The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines

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The active efflux of toxic compounds by (multi)drug transporters is one of the mechanisms that bacteria have developed to resist cytotoxic drugs. The authors describe the role of the lactococcal secondary multidrug transporter LmrP in the resistance to a broad range of clinically important antibiotics. Cells expressing LmrP display an increased resistance to the lincosamide, streptogramin, tetracycline and 14- and 15-membered macrolide antibiotics. The streptogramin antibiotic quinupristin, present in the fourth-generation antibiotic RP 59500, can inhibit LmrP-mediated Hoechst 33342 transport, but is not transported by LmrP, indicating that quinupristin acts as a modulator of LmrP activity. LmrP-expressing *Lactococcus lactis* cells in which a proton-motive force is generated accumulate significantly less tetracycline than control cells without LmrP expression. In contrast, LmrP-expressing and control cells accumulate equal amounts of tetracycline in the absence of metabolic energy. These findings demonstrate that the increased antibiotic resistance in LmrP-expressing cells is a result of the active extrusion of antibiotics from the cell.

Keywords: multidrug resistance, multidrug transport, *Lactococcus lactis*, antibiotic resistance

INTRODUCTION

The successful introduction of antibiotics in the late 1940s led many to believe that infectious diseases, such as tuberculosis, could be conquered once and for all. The number of multidrug-resistant pathogenic microorganisms, however, demonstrates that microbial antibiotic resistance is a serious threat to public health worldwide (Cohen, 1992; Travis, 1994; Levy, 1998). The ease with which bacteria can exchange genetic material plays an important role in the emergence and spread of multidrug resistance (Davies, 1994; Baquero & Blázquez, 1997; Saier *et al.*, 1998; Perreten *et al.*, 1997; Anderson, 1999). Therefore, antibiotic resistance

genes from pathogenic as well as from non-pathogenic organisms are of medical importance.

One of the sophisticated biochemical mechanisms that bacteria have developed to evade the lethal effects of toxic drugs is the active efflux of these compounds. In contrast to specific drug-efflux systems, multidrug transporters can extrude a wide variety of structurally unrelated compounds. At present, four classes of multidrug efflux systems have been characterized in bacteria: (i) the ATP-binding cassette superfamily, (ii) the major facilitator superfamily, (iii) the resistance-nodulation-division family, and (iv) the small multidrug resistance family (Paulsen *et al.*, 1996; van Veen & Konings, 1998). Many organisms can express several multidrug transporters belonging to different classes.

In the Gram-positive bacterium *Lactococcus lactis* at least four drug-extrusion activities have been detected (Molenaar *et al.*, 1992; Bolhuis *et al.*, 1994; Glaasker *et*

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Abbreviation: PMF, proton-motive force.

al., 1996). The PMF-driven extrusion of amphiphilic drugs from the inner leaflet of the membrane is mediated by the multidrug transporter LmrP (Bolhuis *et al.*, 1995, 1996). LmrP is a 408-amino-acid integral membrane protein with 12 putative transmembrane segments, and belongs to the major facilitator superfamily. Extrusion of drugs by LmrP is driven by both the membrane potential and the transmembrane proton gradient, indicating that LmrP mediates an electrogenic proton/drug antiport reaction (Bolhuis *et al.*, 1996; Putman *et al.*, 1999b).

In this paper we describe the role of the secondary multidrug transporter LmrP in the resistance to a wide range of clinically important antibiotics that are used to fight infectious diseases. LmrP confers resistance to clindamycin, 14- and 15-membered macrolides, dalfo-pristin, RP 59500 and tetracycline antibiotics. Hence, LmrP and its endogenous and acquired homologues in pathogenic bacteria are a serious threat to the antibiotic-based treatment of patients with infectious diseases.

