Biofilm development and toxic compound resistance in Lactococcus lactus

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CHAPTER 4

It is established in the sciences that no knowledge is acquired save through the study of its causes and beginnings, if it has had causes and beginnings; nor completed except by knowledge of its accidents and accompanying essentials.

Avicenna [†]

[†] Persian philosopher and scientist (980-1037AD). Excerpt taken from: Charles F. Horne, ed., *The Sacred Books and Early Literature of the East,* (New York: Parke, Austin, & Lipscomb, 1917), Vol. VI: *Medieval Arabia*, pp. 90-91).

Chapter 4

Characterization of Rhodamine 6G-resistant *Lactococcus lactis* strains lacking the LmrCD MDR transporter

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ABSTRACT

Resistance to toxic compounds is a requirement for bacteria to survive environmental stresses. Here, we have analyzed the ability of a *L. lactis* strain that lacks the major drug transporter LmrCD to regain multidrug resistance. Cells readily adapted to high concentrations of rhodamine 6G with considerable cross resistance to many drugs and detergents, most notably daunomycin. Resistance is accompanied with the up-regulation of the known MDR efflux protein LmrP, and the daunomycin-dependent upregulation of another MFS type of transporter *llmg_0631*. Transport assays suggest that the resistant strains are active in Rhodamine 6G transport, but the resistance appears to be derived from multifactorial processes among which an altered oxidative stress response.

INTRODUCTION

The ABC-type multidrug transporter LmrCD plays a crucial role in the intrinsic resistance of *L. lactis* to various drugs and bile acids. In addition, *L. lactis* shows an adaptive resistance wherein exposure to sub-inhibitory concentrations of drugs can transiently induce a multidrug resistance (10). This process also depends on LmrCD. The genome of *L. lactis* contains 40 putative drug transporter genes, only a few of which have been characterized functionally (11). These are the ABC-type MDR transporter LmrA (26); a member of the MFS, LmrP (2); and the heterodimeric ABC-type MDR transporter, LmrCD (12). The involvement of the other MDR-like transporters in the intrinsic and acquired drug resistance in *L. lactis* remains unresolved. Previous work has shown that *L. lactis* mutants that are able to grow in the presence of increasing concentrations of toxic compounds, such as ethidium, rhodamine, and daunomycin, results in the development of strains which are not only resistant to the selection drug but also cross-resistant to structurally unrelated drugs (1). The resistance was shown to be largely due to LmrCD based efflux (11). This raises the question as to whether in the absence of the *lmrCD* genes. *L. lactis* cells are still able to develop multidrug resistance when exposed to various toxic compounds. Moreover, if so, it is of interest to determine if this resistance is transporter

based, possibly revealing the function of cryptic transport genes, or that other resistance mechanisms prevail that are more specific by nature as for instance mutations in target proteins or caused by overall physiological changes. In this work, an LmrCD deleted, drug-susceptible *L. lactis* MG1363 strain (10) was challenged with increasing concentrations of a well-known substrate of LmrCD, Rhodamine 6G. This yielded strains with high and intermediate resistance. The mechanism by which the cells adapted to this compound was evaluated by different methods including transport assays and expression profiling of potential multidrug transporter genes. The data suggests a transporter-based efflux mechanism in acquired drug resistance indicate a high degree of adaptability of the *L. lactis* cells toward notorious compounds in the growth medium.

MATERIALS & METHODS

Microorganisms, media, and culture conditions. *L. lactis* NZ9000 is a derivative of the plasmid-free *L. lactis* MG1363 strain containing *pepN*::*nisRK* (5,6) referred to as the wild-type. *L. lactis* NZ9000 Δ*lmrCD* (13) lack the ABC-type MDR transporter LmrCD. Cells were grown in M17 medium (Difco) containing 0.5% (wt/vol) glucose (GM17) at 30°C. Growth in microtiter plates was monitored by absorbance using a multiscan photometer at specified wavelengths (spectraMax 340, Molecular Devices).

