices, which contains motifs diagnostic for the Major Facilitator Superfamily of the secondary transporters [2]. LmrP has a very broad substrate specificity and extrudes a variety of lipophilic cationic compounds such as ethidium, daunomycin, tetraphenylphosphonium (TPP^+) and Hoechst 33342 [2–4]. It also confers resistance to several broad-spectrum antibiotics [5]. LmrP can mediate ΔpH- and $\Delta\psi$-driven extrusion of monovalent cations such

Abbreviations: MDR, multidrug resistance; pmf, proton motive force; TPP^+, tetraphenylphosphonium; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; His_6-tag, six-histidine tag; DDM, dodecyl-β-maltoside

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as TPP^+ and a fluorescent membrane probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) from cells or right-side-out membrane vesicles and the uptake of these compounds in inside-out membrane vesicles [3]. These observations indicate that LmrP catalyzes electrogenic H^+/drug antiport in which at least two protons are exchanged for one drug molecule. Extensive studies have been focused on the cellular compartment from which LmrP picks up its highly lipophilic substrates [3]. In these studies the compound TMA-DPH was used, a probe that is fluorescent only in the hydrophobic environment of the membrane. The activity of LmrP was found to depend on the concentration of TMA-DPH in the inner leaflet of the lipid bilayer. These and other data provided convincing evidence that LmrP functions as a vacuum-cleaner catalyzing drug efflux from the inner leaflet of the membrane directly into the external medium [6]. Substrate competition experiments [7] revealed that LmrP-mediated Hoechst 33342 efflux is competitively inhibited by quinine and verapamil, noncompetitively by nicardipin and vinblastin, and uncompetitively by TPP^+. These findings indicate the presence of multiple drug binding sites in the multidrug transporter LmrP. The presence of multiple drug binding sites in MDR transporters was later also demonstrated for the Major Facilitator Superfamily H^+/drug antiporter MdfA of *Escherichia coli* [8].

LmrP-mediated efflux of cationic drugs from the inner leaflet of the membrane predicts an electrostatic interaction of the substrates with acidic residues, especially those located in the transmembrane domains of LmrP. Cysteine scanning mutagenesis of LmrP in combination with thiol chemistry identified three membrane-embedded acidic residues: Asp^{142}, Glu^{327} and Glu^{388} [9]. The role of these residues in drug recognition was evaluated in transport experiments with the cationic substrates ethidium and Hoechst 33342 with wild-type (WT) and mutant LmrPs in which each of these residues had been replaced by cysteine, alanine, lysine, glutamate or aspartate. These studies revealed an important but not crucial role of the negative charges at positions 142 and 327 in the transport process by LmrP [9].

To elucidate further the role of Asp^{142} and Glu^{327} in substrate binding and transport, direct substrate binding studies were performed with purified WT and mutant LmrPs. Furthermore, the role of the proton motive force (pmf) and its components, the ΔpH and the $\Delta\psi$, in driving the extrusion of differently charged substrates was evaluated (chemical structures of the substrates are shown in Fig. 1). The results indicate that Glu^{327}, but not Asp^{142}, is an essential residue for the high affinity binding of Hoechst 33342. WT LmrP was found to mediate electrogenic transport of ethidium, consistent with a previous report in which other monovalent substrates of LmrP were studied [3]. In contrast, LmrP-mediated transport of propidium, a compound which carries two positive charges, depends mainly upon the ΔpH. The LmrP mutants D142C and

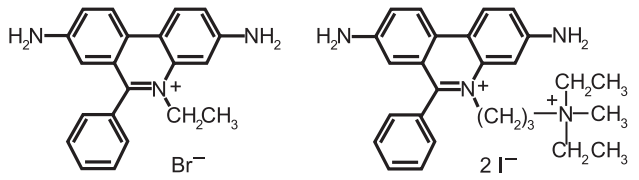

Fig. 1. Chemical structures of ethidium and propidium. Both compounds contain quaternary ammonium which gives them one and two positive charges, respectively.

E327C mediate transport of ethidium in a $\Delta\psi$-independent manner, suggesting a direct role of these two residues in proton translocation.

2. Experimental procedures

2.1. Bacterial strains, plasmids, and growth conditions

L. lactis NZ9000 (Δ*lmrA*) [10], which lacks the gene encoding the ATP-binding cassette-type MDR transporter LmrA, was used in combination with the nisin controlled expression (NICE) system [11,12] for overexpression of WT and mutant LmrP proteins [4,9]. *L. lactis* was grown at 30 °C in M17 medium (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/ml chloramphenicol. Expression of LmrP variants from pNZ8048-derived plasmids was induced by adding approximately 10 ng nisin A/ml at an A_{660} of about 0.6, and cells were harvested 60 min after induction.

2.2. Recombinant DNA techniques

General procedures for cloning and DNA manipulation were performed essentially as described by Sambrook et al. [13]. The PCR overlap extension method [14] was used to introduce the D142C mutation in the *lmrP* gene on the pHLP5 expression plasmid [4], which encodes LmrP with a C-terminal six-histidine tag (His_6-tag). The PCR-amplified DNA fragment was sequenced to verify that only the intended changes were introduced. DNA sequencing was performed at the BioMedical Technology Centre (University of Groningen, Groningen, The Netherlands).

2.3. Expression levels of proteins

Expression levels of His_6-tagged proteins were determined by SDS polyacrylamide gel analysis of membrane vesicles or sonicated cells (20 μg of total protein), followed by transfer to polyvinylidene difluoride membranes, and immunodetection using monoclonal antibodies directed against the His_6-tag (Dianova, Hamburg, Germany). Antibodies were visualized by the Western-light chemiluminescence detection kit (Tropix, Bedford, MA, USA) using a Lumi-Imager F1 (Roche).

2.4. Preparation of membrane vesicles

Inside-out membrane vesicles were prepared from *L. lactis* NZ9000 (Δ*lmrA*) cells expressing LmrP variants by French press treatment [4]. Membranes were frozen in liquid nitrogen and stored at −80 °C at a protein concentration of 15 to 35 mg/ml in 10 mM K-HEPES, pH 7.0, containing 50 mM K_2SO_4, 5 mM $MgSO_4$, 8 mM NaCl and 10% (w/v) glycerol.