METHODS

Antibiotics. Azithromycin (batch 25381-087-02), ceftazidime (batch UCRZ 2500), ciprofloxacin (batch 530949B/1), clarithromycin (batch 33-132VH), clindamycin (batch 44BXC), gentamicin (batch USQ-6-GMF-N-6029) and meropenem (batch 00047N) were generous gifts from Pfizer, Glaxo Wellcome Research and Development, Bayer, IDC Abbott Laboratories, Pharmacia & Upjohn, Schering-Plough, and Zeneca Pharmaceuticals, respectively. Dalfo-pristin (batch GRV 1204), quinupristin (batch GRV 1205), RP 59500 (batch CB06274) and spiramycin (batch CA9502600) were gifts from Rhône-Poulenc Rorer. Vancomycin (batch X00336 SI05573) was a gift from Eli Lilly & Co. Ofloxacin (batch H900) and roxithromycin (batch 5A 3175B) were gifts from Hoechst Marion Roussel. Ampicillin, chloramphenicol and tetracycline were purchased from Boehringer Mannheim. Chlor-tetracycline, demeclocycline, erythromycin, kanamycin, minocycline, oxytetracycline and [^3H]tetracycline were obtained from Sigma.

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* was grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose (w/v) plus 5 µg chloramphenicol ml⁻¹ when appropriate. *Escherichia coli* was grown at 37 °C in Luria Broth containing 50 µg ampicillin ml⁻¹ when appropriate.

Preparation of membrane vesicles. *L. lactis* NZ9000 harbouring the plasmid pHLP5 (Putman *et al.*, 1999a) was grown at 30 °C to an OD₆₆₀ of about 0.5. LmrP expression was triggered by the addition of 0.1% (v/v) of the supernatant of the nisin-A-producing *L. lactis* strain NZ9700, giving a final concentration of approximately 10 ng nisin A ml⁻¹. After incubation for 1 h at 30 °C the cells were harvested by centrifugation. Inside-out membrane vesicles were prepared using a French pressure cell as described by Putman *et al.* (1999a). The protein concentration was determined by the Lowry method in the presence of 0.5% SDS using bovine serum albumin as a standard.

Hoechst 33342 transport. Hoechst 33342 transport mediated by LmrP was studied as described previously (Putman *et al.*,

1999a, b). Inside-out membrane vesicles (0.5 mg protein ml⁻¹) were resuspended in 50 mM potassium HEPES, pH 7.0, containing 2 mM MgSO₄, 8.5 mM NaCl, 0.1 mg creatine kinase ml⁻¹, plus 5 mM phosphocreatine. After 30 s incubation at 30 °C Hoechst 33342 was added to 1 µM final concentration. LmrP was energized by the generation of a proton-motive force (PMF) by the F₀F₁ H⁺-ATPase, upon the addition of 0.5 mM Mg²⁺-ATP. The amount of membrane-associated Hoechst 33342 was measured fluorimetrically (Perkin Elmer LS50B fluorometer), using excitation and emission wavelengths of 355 and 457 nm, respectively, and slit widths of 5 nm each. The initial rate of Hoechst 33342 transport was determined by linear regression of the fluorescence data obtained in the first 15 s after Mg²⁺-ATP addition.

Measurement of the transmembrane H⁺ gradient (ΔpH) in membrane vesicles. The ΔpH (inside acid) in inside-out membrane vesicles was monitored by fluorescence quenching of acridine orange as described previously (Putman *et al.*, 1999a). The transmembrane potential (Δψ, inside positive) in inside-out membrane vesicles was estimated from the increase in ΔpH upon dissipation of the Δψ by the addition of the K⁺ ionophore valinomycin. To study the effect of antibiotics on the PMF, the drugs were added to a final concentration of 1 and 4 times the IC₅₀ for inhibition of LmrP-mediated Hoechst 33342 transport.

Determination of growth rate. For the determination of growth rates in the presence of various antibiotics, overnight *E. coli* cultures (DH5α/pET302 and DH5α/pHLP1) were diluted into fresh medium and grown to mid-exponential phase. The cells were then diluted in fresh medium to an OD₆₆₀ of 0.025 and 150 µl aliquots of the cell suspension were transferred to sterile low-protein-binding 96-well microplates (Greiner), containing 50 µl of various concentrations of the antibiotics in fresh medium. For the induction of LmrP expression isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 5 µM. Aliquots (50 µl) of sterile silicone oil were pipetted on top of the sample to prevent evaporation. The cells were grown semi-anaerobically at 30 °C, and the cell density was monitored by measuring the absorbance at 690 nm every 15 min for 23 h in a multiscan photometer (Titretek Multiscan MCC/340 MKII). Growth rates were determined by non-linear least-square fitting of the absorbance data to the Gompertz equation describing bacterial growth (Zwietering *et al.*, 1990). The antibiotic concentrations which inhibited the growth rate by 50% (IC₅₀) were determined.