Adaptation conditions and measurement of rhodamine 6G resistance. Rhodamine 6G-resistant cells were selected by growth of *L. lactis* NZ9000 Δ*lmrCD* in GM17 medium containing increasing concentrations of rhodamine 6G. Exponentially growing cells were diluted 1:100 in 5 ml of fresh GM17 containing rhodamine 6G and grown overnight. This procedure was repeated several times with a concomitant stepwise increase of the cholate concentration until a significant increase in MIC value had occurred. The final concentration of rhodamine used was 22 μM. Growth curves were determined at each transfer during the adaptation period. To obtain single colonies, the adapted cultures were spread-plated on GM17 medium with 1.8% (wt/vol) agar containing 12 and 22 μM rhodamine 6G. Two colonies from each plate were selected and subcultured in fresh GM17 broth without

rhodamine 6G. Cultures were supplemented with glycerol at a final concentration of 10 % (vol/vol) and stored at -80°C. One of the rhodamine-resistant variants of *L. lactis ΔlmrCD* isolated at an early and advanced stage of adaptation was designated as *ΔlmrCD(rho12)* and *ΔlmrCD(rho22)*, respectively. These were used for further characterization.

Growth inhibition bioassays. *Planktonic cells:* Overnight cultures of indicated *L. lactis* strains were diluted in fresh GM17 and grown to an $OD_{660\ nm}$ of 0.6. Aliquots of 150 µl were transferred to 96-wells microtiter plates that contained 50 µl of GM17 medium and when indicated drugs at a given concentration. Plates were sealed with parafilm and incubated for 12 hrs at 30°C after which the $OD_{660\ nm}$ was measured. Concentrations that inhibited growth by 50% (IC_{50}) and 100% (MIC) were determined. Experiments were carried out in triplicate, and data shown are averaged with indicated standard error of the mean. To carry out growth inhibition assays under drug challenged conditions, same procedure was followed except that the overnight cultures were diluted in fresh GM17 supplemented with subinhibitory concentration of daunomycin (1µM) and grown as above.
Biofilm cells: The drug susceptibility of *L. lactis* biofilms was determined as described previously with minor modifications (7). Cells grown for 12 hrs in GM17 were diluted in fresh GM17 to an $OD_{660\ nm}$ of 0.6-0.8. Aliquots of 200 µl were transferred to each well of a 96-well flat-bottom polystyrene microtiter covered with a 96-peg lid of polystyrene plate (Nunc, Denmark). Cells were incubated for 24 hrs to allow for biofilm formation on the pegs. To remove non-adherent cells, the peg lid was dipped into a series of three 96-well plates containing 200 µl of sterile phosphate buffered saline (PBS) per well. The peg lid was then placed on a fresh plate that contained in each well 200 µl GM17 with the indicated drug. GM17 medium without drugs served as positive growth control. Biofilms were incubated overnight at 30°C, rinsed three times in PBS as described above and placed into a final 96-well plate containing 200 µl of GM17 along with various drug concentrations. Biofilm cells were then dislodged from the pegs by bath sonication of the microtiter plate for 5 min at 40 kHz (Zenith Ultrasonics, Norwood, NJ). The peg lid was removed and the $OD_{660\ nm}$ of the remaining suspension in the wells was determined. The plate was covered by a normal lid and incubated

for 24 hrs, where after a second $OD_{660\ nm}$ reading was performed. The IC_{50} was defined as the drug concentration that showed 50 % of the growth reduction as compared to the growth in control wells, no drugs. Wells containing sterile GM17 served as spectrophotometric blanks.