2.5. Binding of Hoechst 33342

Binding of Hoechst 33342 to LmrP was quantified using the enhancement of Hoechst 33342 fluorescence upon binding to the protein, essentially as described previously by Qu and Sharom [15] and Qu et al. [16]. His_6-tagged LmrP was purified on Ni-NTA agarose as described elsewhere [4]. The protein was eluted with 50 mM potassium phosphate pH 7.0, containing 250 mM imidazole, 100 mM NaCl and 0.05% dodecyl-β-maltoside (DDM). Elution fractions containing mainly LmrP were concentrated on a Microcon® centrifugal filter device (Millipore Corporation, Bedford, MA, USA) with the nominal molecular weight limit of 30 kDa. The concentration step was followed by two washing steps with the binding buffer (50 mM Tris–Cl pH 7.0 containing 100 mM NaCl and 0.05% DDM). The LmrP concentration in all binding experiments was 25 μg/ml. The increase of Hoechst 33342 fluorescence during titration was recorded at 20 °C with a Perkin-Elmer LS 50B fluorimeter, using excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 2.5 nm each. To correct the fluorescence enhancement data, the following equation was used:

$$F_{corr} = (F_i - B_i)(V_i/V_0) \times 10^{0.5b(A_{\lambda ex}+A_{\lambda em})} \quad (1)$$

where F_{corr} is the corrected value of the fluorescence intensity, F_i the experimentally measured fluorescence intensity, B_i the fluorescence intensity of free Hoechst 33342 in the binding buffer, V_0 the initial volume of the sample, V_i the volume of the sample at a given point of titration, b the optical path length of the cuvette in centimeters, and $A_{\lambda ex}$ and $A_{\lambda em}$ the absorbance of the sample at the excitation and emission wavelengths, respectively. To estimate the K_d values for Hoechst 33342 binding to LmrP, the obtained data were fitted to the equation:

$$\Delta F = \Delta F_{max}[H]/(K_d + [H]) \quad (2)$$

where ΔF is the increase in fluorescence relative to the initial value after addition of Hoechst 33342 at a concentration [H], and ΔF_{max} the maximum enhancement in fluorescence intensity that occurs upon saturation of the substrate binding site.

Quinine titration data were corrected for the fluorescence of Hoechst 33342 in the absence of LmrP. The concentration of quinine which inhibits Hoechst 33342 binding by 50% (IC_{50}) was calculated with Sigma Plot software (SPSS Inc., Chicago, IL, USA) using the following equation:

$$F_h = F_{min} + (F_{max} - F_{min})/\left(1 + 10^{\log[I]-\log(IC50)}\right) \quad (3)$$

where F_h is the measured Hoechst 33342 fluorescence in the presence of various concentrations of quinine, F_{max} the maximal fluorescence in the absence of quinine, F_{min} the minimal fluorescence in the presence of saturating concentrations of quinine, and log[I] the logarithm of the quinine concentration. The obtained IC_{50} value was used to calculate K_i for quinine by using the equation of Cheng and Prusoff [17]:

$$K_i = IC_{50}/(1 + [H]/K_d) \quad (4)$$

where K_d is the apparent affinity of Hoechst 33342 for LmrP.

2.6. Binding of ethidium

Ethidium binding was measured as described by Muth and Schuldiner [18]. Membrane vesicles containing 30 mg of total protein were solubilized as described [4]. The supernatant obtained after ultracentrifugation was mixed with 1.0 ml of equilibrated Ni-NTA agarose resin followed by 45 min incubation at 4°C. The unbound fraction was removed by centrifugation for 10 s at the top speed of a table centrifuge, and the resin was washed once with wash buffer containing 20 mM imidazole. Washed resin with bound LmrP (LmrP-resin) was resuspended in 50 mM potassium phosphate pH 7.0 containing 100 mM NaCl and 0.05% DDM in a volume of 1 ml. LmrP-resin (100 μl) was incubated at 4°C with 400 μl of 50 mM potassium phosphate pH 7.0, 100 mM NaCl and 0.05% DDM containing 5 μM ethidium. As a negative control, 1 mM quinine was added to inhibit ethidium binding. Binding reactions were terminated by separating the beads from the supernatant by centrifugation. The pellet resin fraction was collected and incubated for 5 min at room temperature with 200 μl of 50 mM potassium phosphate pH 7.0 containing 100 mM NaCl, 0.05% DDM and 250 mM imidazole to release the His_6-tagged LmrP and ethidium from the resin. After centrifugation, 150 μl of supernatant was used to determine the amount of His_6-tagged-LmrP-bound ethidium by measuring fluorescence with a Perkin-Elmer LS 50B fluorimeter, using excitation and emission wavelengths of 500 and 580 nm, respectively, and slit widths of 10 nm each. All binding reactions were performed in triplicate. The amount of LmrP bound to Ni-NTA agarose was determined with the RC-DC protein assay kit from Bio-Rad (100 μg of LmrP per 100 μl of Ni-NTA suspension).

2.7. Ethidium and propidium transport

Ethidium transport experiments were performed as reported previously [9,19]. Control cells harboring vector

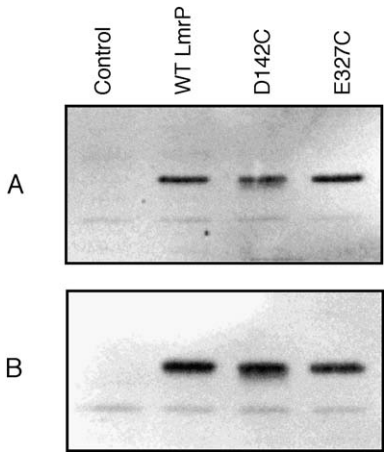

Fig. 2. Overexpression of LmrP in cells (A) and inside out-membrane vesicles (B) of *L. lactis*. Cells harboring an empty vector pNZ8048 and plasmids encoding for the WT and mutants of LmrP were cultured as described in Experimental procedures. Cells were sonicated and proteins separated by SDS-PAGE. Membrane vesicles were prepared as described in Experimental procedures. Each lane was loaded with 20 μg of protein. His_6-tagged proteins were detected with monoclonal antibodies directed against the tag.

pNZ8048 and cells expressing LmrP variants were harvested by centrifugation, washed twice with 50 mM potassium phosphate pH 7.0, containing 5 mM $MgSO_4$, and suspended in the same buffer to an A_{660} of 0.5. Transport assays were performed at 30 °C. Cells (2 ml) were pre-energized by the addition of 25 mM glucose. After 5 min, 10 μM ethidium was added to the cell suspension and the accumulation of ethidium was measured indirectly by following the fluorescence of the ethidium–polynucleotide complex in the cells. Fluorescence was monitored with a Perkin-Elmer LS 50B fluorimeter, using excitation and emission wavelengths of 500 and 580 nm, respectively. To determine the rate of passive ethidium influx into *L. lactis* cells, ethidium transport assays were performed in the absence of glucose. Propidium (Molecular Probes Inc., Eugene, OR, USA) transport assays were carried out with 10 μM propidium as described for ethidium.