Tetracycline accumulation in whole cells. *L. lactis* NZ9000 cells harbouring the control plasmid (pNZ8048) or the LmrP-expression plasmid (pHLP5) were grown at 30 °C to mid-exponential phase (OD₆₆₀ 0.5). Nisin A was added to a concentration of approximately 10 ng ml⁻¹, to trigger the transcription of the *lmrP* gene. Subsequently, the cells were incubated for 1 h at 30 °C, harvested by centrifugation, washed three times in 50 mM potassium phosphate, pH 7.0, containing 0.2% glucose and 1 mM MgSO₄, and resuspended in the same buffer to a protein concentration of approximately 0.75 mg ml⁻¹. After a 10 min preincubation of 100 µl aliquots of the cell suspension at 30 °C, [^3H]tetracycline was added to a final concentration of 5 µM. At the time points indicated in the legend to Fig. 4, the reaction was stopped by the addition of 2 ml ice-cold 100 mM potassium phosphate, pH 7.0, containing 100 mM LiCl. The samples were rapidly filtered through 0.45 µm pore-size cellulose acetate filters (Schleicher & Schuell). The filters were washed once with 2 ml ice-cold

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
Strains		
<i>L. lactis</i>		
NZ9000	MG1363 derivative, <i>pepN::nisRK</i>	Putman <i>et al.</i> (1999a)
NZ9700	Nisin-producing transconjugant containing TN5276	Kuipers <i>et al.</i> (1993)
<i>E. coli</i>		
DH5 α	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 ΔlacU169 (ϕ80 <i>lacZ</i>ΔM15)</i>	BRL
Plasmids		
pNZ8048	Cm ^R , pSH71 replicon, inducible <i>nisA</i> promoter	Putman <i>et al.</i> (1999a)
pHLP5	pNZ8048 carrying His-tagged LmrP of <i>L. lactis</i>	Putman <i>et al.</i> (1999a)
pET302	pTRC99A carrying a His-tag coding region and <i>trc</i> promoter	van der Does <i>et al.</i> (1998)
pHLP1	pET302 carrying <i>lmrP</i> on a 1.39 kb <i>NcoI</i> - <i>XbaI</i> fragment	Bolhuis <i>et al.</i> (1996)

100 mM potassium phosphate, pH 7.0, plus 100 mM LiCl. The radioactivity on the filters was determined by liquid scintillation counting.

RESULTS

Inhibition of LmrP-mediated drug transport

To study the specificity of antibiotic binding to LmrP, several classes of clinically important antibiotics were tested for their ability to inhibit the LmrP-mediated transport of Hoechst 33342. The bisbenzimidazole dye Hoechst 33342 is fluorescent in a lipid environment, but

essentially non-fluorescent in aqueous solution. Therefore, the LmrP-mediated transport of Hoechst 33342 from the membrane into the lumen of inside-out membrane vesicles of *L. lactis* can be monitored as a loss of fluorescence in time (Fig. 1a). Antibiotics that are substrates of LmrP compete with Hoechst 33342 for binding to LmrP, and consequently inhibit Hoechst 33342 transport. A typical result, obtained with azithromycin, is shown in Fig. 1(a). From the dose-response curve (Fig. 1b) the concentration giving 50% inhibition of the initial rate of LmrP-mediated Hoechst 33342 transport (IC_{50}) was determined for the various anti-

Fig. 1. Inhibition of LmrP-mediated Hoechst 33342 transport by azithromycin. (a) Inside-out membrane vesicles prepared from LmrP-expressing *L. lactis* were diluted to a concentration of 0.5 mg ml⁻¹ in 50 mM potassium HEPES (pH 7.0) containing 2 mM MgSO₄, 8.5 mM NaCl, 0.1 mg creatine kinase ml⁻¹ and 5 mM phosphocreatine. After incubation for 30 s, 1 μM Hoechst 33342 was added. Transport was initiated upon the addition of 0.5 mM Mg²⁺-ATP. The rate of Hoechst 33342 transport was measured in the absence or presence of various concentrations of azithromycin. (b) Determination of the azithromycin concentration giving 50% inhibition of the initial rate of LmrP-mediated Hoechst 33342 transport (IC_{50}). The initial rate was determined over the first 15 s after addition of 0.5 mM Mg²⁺-ATP. The IC_{50} was determined by non-linear regression analysis using the general dose-response equation.