Biofilm formation. The ability of *L. lactis* to form biofilms was assessed by a previously described method with minor modifications (3). Cells were grown for 24 h in GM17 and subsequently diluted to an $OD_{660\ nm}$ of 1 in fresh GM17 supplemented with drugs at the indicated concentrations. Next, aliquots of 200 µl were transferred into the wells of a 96-well flat-bottom microtiter plate. For each drug concentration, 16 wells were used. Plates were then sealed with parafilm and incubated for 24 h at 30°C. Planktonic cells were transferred to a new microtiter plate and the $OD_{660\ nm}$ was measured. For each drug concentration, the biofilm of a eight wells was thoroughly scraped from the walls of the well using a pipette tip and cells were resuspended in 200 µl of fresh GM17. The $OD_{660\ nm}$ was used as a measure of the biofilm cell density. The biofilms in the remaining eight wells were washed twice by fully submerging the plate in deionized water, air dried overnight, stained for 15 min with an aqueous solution of 0.1 % (w/v) crystal violet, rinsed with water and air dried overnight. The crystal violet-stained biofilm was suspended to homogeneity in 200 µl of DMSO and the $OD_{575\ nm}$ was measured for each of the wells and averaged. Wells containing sterile GM17 served as controls and were used for background subtraction. To relate biofilm formation to cell density, the ratio of biofilm $OD_{575\ nm}$ to OD_{660} was calculated. Assays were repeated three times with independent cultures.

MDR transport activity. MDR transport activity of the various *L. lactis* strains was assessed with daunomycin, Hoechst 33342 and Hoechst 33258 using previously established protocols (12).

Analysis of expression of putative MDR transporter genes. Real time qPCR was used to analyze of expression of putative efflux pump genes using the housekeeping gene *tufA* as a control following exposure or not of the above mentioned strains to daunomycin. Primer pairs used are listed in Table 1. Total RNA was isolated as described previously (27). Following elution,

TABLE 1. Oligonucleotide primers used for qPCR analysis

Primer name		Primer sequence 5'→ 3'
llmg_0631	F	GACCAGGCATGCGGCTAGGATAATCAAAGAG
	R	CATCGCGAGCTCTCATTCCATAATACTCCGTTTCCG
llmg_1202	F	CGAGGCGCATGCATGAAAATTTTTAAAGATTTGTGG
	R	CAGCATGAGCTCTCAGTAGATTTCTCCATTTTC
llmg_1210	F	AGCCTCTCCACAGCACAAG
	R	CCATCGCCATCACTACACG
llmg_140	F	CATCTAGCATGCGGGACATTTATGAC
	R	CATCGAGAGCTCCGATTAAGAAGCAAATTGAAC
llmg_133	F	CATCGAGCATGCATAGCAGTAGCTGAGTCTATT
	R	CATCTAGAGCTCCTACGAATGACCATAGAC
llmg_1856	F	CATTGCACGGGCTTTCTTACG
	R	GTTCATTGTGTTTCCCACTACCTG
llmg_2446	F	GGAGCAGCTTTAGCTATTGCC
	R	CCAAGCACCTAATGCTGCCC
llmg_1322	F	CCTTTGCCAATCGAAGTACAACC
	R	GAATTGTATAATGCAGTACCGGCCC
llmg_2386	F	GACACGTTCTCCTAAACGGAC
	R	CGGCACTTGGAGCAGTTC
llmg_0320	F	AGACCGCTTTGGTCGCAAAC

Primer name		Primer sequence 5'→ 3'
	R	AGCAGCAGCTGTCCATCC
TufA2	F	TGACGAAATCGAACGTGGTCAAG
	R	GTCACCAGGCATTACCATTTCAG
llmg_0421	F	CCTCACGAAGTTGGGTTCC
	R	TTATCGTCATGGCCAAGCG
llmg_2513	F	GGACACGTTGTGCCAGCTTC
	R	GCATAGGCACGCTCTGCATC
llmg_0856	F	GTGCGTGATGAGAAGAGATATGC
	R	AAAGAATGACTCCAGTCCAATCC
llmg_1015	F	TCTTAAGGAAGCGCCCATCAG
	R	CAAGCGAGTAATGAAGGTTCCAC