3. Results

3.1. Construction and expression of D142C LmrP mutant

LmrP contains two negatively charged amino acids in its membrane domain which are important for substrate transport [9]. One of these residues Glu^{327} was replaced by cysteine. This E327C[2] mutant was found to transport Hoechst 33342 at a lower rate than WT LmrP but at a higher rate than the double mutant E327C-C270A in which also the endogenous Cys^{270} was replaced by alanine [19].

[2] It should be noted that the annotation of the cysteine mutants used in this manuscript differs from the annotation used in the Ref. [9] (E327C-C270A was E327C; D142C-C270A was D142C).

To evaluate also the role of the second critical negatively charged residue Asp^{142}, a single mutant D142C of LmrP was constructed. Western blot analysis with anti-His_6-tag antibodies showed that the D142C mutant was expressed to similar levels as WT and E327C LmrP (Fig. 2). The transport activity of D142C LmrP was similar to the double mutant D142C-C270A but lower than WT LmrP (see Fig. 4, Table 1 and data not shown).

3.2. Hoechst 33342 binding to purified wild-type and mutant LmrP

The fluorescent dye Hoechst 33342 is a well-studied model substrate for LmrP as its transport can be measured both in proteoliposomes reconstituted with purified LmrP and in inside-out membrane vesicles of *L. lactis* overexpressing LmrP [4]. Binding of Hoechst 33342 to WT and mutant LmrP protein was determined with the method developed by Qu et al. [16]. This method is based on the enhancement of Hoechst 33342 fluorescence upon transfer from a polar (aqueous) environment to a less polar binding site on the protein. Titration of purified LmrP with Hoechst 33342 resulted in an increase of the dye fluorescence up to saturation levels (see Fig. 3A and B). The experimental data could be fitted with a binding equation for a single binding site (see Eq. (2)) and yielded an apparent K_d value of 1.3 μM (Table 1). The specificity of Hoechst 33342 binding was assessed by the addition of the competitive inhibitor quinine to chase bound Hoechst 33342. The fluorescence of Hoechst 33342 bound to LmrP decreased with increasing concentrations of quinine (Fig. 3C). From the data obtained, an apparent K_i of 3 μM of quinine could be estimated.

Subsequently, the binding was measured of Hoechst 33342 to the E327C and D142C LmrP mutants (Table 1). The D142C LmrP mutant showed a similar Hoechst 33342 binding as WT LmrP, with an estimated K_d value of 0.4 μM. Likewise, a similar result was obtained with the D142A

Table 1
Affinity of Hoechst 33342 binding and transport activity of the WT and mutants of LmrP

	K_d Hoechst (μM)	Transport activity	
		Eth	Hoechst
WT	1.3±0.1	+++	+++
C270A	1.8±0.1	++	++
D142C-E327C-C270A	7.0±0.4	–	–
D142A	1.2±0.3	–/+	–/+
D142C	0.4±0.06	++	+
E327A	65.0#	++	–
E327C	12.8#	++	+

K_d values are an average from three measurements (for details see Experimental procedures); ± indicates standard errors. K_d values marked with # are only an approximate estimation since the saturation of Hoechst 33342 binding was not reached. Transport activities were measured in *L. lactis* cells, expressing WT or mutant LmrP, with glucose as energy source. The transport activities with the exception of these for the D142C mutant come from Refs. [9] and [19]. (+++) stands for the WT activity, and (–) for the lack of the transport activity.

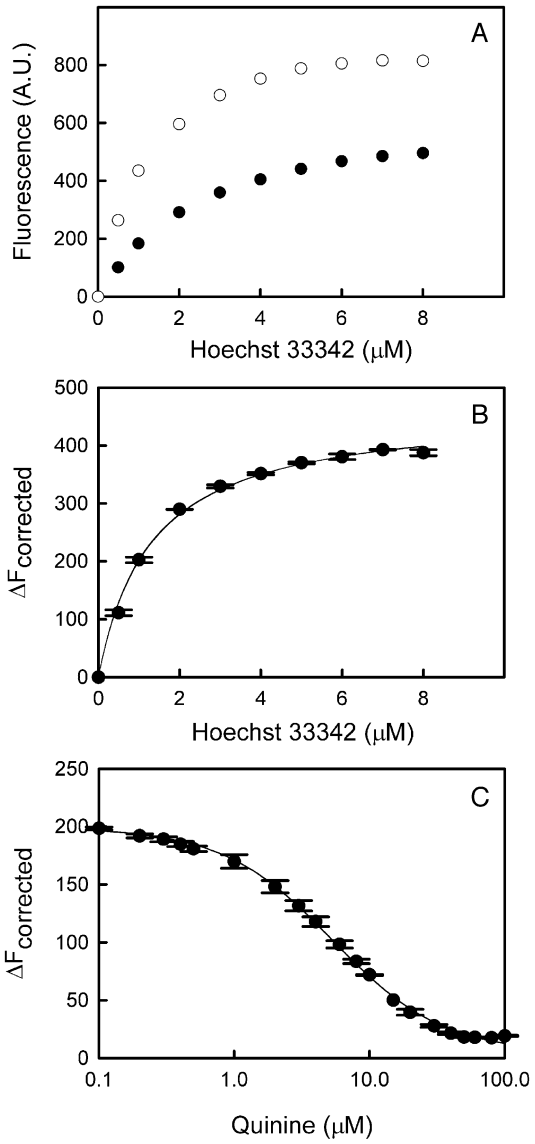

Fig. 3. Purified LmrP binds specifically Hoechst 33342. (A) An example of raw data of enhancement of Hoechst 33342 fluorescence upon binding to LmrP (F_i, open circles) and Hoechst 33342 fluorescence in the buffer (B_i, closed circles); A.U., arbitrary units. LmrP solutions (25 μg/ml protein) were titrated with increasing concentrations of Hoechst 33342 at 20 °C. (B) Hoechst 33342 binding to WT LmrP. The data are corrected for fluorescence of free Hoechst in the buffer, light scattering, and inner filters effects according to Eq. (1). The curve was obtained by fitting the data points to Eq. (2). The data points are an average from three measurements. (C) Inhibition of Hoechst 33342 binding to LmrP by quinine. LmrP solutions (25 μg/ml protein) containing 1 μM Hoechst 33342 were titrated with increasing concentrations of quinine. The curve was obtained by fitting the data points to Eq. (3). The data points are the average of two experiments.

mutant. In contrast, the E327C and E327A mutants of LmrP had a drastically reduced binding affinity of Hoechst 33342. Since saturation of Hoechst 33342 binding could not be reached, the K_d values could only be roughly estimated (Table 1). These observations clearly demonstrate that Glu^{327} is a critical residue for the high affinity binding of Hoechst 33342.