Table 2. Inhibition of LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles by antibiotics

Class	Antibiotic	Apparent IC ₅₀ (μ M)
Lincosamides	Clindamycin	90.1
Macrolides	Azithromycin	2.0
	Clarithromycin	0.9
	Erythromycin	2.6
	Roxithromycin	0.4
	Spiramycin	60.3
Streptogramins	Dalfopristin	3.5
	Quinupristin	11.2

Fig. 2. Effect of antibiotics on the PMF in inside-out membrane vesicles. The Δ pH-dependent fluorescence of acridine orange in inside-out membrane vesicles of *L. lactis* was measured in the absence (solid black trace) or presence of 90 μ M clindamycin (solid grey trace) or 60 μ M spiramycin (dotted black trace). For clarity of presentation, the traces are offset 100 and 200 units from the solid black trace, respectively. Inside-out membrane vesicles were diluted to a concentration of 0.5 mg protein ml⁻¹ in 50 mM potassium HEPES (pH 7.0) containing 2 mM MgSO₄, 8.5 mM NaCl, 0.1 mg creatine kinase ml⁻¹ and 5 mM phospho-creatine. Acridine orange was added to a final concentration of 1.25 μ M (a). A PMF (inside acid and positive) was generated by the F₁F₀ H⁺-ATPase upon the addition of 0.5 mM Mg²⁺-ATP (b). Valinomycin (c) and nigericin (d) were added to a final concentration of 1 μ M each, to interconvert $\Delta\psi$ into Δ pH, and to dissipate the Δ pH, respectively.

biotics (Table 2). Since tetracyclines and the quinolones ciprofloxacin and ofloxacin strongly interfered with Hoechst 33342 fluorescence (data not shown) these compounds were not tested. Ampicillin, chloramphenicol, ceftazidime, gentamicin, kanamycin, meropenem, sulfamethoxazol, trimethoprim and vancomycin, added in a 35- to 200-fold excess of Hoechst 33342, did not affect LmrP-mediated Hoechst 33342 transport in inside-out membrane vesicles, indicating

Fig. 3. Effect of erythromycin on the growth rate of *E. coli* DH5 α with and without expression of LmrP. Control cells (DH5 α /pET302, ●) and LmrP-expressing cells (DH5 α /pHLP1, □), were grown semi-anaerobically at 30 °C on Luria Broth, supplemented with 50 μ g ampicillin ml⁻¹, 5 μ M IPTG and erythromycin at various concentrations as indicated. The relative growth rate is plotted as a function of the erythromycin concentration.

that these antibiotics do not bind to LmrP. Lincosamide, macrolide and streptogramin antibiotics significantly inhibited LmrP-mediated Hoechst 33342 transport (Table 2).

The antibiotics could inhibit the Hoechst 33342 transport by a direct interaction with LmrP or by interfering with the driving force for LmrP-mediated transport. To analyse the effect of the antibiotics on the PMF, the fluorescent probe acridine orange was used (Fig. 2). Generation of a Δ pH (inside acid) causes quenching of acridine orange fluorescence. Upon addition of valinomycin, the fluorescence further decreases, due to the interconversion of $\Delta\psi$ into Δ pH. The subsequent dissipation of the pH gradient, by the addition of nigericin, leads to the release of acridine orange from the vesicles, with a concomitant increase in fluorescence (Ramawamy *et al.*, 1989). This increase is indicative of the magnitude of the PMF that was generated. The acridine orange measurements revealed that azithromycin, clarithromycin, erythromycin, roxithromycin, dalfopristin and quinupristin, at concentrations of up to 4 times their IC₅₀, did not significantly affect the magnitude and composition of the PMF. However, as shown in Fig. 2, clindamycin and spiramycin inhibited the generation of a PMF in the inside-out membrane vesicles at a concentration equal to their respective IC₅₀. These results indicate that clindamycin and spiramycin inhibit LmrP-mediated Hoechst 33342 transport indirectly by interfering with the generation of the driving force for transport, whereas the other macrolide and streptogramin antibiotics interact with LmrP directly.