nucleic acid concentrations were determined by spectrophotometry (NanoDrop) and residual DNA contamination was removed by incubating the samples with 4 units of TURBO DNase (Ambion). After DNase inactivation, the RNA was recovered, quantified, and used as a template for PCR to confirm inactivation of contaminating DNA. The PCR-negative RNA was used for first-strand cDNA synthesis by using SuperScript II reverse transcriptase (Invitrogen). For cDNA synthesis, the manufacturer's protocol was followed, starting with 1 µg total RNA template and 50 ng random hexamers. RNA transcripts were quantified in triplicate on an iCycler iQ 96-well PCR plate (Bio-Rad, Hercules, CA) by using the Brilliant SYBR green QPCR mix (Stratagene) in a 25 µl volume containing 50 ng cDNA. Optimal primer concentrations were determined empirically. The efficiency of each primer pair was determined with a dilution series of chromosomal DNA. The 96-well plate was sealed with optical tape, and samples were quantified with the iCycler (Bio-Rad) using a standard thermal cycling program. Real-time results were analyzed using the iCycler iQ optical system software, version 3.0a (Bio-Rad). The melting curve profile was analyzed and it was verified that there was a single product for each sample.

RESULTS

Development of adaptive resistance against rhodamine 6G by *Lactococcus lactis*. *L. lactis* has previously been shown to rapidly develop resistance against rhodamine 6G and other toxic compounds when exposed to these drugs. This resistance has been associated with the elevated expression of the ABC-type MDR transporter LmrCD. To determine the level of adaptiveness of *L. lactis* to drugs, a strain lacking the *lmrCD* genes (10) was challenged with increasing concentrations of rhodamine 6G. Remarkably, cells quickly acquired a significant level of resistance (Fig. 1A). To document the progress of adaptation of the Δ*lmrCD* strain to high concentrations of rhodamine 6G, the resistance was assessed at each stage of the drug exposure. The starting rhodamine 6G concentration of ~7.5 µM was slightly above the concentration which inhibits the growth rate of the unadapted Δ*lmrCD* strain by about 50 %. By day 12, the growth rate of the rhodamine 6G resistant cells was two-fold higher than that of the parental Δ*lmrCD* strain i.e., 0.58 h^{-1}

instead of 0.24 h^{-1} (Fig. 1A). These adapted cells were designated as *ΔlmrCD (rho12)* as they showed colony formation on plates containing up to 12 µM rhodamine. Adaptation to even higher rhodamine 6G levels was continued and the most resistant cells isolated at 22 µM rhodamine were designated as *ΔlmrCD(rho22)*. At the indicated concentrations, the lag phase and the growth rate of *L. lactis ΔlmrCD(rho12)* and *ΔlmrCD(rho22)* were essentially unaffected as compared to the parental strain in the absence of rhodamine. However, adaptation to higher rhodamine 6G levels (28 µM) resulted in cultures with an extended lag phase and poor growth. These were not further examined.