3.3. Binding of ethidium to LmrP

To measure the binding of ethidium to LmrP, the method developed by Muth and Schuldiner [18] was used. WT and mutants of LmrP were purified and immobilized on Ni-NTA agarose. Immobilized LmrP was incubated with 5 μM ethidium, and Ni-NTA-LmrP with bound ethidium was collected by centrifugation. LmrP with bound ethidium was released from the resin by imidazole and the fluorescence of ethidium in the eluate was measured (see Experimental procedures). Ethidium was found to bind specifically to immobilized WT LmrP, and this binding was significantly inhibited by a 200-fold excess of quinine (Fig. 4). Surprisingly, ethidium binding by immobilized D142C and E327C LmrP was only slightly lower than observed for WT LmrP (Fig. 4). This suggests that neither Asp^{142} nor Glu^{327} is critical for binding of ethidium at the concentration (10 μM) used in transport studies.

3.4. Transport of ethidium

Accumulation of ethidium in cells can be measured fluorimetrically by following in time the changes of the fluorescence of the intracellular ethidium-polynucleotide complex [4,9,19,20]. Under non-energized conditions, *L. lactis* not expressing LmrP (i.e., control cells) slowly accumulate ethidium (Fig. 5A, solid line). In contrast, a rapid influx of ethidium occurred in WT LmrP-expressing cells (Fig. 5B, solid line). This ethidium influx is LmrP-mediated [19]. Cells expressing the E327C or D142C LmrP mutants (Fig. 5C and D, solid lines) showed only moderate rates of ethidium influx under non-energized conditions, although significantly above the rate of ethidium influx into control cells (see also Table 1).

Upon addition of glucose, *L. lactis* cells generate a pmf by proton pumping coupled to ATP hydrolysis by the

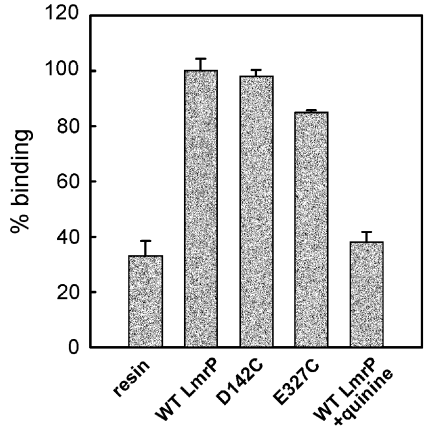

Fig. 4. Ethidium binding by WT and mutants of LmrP. Purified and immobilized on Ni-NTA agarose His_6-tagged LmrP variants were incubated in a buffer containing 5 μM ethidium or 5 μM ethidium and 1 mM quinine. Ethidium retained by the resin and resin-bound LmrP was assayed fluorimetrically as described in Experimental procedures. Ethidium binding to resin loaded with WT LmrP is taken as 100%.

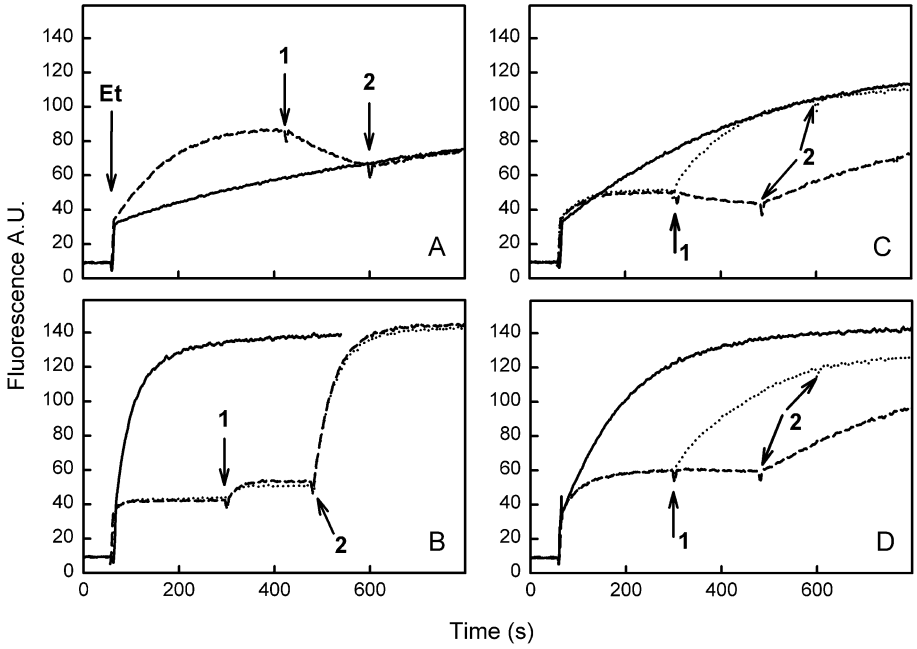

Fig. 5. Ethidium accumulation under different energetic conditions by *L. lactis* cells expressing WT or mutant LmrP. (A) Control cells not expressing LmrP; (B) WT LmrP-expressing cells; (C) D142C mutant LmrP-expressing cells; (D) E327C mutant LmrP-expressing cells. Et indicates addition of 10 μM ethidium. Solid lines represent traces obtained under non-energized conditions. Energized conditions: dashed lines, valinomycin was added at time point (1) and nigericin at time point (2); dotted lines, nigericin was added at time point (1) and valinomycin at time point (2). Valinomycin and nigericin were added to final concentrations of 2 μM. A.U., arbitrary units. For details see Experimental procedures.