Table 3. Antibiotic susceptibilities of *E. coli* DH5 α harbouring the LmrP-encoding plasmid pHLP1 or the control plasmid pET302

Class	Antibiotic	IC ₅₀ ($\mu\text{g ml}^{-1}$)	
		DH5 α /pHLP1	DH5 α /pET302
Aminoglycosides	Gentamicin	1.55 $\pm$ 0.21	3.00 $\pm$ 0.76
	Kanamycin	1.29 $\pm$ 0.18	1.41 $\pm$ 0.28
β -Lactams	Ceftazidime	2.18 $\pm$ 0.87	2.78 $\pm$ 1.86
	Meropenem	0.04 $\pm$ 0.02	0.04 $\pm$ 0.01
Glycopeptides	Vancomycin	49.6 $\pm$ 2.5	51.1 $\pm$ 6.4
Lincosamides	Clindamycin	147.9 $\pm$ 21.4	65.3 $\pm$ 24.1
Macrolides	Azithromycin	4.00 $\pm$ 1.04	2.51 $\pm$ 1.10
	Erythromycin	64.1 $\pm$ 7.2	32.5 $\pm$ 6.8
	Clarithromycin	55.3 $\pm$ 0.6	30.8 $\pm$ 0.6
	Roxithromycin	102.4 $\pm$ 22.0	62.4 $\pm$ 23.49
	Spiramycin	89.5 $\pm$ 9.6	91.7 $\pm$ 8.5
Quinolones	Ciprofloxacin	0.10 $\pm$ 0.11	0.18 $\pm$ 0.10
	Ofloxacin	0.04 $\pm$ 0.01	0.04 $\pm$ 0.01
Streptogramins	Dalfopristin	71.9 $\pm$ 22.9	41.6 $\pm$ 13.2
	Quinupristin	483.1 $\pm$ 14.1	426.8 $\pm$ 47.8
	RP 59500	83.7 $\pm$ 3.5	58.7 $\pm$ 17.0
Tetracyclines	Chlortetracycline	0.55 $\pm$ 0.40	0.13 $\pm$ 0.01
	Demeclocycline	0.30 $\pm$ 0.11	0.11 $\pm$ 0.01
	Minocycline	0.63 $\pm$ 0.16	0.41 $\pm$ 0.15
	Oxytetracycline	0.22 $\pm$ 0.08	0.12 $\pm$ 0.01
	Tetracycline	0.37 $\pm$ 0.25	0.12 $\pm$ 0.08
Others	Chloramphenicol	0.81 $\pm$ 0.60	0.52 $\pm$ 0.33

Antibiotic transport by LmrP

The antibiotic specificity of LmrP was further studied in cytotoxicity assays. This type of experiment allowed the use of antibiotics which interfered with Hoechst 33342 fluorescence in the previous experiment. In cytotoxicity assays, the antibiotic susceptibilities of cells over-expressing the drug transporter are compared with those of non-LmrP-expressing control cells. Unfortunately, previous work showed that the overexpression of LmrP is detrimental to the cells (Putman *et al.*, 1999a). A positive effect of LmrP on antibiotic resistance is hard to interpret against a background of growth inhibition by LmrP expression. Therefore the experiments were performed in *E. coli* cells with a low level of LmrP expression (DH5 α /pHLP1, induced with 5 μM IPTG), which have a growth rate comparable to that of the control cells (DH5 α /pET302).

The growth rates of DH5 α /pET302 and DH5 α /pHLP1 were determined in the presence of a variety of antibiotics. A typical result, obtained with erythromycin, is shown in Fig. 3. The dose-response curve of the LmrP-expressing cells is shifted towards higher erythromycin concentrations, compared to the curve of the control cells, indicating that LmrP increases the resistance to this antibiotic, even at this low level of expression of LmrP. The concentration of the various antibiotics required to inhibit the growth rate by 50% (IC₅₀) is shown in Table 3. Because of the presence of an ampicillin-resistance marker on plasmids pET302 and

pHLP1, ampicillin was not included in the cytotoxicity experiments. Since the sulfonamide antibiotic sulfamethoxazol did not significantly inhibit *E. coli* growth at a concentration of 400 $\mu\text{g ml}^{-1}$ (data not shown), this compound was omitted from further studies.