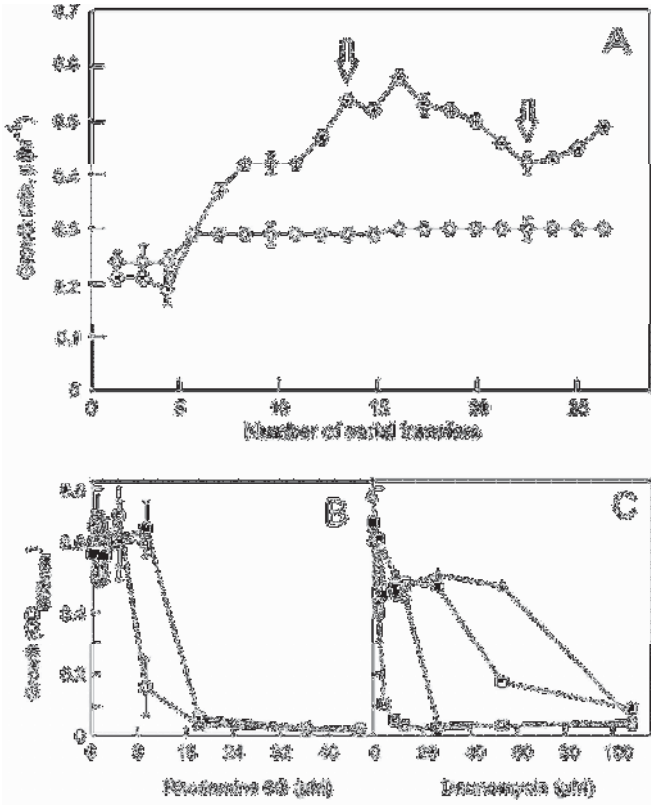

FIG. 1. Adaptive resistance of *L. lactis* Δ*lmrCD* to rhodamine G. (A) *L. lactis* Δ*lmrCD* cells were exposed to increasing concentrations of rhodamine 6G resulting in the progressive appearance of resistant strains with an increased growth rate in the presence of 4 µM of rhodamine 6G. The rhodamine adapted strains selected after 12 and 20 serial transfers (indicated by arrows) were used in the further experiments. (B) Daunomycin

and (C) Rhodamine 6G resistance of the wild-type (closed squares), Δ*lmrCD* (open squares), Δ*lmrCD(rho22)* (open triangles), and Δ*lmrCD(rho12)* (closed triangles) strain. Cells were grown for 12 hrs in GM17 medium containing varying drug concentrations in 96-well microtiter plates.

Adaptation to Rhodamine 6G confers multidrug resistance. *L. lactis ΔlmrCD(rho22)* and *ΔlmrCD(rho12)* cells were tested for resistance to structurally and functionally diverse toxic compounds. To this end, the rhodamine-adapted cells were grown in the presence of a variety of drugs and growth rates were determined. The drugs tested ranged from anthracylines, structurally unrelated fluorescent dyes like rhodamine 6G, ethidium bromide, and Hoechst dyes (Table 2) and various cationic drugs. As compared to the parental cells, both adapted strains exhibited an enhanced resistance to rhodamine 6G (Fig. 1B) and daunomycin (Fig. 1C). The *ΔlmrCD(rho22)* strain showed higher resistance levels than the *ΔlmrCD(rho12).* Interestingly, the acquired resistance of *ΔlmrCD(rho22)* to several of the tested drugs, all of which are known MDR substrates even exceeded that of wild-type cells. These data suggest that even in the absence of LmrCD, *L. lactis* cells can regain a multidrug resistance phenotype.

Biofilm formation and resistance of rhodamine 6G-adapted *L. lactis* cells. The ability of the various *L. lactis* strains to form biofilms was investigated using a crystal violet based microtiter plate assay. This stain binds to negatively charged molecules such as peptidoglycan, acidic polysaccharides and DNA and its absorption therefore serves as a measure for biomass at the surface, i.e., biofilms (3). *L. lactis* was grown for 24 hrs in wells of polystyrene (hydrophobic) microtiter plates. Next, the planktonic cells were removed and the remaining wall associated biofilms were stained with crystal violet and quantitated.

The *L. lactis ΔlmrCD(rho22)* and *ΔlmrCD(rho12)* strains showed largely a similar pattern of biofilm development as the parental and wild type strains when grown in the absence of added drug (Fig. 2A). We also compared the effect of daunomycin on the ability of the tested strains to maintain a biofilm, independent of their effect on cell growth. Because of the spectral properties, rhodamine 6G could not be used as it interfered with the crystal violet

TABLE 2. Susceptibility of selected *L. lactis* strains to various dyes and drugs

Drugs/dyes	*L. lactis**			
	Δ*lmrCD* (*rho12*)	Δ*lmrCD* (*rho22*)	Δ*lmrCD*	Wild-type
	IC 50 (μM)			
Cholate	1950	3200	1800	2600
Deoxycholate	270	262	200	198
Benzalkonium Chloride	51	49	51	51.5
N-Lauroylsarcosine	190	255	240	270
Quinine	1.8	1.7	1.3	1.7
Daunomycin	19	68	3	41
Rhodamine 6G	11.9	12.5	6.9	13
Rhodamine 123	53.2	>64	48.5	>64
Hoechst 33342	1.8	5.8	0.75	4.65
Hoechst 33258	26	142	23.8	89
Acridine orange	25.7	24.5	24.7	24.7
Ethidium bromide	12.5	11	11.8	11.8
9-aminoacridine	26	29	22.8	23.5
2-aminoanthracene	775	520	800	665
Mitoxantrone	>50	49	45	43.5

*Cells were grown in liquid culture as described in the Materials and Methods

detection (not shown). When cells were exposed to daunomycin, biofilms of the *ΔlmrCD(rho22)* strain were about as stable as that of the wild type but both the parental and *ΔlmrCD(rho12)* strain showed a decreased biofilm maintenance in the presence of increasing concentrations of daunomycin (Fig. 2A).