membrane-bound F_0F_1-ATPase. The components of the pmf, the membrane potential ($\Delta\psi$) and the proton gradient (ΔpH), can be dissipated by the ionophores valinomycin and nigericin, respectively. 2-Aminobutyric acid is a non-metabolisable analogue of alanine, which has been shown to be transported in symport with protons. Both components of the pmf, the $\Delta\psi$ and the ΔpH, function as driving forces for its transport [21,22]. The generation of a $\Delta\psi$ and a ΔpH upon glucose addition to WT and E327C or D142C LmrP mutant overexpressing cells of *L. lactis* was demonstrated by the effects of valinomycin and nigericin on the pmf-driven uptake of 2-aminobutyric acid (data not shown). The effects of these ionophores on 2-aminobutyric acid transport were compared with pmf-driven transport of monovalent, positively charged ethidium in cells of *L. lactis*. Glucose-energized control cells accumulated ethidium at a significantly higher rate than non-energized cells (Fig. 5A). Dissipation of the $\Delta\psi$ by valinomycin reduced the accumulation of ethidium, while subsequent dissipation of the ΔpH by nigericin did not further affect the ethidium uptake. These results are in agreement with previous suggestions that $\Delta\psi$ (inside negative) drives passive diffusion of lipophilic cationic compounds across the membrane [19,23,24]. *L. lactis* cells overexpressing WT LmrP accumulated under energized conditions significantly less ethidium than control cells (Fig. 5A and B). Dissipation of the $\Delta\psi$ inhibited the efflux process and resulted in an increased rate and extent of ethidium accumulation. Subsequent dissipation of the ΔpH completely inhibited efflux resulting in an increased ethidium influx rate and uptake level similar to the non-energized cells (Fig. 5B). Similarly, a partial inhibition of ethidium efflux was obtained when only the ΔpH was dissipated, and ethidium accumulation level was further increased by the subsequent dissipation of the $\Delta\psi$ (Fig. 5B, dashed and dotted lines). Taken together, these results demonstrate that ethidium efflux by WT LmrP is driven by both $\Delta\psi$ and ΔpH.

The effect of the ionophores was also determined on pmf-driven ethidium efflux from *L. lactis* cells expressing the D142C or E327C LmrP protein. In the presence of glucose, the LmrP mutant cells (Fig. 5C and D, dashed lines) accumulated ethidium to a higher level than cells expressing WT LmrP (Fig. 5B), but significantly lower than energized control cells (Fig. 5A). This demonstrates that both mutants catalyzed ethidium efflux at a lower rate then WT LmrP-expressing cells, despite their ability to bind ethidium. Dissipation of the $\Delta\psi$ hardly affected the ethidium efflux activity, while subsequent dissipation of the ΔpH completely abolished efflux (Fig. 5C and D, dashed lines). A similar result was obtained when the ΔpH was first dissipated, whereas subsequent dissipation of the $\Delta\psi$ had no further effect (Fig. 5C and D, dotted lines). These observations suggest that ethidium efflux by the D142C and E327C mutant LmrP proteins is driven mainly by the ΔpH and hardly or not at all by the $\Delta\psi$.

3.5. Transport of propidium

Propidium, a fluorescent DNA/RNA stain, is structurally similar to ethidium (Fig. 1) but carries two positive charges

instead of one, while being less membrane-permeable than ethidium [25]. In the absence of a pmf, LmrP facilitates diffusion of substrates in the direction dictated by the substrate concentration gradient [19]. The transport of propidium can be followed by measuring the fluorescence of the transporting cells. To test if propidium is a substrate of LmrP, the diffusion of propidium into non-energized control and LmrP-overexpressing cells was monitored. Non-energized control cells accumulated propidium very slowly (Fig. 6A, solid line), consistent with an efficient barrier function of the cell membrane of *L. lactis*. Upon solubilization of the cell membrane by addition of the detergent dodecyl-β-maltoside (DDM), a rapid increase of the propidium fluorescence occurred (Fig. 6A). In contrast, LmrP-overexpressing non-energized cells took up propidium very rapidly and the uptake was only slightly stimulated by the subsequent addition of the detergent (Fig. 6A, dotted line). This fast increase of propidium uptake by LmrP overexpressing cells could be inhibited by quinine (0.5 mM), an inhibitor of LmrP, [7], added prior to propidium (Fig. 6A, dashed line). These results demonstrate that propidium is a substrate of LmrP.

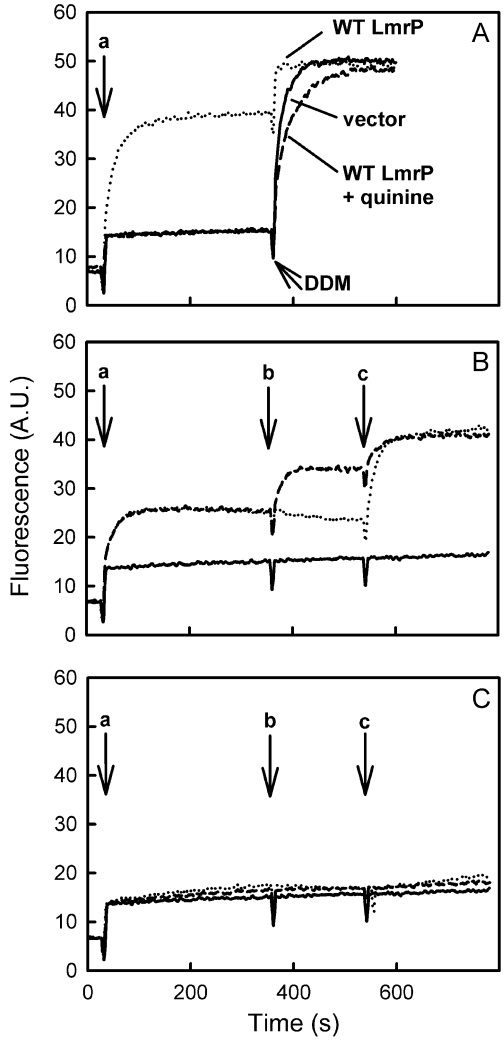

Fig. 6. Propidium accumulation under different energetic conditions by cells of *L. lactis*. Propidium was added to the cell suspension of A_{660}=0.5 at the time point (a) to a final concentration of 10 μM. (A) Passive influx of propidium into non-energized control (vector, solid line) and LmrP-expressing cells (dotted line). Quinine (0.5 mM) was used to block LmrP-facilitated diffusion of propidium (dashed line). DDM (0.1%) was added to permeabilize the cell membrane (DDM). (B) Accumulation of propidium by cells pre-energized with 25 mM glucose. Solid line, cells with empty vector pNZ8048, (b) addition of valinomycin, (c) addition of nigericin; dotted line and dashed line, cells expressing WT LmrP. For the dotted line, (b) addition of valinomycin, (c) addition of nigericin; for the dashed line, (b) addition of nigericin, (c) addition of valinomycin. (C) Transport activity of LmrP mutants D142C (dotted line) and E327C (dashed line) relative to cells harboring empty vector (solid line), (b) addition of valinomycin, (c) addition of nigericin. Both ionophores were added to final concentrations of 2 μM. A.U., arbitrary units.