Comparison of the IC₅₀ values for DH5 α /pHLP1 and DH5 α /pET302 revealed that the observed differences between the LmrP-expressing and control cells are small. This is due to the very low level of LmrP expression under the conditions used. Only for lincosamides, tetracyclines and a number of macrolide and streptogramin antibiotics was a shift in the dose-response curve (Fig. 3) observed in each single measurement. Although after averaging the measurements some of the data (Table 3) actually overlap in their standard deviation, we consider these antibiotics to be substrates for LmrP.

LmrP consistently increased the resistance to the macrolides azithromycin, clarithromycin, erythromycin and roxithromycin. These macrolide antibiotics contain a 14- or 15-membered lactone ring that has one or more deoxysugars attached (Woodward, 1957; Ballow & Amsden, 1992). Interestingly, LmrP did not seem to confer resistance to the 16-membered macrolide spiramycin. LmrP expression increased the resistance to the streptogramins dalfopristin and RP 59500, but did not affect the resistance to quinupristin. The fourth-generation antibiotic RP 59500 is a combination of dalfopristin and quinupristin, which demonstrates synergistic and bactericidal activity against Gram-positive

Fig. 4. Effect of ionophores on tetracycline accumulation in *L. lactis* cells. Tetracycline accumulation in *L. lactis* NZ9000/pNZ8048 (control cells, ●) and NZ9000/pHLP5 (LmrP-expressing cells, □) was measured in cells suspended in 50 mM potassium phosphate, pH 7.0, containing 0.2% glucose and 1 mM MgSO_4 . The cells ($0.75 \text{ mg protein ml}^{-1}$) were incubated for 10 min at 30°C , prior to the addition of $5 \mu\text{M}$ [^3H]tetracycline. The accumulation of tetracycline was determined after 0, 1, 3, 5, 10 and 15 min. The experiments were performed in the absence of ionophores (a; $\Delta\text{pH} + \Delta\psi$) or in the presence of $5 \mu\text{M}$ valinomycin (b; ΔpH increased, $\Delta\psi$ dissipated), $5 \mu\text{M}$ nigericin (c; ΔpH dissipated, $\Delta\psi$ increased) or both valinomycin and nigericin, each at $5 \mu\text{M}$ (d; both ΔpH and $\Delta\psi$ dissipated).

pathogens including methicillin-resistant *Staphylococcus aureus* (Leclercq & Courvalin, 1991; Aumercier *et al.*, 1992; Kang & Ryback, 1995). The LmrP-expressing strain showed higher resistance to all tetracycline antibiotics tested, indicating that substitutions on carbon atoms 5, 6 and 7 do not preclude the interaction between LmrP and the tetracycline antibiotics. LmrP expression did not increase the resistance to aminoglycosides, β -lactams, glycopeptides, quinolones, and chloramphenicol.

Electrogenic tetracycline transport by LmrP

To determine whether the observed resistance to tetracycline antibiotics in LmrP-expressing cells is indeed caused by an active extrusion process, the accumulation of tetracycline in LmrP-expressing and control *L. lactis* cells was measured. In the presence of an energy source the control cells (NZ9000/pNZ8048) accumulated sixfold more tetracycline than the LmrP-expressing

NZ9000/pHLP5 cells (Fig. 4a). Dissipation of the membrane potential by the addition of valinomycin (Fig. 4b) resulted in a slight increase in the amount of tetracycline in the control cells. This is most likely due to an increase in ΔpH , which is a driving force for the passive influx of tetracycline into the cell (Munske *et al.*, 1984). The accumulation of tetracycline in the LmrP-expressing cells was hardly affected by the addition of valinomycin, indicating that the ΔpH is a driving force for LmrP-mediated tetracycline extrusion. Dissipation of the ΔpH by the addition of nigericin slightly reduced the high level of tetracycline accumulation in control cells, but did not affect the low level of tetracycline accumulation in LmrP-expressing cells (Fig. 4c). When both components of the PMF were dissipated by the addition of both valinomycin and nigericin (Fig. 4d), equal tetracycline accumulation in both the control and LmrP-expressing cells was observed. These results demonstrate that the increased tetracycline resistance in LmrP-expressing cells is a result of the active extrusion of tetracycline by LmrP. Both the ΔpH and $\Delta\psi$ can drive the LmrP-mediated tetracycline efflux, indicative of an electrogenic tetracycline/ H^+ antiport reaction.