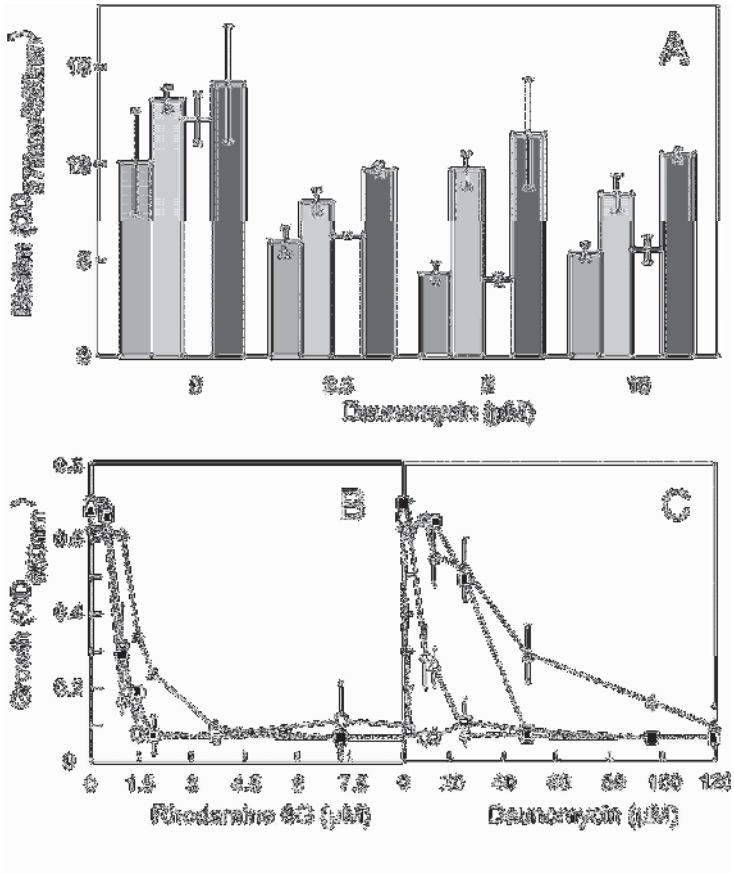

FIG. 2. Biofilm formation by selected *L. lactis* strains. (A) Wild-type (black bar), Δ*lmrCD* (white bar), *ΔlmrCD(rho12)* (light grey bar) and the *ΔlmrCD(rho22)* (dark grey bar) strains were grown for 24 hrs in GM17 medium containing varying concentrations of daunomycin in 96-well microtiter plates. planktonic cells were removed, and biofilms were collected and resuspended whereupon the OD_{650nm} was measured. Next, cells were stained with crystal violet, analyzed for OD_{575nm} and the ratio of OD_{575nm} to OD_{650nm} was used as a relative measure for biofilm formation. (B) Rhodamine 6G and (C) daunomycin susceptibility of the biofilms of *L. lactis* wild-type (solid squares), Δ*lmrCD* (open squares), *ΔlmrCD(rho12* (solid triangles) and *ΔlmrCD(rho22)* (open triangles)

cells. Cells were grown for 12 hrs in 96 well microtiter plates in GM17 medium with various drug concentrations. Growth was determined as $OD_{660nm.}$

Because of the difference in biofilm development in the presence of daunomycin, the drug susceptibility of the biofilms was further examined. Biofilm cells typically exhibit an increased resistance to toxic compounds when compared to the planktonic cells (4). This increased resistance has been attributed to a variety of phenomena, including a reduced penetration of antimicrobials, and the production of efflux pumps and enzymes for drug detoxification (20). *L. lactis* biofilms were allowed to develop on a polystyrene surface whereupon they were exposed to rhodamine 6G (Fig. 2B) and daunomycin (Fig. 2C) for 18 hrs. Next, the biofilm cells were harvested and grown in liquid medium for 24 hrs. Growth was used as a measure of survival. Likewise, planktonic cells were harvested from the liquid medium serving as controls.