The impact of dissipating the $\Delta\psi$ and/or the ΔpH on the propidium fluxes into *L. lactis* cells was investigated is the same way as for ethidium. Under energized conditions, control cells accumulated propidium at a rate similar or slightly higher than non-energized cells while dissipation of the $\Delta\psi$ and/or the ΔpH did not affect the rate of propidium accumulation (Fig. 6B, solid line). Strikingly, energized cells overexpressing LmrP accumulated propidium during the first 40 s at a significantly higher rate than control cells (Fig. 6B, dotted line) but the steady-state level of accumulation was significantly lower than in non-energized LmrP-overexpressing cells (Fig. 6A, dotted line). Unlike the accumulation of ethidium, the uptake of propidium was only slightly reduced upon the dissipation of the $\Delta\psi$ while subsequent dissipation of the ΔpH triggered a fast influx of propidium to levels similar to those observed under non-energized conditions (Fig. 6B, dotted line). When the ΔpH was dissipated first, a fast development of propidium fluorescence was observed, which was further stimulated by addition of valinomycin (Fig. 6B, dashed line). Cells expressing the D142C and E327C LmrP mutants behaved essentially as control cells and showed hardly any propidium efflux activities (Fig. 6C).

4. Discussion

LmrP is a secondary MDR transporter that mediates the extrusion of lipophilic drugs in exchanges for protons. In a previous report, three membrane-embedded carboxylic residues were identified with a critical role in the transport mechanism [9]. Two of these residues, i.e., Asp^{142} and Glu^{327}, are important for the LmrP transport activity [9], but their exact role in the transport process has remained obscure. To obtain insight into the role of these residues, substrate binding, transport and the energetics of drug extrusion were analyzed for the side-directed LmrP mutants at positions Asp^{142} and Glu^{327} and compared with WT LmrP. For these experiments, transport of two cationic substrates that differ in the number of positive charges, i.e., ethidium with one, and propidium with two positive charges, was analyzed (Fig. 1).

WT LmrP was found to bind Hoechst 33342 with a K_d of about 1.3 μM (Table 1). Hoechst 33342 binding could competitively be inhibited by quinine (Fig. 3C), a known inhibitor of LmrP-mediated Hoechst 33342 transport [7]. The measured K_d for Hoechst 33342 binding is close to the apparent K_m of 0.63 μM for LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles, while the K_i for quinine (apparent K_i of 3.0 μM) is in the same range as the IC_{50} of quinine of ~4.8 μM for Hoechst 33342 transport [7]. Substitution of Glu^{327} but not of Asp^{142} by alanine or cysteine drastically reduced the affinity of LmrP for Hoechst 33342. Nevertheless, both the D142A and D142C LmrP mutants were strongly impaired in Hoechst 33342 transport [9]. As expected, also the triple mutant D142C-E327C-C270A displayed a decreased affinity for Hoechst 33342 binding. This is most likely due to the E327C substitution, since the mutations D142C and C270A have no effect on Hoechst 33342 binding. This triple mutant is unable to transport Hoechst 33342 although it still has affinity for Hoechst 33342, suggesting that also hydrophobic interactions play a role in Hoechst 33342 binding. Taken together, these results indicate that Glu^{327}, but not Asp^{142}, plays a crucial role in binding and transport of Hoechst 33342 to LmrP. (Fig. 4). Both the D142C and E327C LmrP mutants had reduced ethidium transport activity (Fig. 5C and D). At the relatively high ethidium concentration (10 μM) used in the transport assay, the negatively charged residues at position 142 or 327 were found not to be essential for ethidium binding. The strong decrease of transport activity can thus not be explained by decreased binding. This observation indicates that these two membrane-embedded residues may not be involved in ethidium binding but may play additional roles, possibly in proton translocation in the transport process.

The substrates of LmrP used in this study can enter the cells via passive diffusion and by LmrP-mediated facilitated diffusion [19] and are excreted by pmf-driven active efflux by LmrP. The driving force for passive diffusion across the membrane of non-energized cells is the chemical gradient of a substrate [26]. The rate of diffusion in non-energized and energized control cells, lacking LmrP, is relatively high for ethidium (Fig. 5A, solid line) but low for propidium (Fig. 6A, solid line). In cells expressing LmrP, facilitated influx driven by the chemical substrate gradient occurs via LmrP in the absence of a pmf. The rate of facilitated influx is high for ethidium (Fig. 5B, solid line) and propidium (Fig. 6A, dotted line). In energized, LmrP-expressing cells, the pmf will influence the distribution of the cationic substrates in two ways: (i) the transmembrane potential (inside negative) will stimulate passive influx across the membrane [23,24,26], and (ii) the pmf will drive the active extrusion. Due to this passive influx (leak), a continuous action of the efflux system is required to keep the cells free of the substrates. The relative roles of the $\Delta\psi$ and the ΔpH as driving forces for active extrusion will differ for the differently charged substrates and will depend on the net translocation of charge and protons in the exchange process. The $\Delta\psi$ can only be a driving force for efflux if the excreted cationic substrate has less charge than the number of protons translocated per substrate molecule [26]. In growing cells of *L. lactis* suspended in medium of pH 7.0, the $\Delta\psi$ component of the pmf is significantly higher than the ΔpH component ($-Z\Delta$pH). Poolman et al. [27] observed in such cells a $\Delta\psi$ of about −55 mV and a $-Z\Delta$pH of around −20 mV. The roles of these two components of the pmf in LmrP-mediated transport of ethidium and propidium were analyzed for WT LmrP and mutants D142C and E327C. Since Hoechst 33342 can undergo deprotonation, which also alters its fluorescence intensity, the charge of the transported species is not precisely defined under the experimental conditions. Therefore, we could not use this substrate to investigate the role of the $\Delta\psi$ or ΔpH in energizing its transport. Transport of ethidium by WT LmrP is partially inhibited by dissipation of the $\Delta\psi$ or pmf. Full inhibition is observed only upon dissipation of the entire pmf. The remaining ethidium transport activity in the presence of only the $\Delta\psi$ (in presence of nigericin) or the ΔpH (in presence of valinomycin) can be explained by the concomitant increase of one component upon the dissipation of the other component of the pmf. As a result, the driving force for the extrusion process will hardly change [22]. These results are in agreement with an electrogenic nH^+/substrate^{z+} antiport mechanism previously proposed for substrates like TPP^+ and TMA-DPH [3]. This implies that at least two protons (n=2) are translocated per molecule of a monovalent substrate (z=1). In contrast, transport of propidium was not inhibited by the dissipation of the $\Delta\psi$ alone, while dissipation of the ΔpH alone resulted in very strong inhibition of transport of this compound. Dissipation of the $\Delta\psi$ after dissipation of ΔpH had a small additional inhibitory effect. These findings indicate that transport of propidium, a substrate with two positive charges, is predominantly driven by the ΔpH. Interestingly, within the first 40 s after addition of propidium, the cells expressing WT LmrP displayed under energized conditions a faster influx rate than the control cells which are essentially impermeable to propidium (Fig. 6B, dotted and dashed lines). This rapid influx in the energized and LmrP-expressing cells must, therefore, be LmrP-mediated. The increased accumulation of propidium indicated that the driving force for uptake, supplied by the chemical gradient of propidium, exceeds in absolute value the outward directed driving force supplied by the pmf. Summarizing, these observations show that LmrP-mediated transport is electrogenic for monovalent drugs and electroneutral for divalent substrates. Lewinson et al. [28] also observed that the electrogenicity of the transport reactions by for the *E. coli* MDR transporter MdfA depended on the charge of the substrate. MdfA mediates electrogenic transport of uncharged substrates and electroneutral efflux of substrates with a single positive charge.