DISCUSSION

Despite the very low level of expression of LmrP in the cytotoxicity experiments, a consistently increased resistance to four classes of antibiotics was observed. The involvement of LmrP in the resistance to these compounds is supported by the inhibition of LmrP-mediated Hoechst 33342 transport by macrolide and streptogramin antibiotics, and the active transport of tetracycline by LmrP. Altogether, these results indicate that the antibiotic specificity of LmrP includes lincosamides, 14- and 15-membered macrolides, streptogramins and the tetracycline class of antibiotics. Unlike other secondary multidrug transporters, such as AcrAB, Bmr, MdfA, MexAB, NorA, NorM and Tap, LmrP did not confer resistance to aminoglycosides, β -lactam antibiotics, quinolones or chloramphenicol (Yoshida *et al.*, 1990; Neyfakh, 1992; Li *et al.*, 1995; Edgar & Bibi, 1997; Aínsa *et al.*, 1998; Morita *et al.*, 1998; Nikaido *et al.*, 1998).

Although macrolides, lincosamides and streptogramins differ structurally, these antibiotics are often grouped together (MLS antibiotics) because of their similar mechanism of action, which involves the inhibition of protein synthesis through the binding to the 50S subunit of the bacterial ribosome (Contreras & Vásquez, 1977). Although the most widespread mechanism of resistance to MLS-type antibiotics is target-site alteration (Weisblum, 1995), this work and other studies (Leclercq & Courvalin, 1991; Clancy *et al.*, 1996) demonstrate that the resistance to MLS-type antibiotics can also result from enhanced antibiotic efflux. LmrP confers resistance only to the 14- and 15-membered macrolides. The macrolide specificity of LmrP resembles that of the multidrug transporter MefA from *Streptococcus pyogenes*, which confers resistance to 14- and 15- but not to 16-membered macrolide antibiotics (Clancy *et al.*,

1996). LmrP expression increased the resistance to dalbopristin and RP 59500, but not to quinupristin, suggesting that quinupristin is not transported by LmrP. However, the observed inhibition of LmrP-mediated Hoechst 33342 transport by quinupristin demonstrated that this streptogramin can bind to LmrP. The ability of quinupristin to modulate the activity of multidrug efflux systems may be one of the mechanisms responsible for the synergistic activity of the combination of dalbopristin and quinupristin in antibiotic-based therapy.

Cells expressing LmrP showed an increased resistance to the tetracycline antibiotics. Studies with radio-labelled tetracycline demonstrated that LmrP-expressing cells accumulate significantly less tetracycline than control cells, indicating that the increased tetracycline resistance is due to the active extrusion of tetracycline by LmrP. Dissipation of the ΔpH or $\Delta\psi$ only did not increase the accumulation of tetracycline in the LmrP-expressing cells, suggesting that the LmrP-mediated transport of tetracycline is an electrogenic process. This observation is similar to previous observations on the LmrP-mediated transport of TPP^+ (Bolhuis *et al.*, 1996) and Hoechst 33342 (Putman *et al.*, 1999b) and the TetL-mediated transport of tetracycline (Guffanti & Krulwich, 1995). Specific tetracycline transporters, such as TetB of *E. coli* and TetL of *Bacillus subtilis*, extrude a complex of tetracycline and a divalent cation in an exchange with one (TetB; Yamaguchi *et al.*, 1990, 1991) or more protons (TetL; Guffanti & Krulwich, 1995). The role of divalent cations in the LmrP-mediated tetracycline transport will be a subject of further research.

The antibiotic specificity of LmrP indicates that PMF-dependent multidrug transporters can affect the efficacy of a number of clinically relevant antibiotics. Although *L. lactis* is occasionally found as a pathogen in immunocompromised hosts, this bacterium is generally considered to be non-pathogenic and safe to use in starter cultures for cheese production (Gasser, 1994). Given the broad antibiotic specificity of the lactococcal secondary multidrug transporter LmrP and the ATP-binding cassette transporter LmrA (Putman *et al.*, 2000), additional studies are required to determine the probability of transfer of chromosomal resistance genes, such as *lmrA* and *lmrP*, to other bacteria in food or the digestive tract.

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