A comparison of transport properties of WT LmrP and the D142C and E327C mutants revealed some interesting differences. Despite similar levels of protein expression and ethidium binding, the rates of facilitated ethidium influx via these mutant LmrP proteins under non-energized conditions were much slower than for WT LmrP. Apparently, the lack of a negative charge at position 142 or 327 reduces the transporter turnover. Under energized conditions, ethidium transport mediated by the mutant LmrPs D142C and E327C is $\Delta\psi$-independent. This strongly suggests that these mutants catalyze electroneutral H^+/ethidium exchange with one-to-one stoichiometry. Both mutants showed a very low propidium efflux activity and, therefore, the energetics of transport of propidium was therefore not further analyzed. The observations point towards a role of Asp^{142} or Glu^{327} in proton translocation in the antiport process. Glu^{327} was also found to be essential for high affinity binding of Hoechst 33342 but not of ethidium. Such a double role of Glu^{327} would be similar to the function of Glu^{14} in the transport mechanism of EmrE [29–31]. *E. coli* EmrE is a member of the Small Multidrug Resistance family, and Glu^{14} was proposed to be an essential part of a binding domain shared by substrates and protons but mutually exclusive in time providing the molecular mechanism for the obligatory exchange catalyzed by EmrE [29,31,32]. In this respect, it would be interesting to evaluate the pH dependence of Hoechst 33342 binding by D142C and E327C mutants. Since our data indicate that only Glu^{327} is directly interacting with Hoechst 33342, the D142C mutant should display a more pronounced pH dependency of Hoechst 33342 binding than the E327C mutant. Unfortunately the strong pH dependency of the protonation state and fluorescence of Hoechst 33342 prevented us from determining the pH dependence of Hoechst 33342 binding. Previously, we speculated that Asp^{142} and Glu^{327} both interact with substrates [9]. However, the data presented here show that the reduced activity of LmrP lacking Asp^{142} does not originate from a decreased substrate binding affinity but from altered energetics of transport. The observation that Asp^{142} and Glu^{327} are not strictly required for substrate binding is in agreement with data obtained from the crystal structures of QacR, a transcriptional regulator of the *qacA*, the MDR transporter gene of *Staphylococcus aureus* [33,34], and of AcrB, a drug transporter of *E. coli* [35]. Both QacR and AcrB were crystallized in the presence of various inducers and substrates. Both proteins possess several aromatic and acidic amino acids in the drug binding sites, subsets of which interact with different substrates. Interestingly, binding of ethidium by AcrB does not involve carboxylic residues [35], and QacR uses oxygen atoms of the peptide backbone instead of acidic residues to neutralize the positive charge of pentamidine [34]. Therefore, the presence of charged residues in the membrane domain of transporters does not seem to be a prerequisite of substrate binding but may be critical for proton translocation.

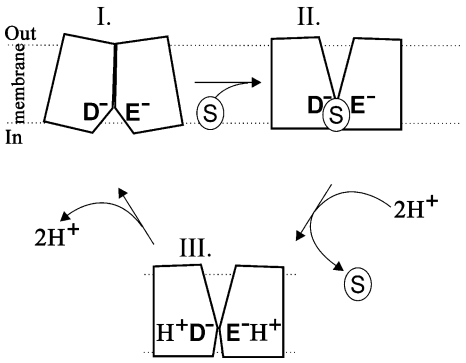

Fig. 7. Proposed mechanism for transport by LmrP. The hydrophobic binding pocket facing the membrane exposes deprotonated Asp^{142} (D) and Glu^{327} (E) (I) which enables binding of a substrate (II); alternatively, binding of substrates induces deprotonation of the indicated residues. When occupied by the substrate, the binding site becomes accessible to the other side of the membrane (III), whereupon binding of protons releases the substrate. The protonated transporter returns to the inward facing conformation, protons are released to the cytoplasm and a new cycle can start. The proposed mechanism suggests that the pH will influence the protonation state of Asp^{142} and Glu^{327} and, therefore, binding of substrates should be pH-dependent.

Previously, we have shown that upon interaction with substrates, the membrane-embedded residues D142C and E327C become more accessible to solvent [9]. This change in solvent exposure may be related to the transfer of these residues from a hydrophobic substrate binding site to a more hydrophilic substrate translocation pathway. On the basis of these findings, we propose for LmrP a transport mechanism as depicted in Fig. 7. Deprotonation of Asp^{142} and Glu^{327} results in the inward-facing conformation of LmrP, at which stage the protein can accept a substrate from the membrane (I). The substrate binding triggers a structural change in LmrP resulting in an outward-facing conformation with Asp^{142} and Glu^{327} exposed to more hydrophilic environment (II). At this step the protonation of Asp^{142} and Glu^{327} occurs, which is linked to the release of the substrate to the outside medium. Protonated LmrP (III) returns to the inward-facing state (I) with concurrent release of protons to the cytoplasm. The energetics of LmrP-mediated transport suggests that the transporter utilizes two protons to extrude one molecule of either ethidium or propidium. In other words, the H^+/substrate stoichiometry is invariant to the charge of the substrate transported.

References

[1] M. Putman, H.W. van Veen, W.N. Konings, Molecular properties of bacterial multidrug transporters, Microbiol. Mol. Biol. Rev. 64 (2000) 672–693.
[2] H. Bolhuis, G. Poelarends, H.W. van Veen, A.J.M. Driessen, W.N. Konings, The Lactococcal lmrP gene encodes a proton motive force-dependent drug transporter, J. Biol. Chem. 270 (1995) 26092–26098.
[3] H. Bolhuis, H.W. van Veen, J.R. Brands, M. Putman, B. Poolman, A.J.M. Driessen, W.N. Konings, Energetics and mechanism of drug transport mediated by the lactococcal multidrug transporter LmrP, J. Biol. Chem. 271 (1996) 24123–24128.

[4] M. Putman, H.W. van Veen, B. Poolman, W.N. Konings, Restrictive use of detergents in the functional reconstitution of the secondary multidrug transporter LmrP, Biochemistry 38 (1999) 1002–1008.
[5] M. Putman, H.W. van Veen, J.E. Degener, W.N. Konings, The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines, Microbiology 147 (2001) 2873–2880.
[6] M.M. Gottesman, I. Pastan, Biochemistry of multidrug resistance mediated by the multidrug transporter, Annu. Rev. Biochem. 62 (1993) 385–427.
[7] M. Putman, L.A. Koole, H.W. van Veen, W.N. Konings, The secondary multidrug transporter LmrP contains multiple drug interaction sites, Biochemistry 38 (1999) 13900–13905.
[8] O. Lewinson, E. Bibi, Evidence for simultaneous binding of dissimilar substrates by the *Escherichia coli* multidrug transporter MdfA, Biochemistry 40 (2001) 12612–12618.
[9] P. Mazurkiewicz, W.N. Konings, G.J. Poelarends, Acidic residues in the lactococcal multidrug efflux pump LmrP play critical roles in transport of lipophilic cationic compounds, J. Biol. Chem. 277 (2002) 26081–26088.
[10] O. Gajic, G.B. Buist, M. Kojic, L. Topisirovic, O.P. Kuipers, J. Kok, Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal MDR proteins, J. Biol. Chem. 278 (2003) 34291–34298.
[11] P.G. de Ruyter, O.P. Kuipers, W.M. de Vos, Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin, Appl. Environ. Microbiol. 62 (1996) 3662–3667.
[12] O.P. Kuipers, P.G.G.A. de Ruyter, M. Kleerebezem, W.M. de Vos, Quorum sensing-controlled gene expression in lactic acid bacteria, J. Biotechnol. 64 (1998) 15–21.
[13] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
[14] R. Higuchi, B. Krummel, R.K. Saiki, A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions, Nucleic Acids Res. 16 (1988) 7351–7367.
[15] Q. Qu, F.J. Sharom, Proximity of bound Hoechst 33342 to the ATPase catalytic sites places the drug binding site of P-glycoprotein within the cytoplasmic membrane leaflet, Biochemistry 41 (2002) 4744–4752.
[16] Q. Qu, J.W. Chu, F.J. Sharom, Transition state P-glycoprotein binds drugs and modulators with unchanged affinity, suggesting a concerted transport mechanism, Biochemistry 42 (2003) 1345–1353.
[17] Y. Cheng, W.H. Prusoff, Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction, Biochem. Pharmacol. 22 (1973) 3099–3108.
[18] T.R. Muth, S. Schuldiner, A membrane-embedded glutamate is required for ligand binding to the multidrug transporter EmrE, EMBO J. 19 (2000) 234–240.
[19] P. Mazurkiewicz, G.J. Poelarends, A.J. Driessen, W.N. Konings, Facilitated drug influx by an energy-uncoupled secondary multidrug transporter, J. Biol. Chem. 279 (2004) 103–108.
[20] H. Bolhuis, D. Molenaar, G. Poelarends, H.W. van Veen, B. Poolman, A.J.M. Driessen, W.N. Konings, Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant *Lactococcus lactis*, J. Bacteriol. 176 (1994) 6957–6964.
[21] J. Thompson, Characteristics and energy requirements of an alpha-aminoisobutyric acid transport system in *Streptococcus lactis*, J. Bacteriol. 127 (1976) 719–730.
[22] A.J. Driessen, J. Kodde, S. de Jong, W.N. Konings, Neutral amino acid transport by membrane vesicles of *Streptococcus cremoris* is subject to regulation by internal pH, J. Bacteriol. 169 (1987) 2748–2754.
[23] P.C. Hsieh, S.A. Siegel, B. Rogers, D. Davis, K. Lewis, Bacteria lacking a multidrug pump: a sensitive tool for drug discovery, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6602–6606.
[24] I.I. Severina, M.S. Muntyan, K. Lewis, V.P. Skulachev, Transfer of cationic antibacterial agents berberine, palmatine, and benzalkonium through bimolecular planar phospholipid film and *Staphylococcus aureus* membrane, IUBMB Life 52 (2001) 321–324.
[25] R.P. Haugland, Nucleic acid detection, in: M.T.Z. Spence (Ed.), Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes, Eugene, 1996, pp. 144–178.
[26] B. Poolman, W.N. Konings, Secondary solute transport in bacteria, Biochim. Biophys. Acta 1183 (1993) 5–39.
[27] B. Poolman, E.J. Smid, W.N. Konings, Kinetic properties of a phosphate-bond-driven glutamate-glutamine transport system in *Streptococcus lactis* and *Streptococcus cremoris*, J. Bacteriol. 169 (1987) 2755–2761.
[28] O. Lewinson, J. Adler, G.J. Poelarends, P. Mazurkiewicz, A.J. Driessen, E. Bibi, The *Escherichia coli* multidrug transporter MdfA catalyzes both electrogenic and electroneutral transport reactions, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 1667–1672.
[29] H. Yerushalmi, S. Schuldiner, An essential glutamyl residue in EmrE, a multidrug antiporter from *Escherichia coli*, J. Biol. Chem. 275 (2000) 5264–5269.
[30] H. Yerushalmi, S. Schuldiner, A model for coupling of H(+) and substrate fluxes based on "time-sharing" of a common binding site, Biochemistry 39 (2000) 14711–14719.
[31] H. Yerushalmi, S.S. Mordoch, S. Schuldiner, A single carboxyl mutant of the multidrug transporter EmrE is fully functional, J. Biol. Chem. 276 (2001) 12744–12748.
[32] H. Yerushalmi, S. Schuldiner, A common binding site for substrates and protons in EmrE, an ion-coupled multidrug transporter, FEBS Lett. 476 (2000) 93–97.
[33] M.A. Schumacher, M.C. Miller, S. Grkovic, M.H. Brown, R.A. Skurray, R.G. Brennan, Structural mechanisms of QacR induction and multidrug recognition, Science 294 (2001) 2158–2163.
[34] D.S. Murray, M.A. Schumacher, R.G. Brennan, Crystal structures of QacR–diamidine complexes reveal additional multidrug binding modes and a novel mechanism of drug charge neutralization, J. Biol. Chem. 279 (2004) 14365–14371.
[35] E.W. Yu, G. McDermott, H.I. Zgurskaya, H. Nikaido, D.E. Koshl Jr., Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump, Science 300 (2003) 976–980.