As compared to planktonic cells, biofilm cells of the rhodamine adapted mutant strains showed a decreased resistance to both daunomycin and rhodamine 6G. This is in remarkable contrast to biofilms of wild type cells, which exhibit an increased resistance to these drugs as compared to the planktonic cells. Overall, these data suggest different resistance mechanisms in the rhodamine-adapted cells when comparing their sessile and planktonic states. Also the acquired resistance phenotype is particular pronounced for the planktonic cells.

Analysis of the expression of putative multidrug transporters genes. To determine if multidrug transport contributes to the resistance of the rhodamine 6G adapted strains, the expression of a selected set of putative multidrug transporter genes was measured in exponentially grown cells by qPCR (Table 3). Expression profiles were recorded with cells grown in the absence and presence of sublethal concentrations of daunomycin as the resistance gained with this drug was greater as compared to rhodamine. Gene expression was normalized to that of the TufA2 housekeeping gene. The expression of most tested genes encoding known and putative MDR transporters appeared unchanged in the rhodamine-adapted strains as

TABLE 3. Real-time PCR expression analysis of putative MDR transporters in various *L. lactis* strains grown in the absence and presence of daunomycin.

Locus tag	Function; gene name	Δ*lmrCD** (rho12)		Δ*LmrCD** (rho22)		Wild-type*	
		-	+	-	+	-	+
llmg_0631	MFS permease; ***pmrA***	0.5	**6.3**	0.8	**5.6**	0.8	1.2
llmg_1202	ABC-type transporter	0.9	0.1	**2.9**	0.9	0.9	0.8
llmg_1210	MFS-EmrB/QacA subfamily	0.9	0.3	0.5	0.7	1.3	1.2
llmg_0140	MFS permease; ***pmrB***	**2.7**	1.53	**3.3**	**2.6**	**3.0**	1.0
llmg_0133	MFS permease; ***blt***	0.6	0.9	1.6	1.2	0.9	0.2
llmg_1856	ABC-type transporter; ***lmrA***	**3.1**	0.6	**2.3**	0.3	**3.2**	0.5
llmg_2446	MFS permease; ***lmrP***	**4.3**	0.6	**7.0**	0.8	0.4	1.4
llmg_1322	MFS permease	0.8	0.4	0.8	0.5	1.1	0.4
llmg_2386	Na+-driven permease	0.6	0.5	0.6	0.4	2	1.8
llmg_0320	MFS permease, putative tetracycline resistance; ***napC.***	0.7	0.8	0.7	0.8	1.0	1.0
llmg_0421	ABC-type transporter, daunorubicin resistance; ***drrB***	0.7	0.7	0.9	0.9	1.0	1.1
llmg_0856	MFS permease	1.5	1.3	1.3	1.3	1.2	1.2
llmg_1015	Na+-driven permease; ***ypbC***.	1.0	0.9	0.6	0.7	1.3	1.3
llmg_2513	MFS permease; ***yxbD***	1.1	1.0	0.8	0.8	0.9	0.9

The values depicted are in terms of fold change relative to the gene expression in Δ*lmrCD* strain. + and – indicate growth in the presence and absence of 1μM daunomycin.

compared to the parental strain. Interestingly, the expression of *lmrP*, a known MFS type secondary MDR exporter was significantly elevated in both the mutants, while expression in the parental was similar to that in the wild type strain. The expression of *lmrA*, that encodes an ABC type of transporter (11) also showed a slight increase in expression in both mutants as well as in the wild type as compared to the Δ*lmrCD* strain.

Among the putative MDR type transporter genes, *llmg_0631* that encodes a putative MFS type permease was significantly over-expressed by 6.3 and 5.6-fold in the *L. lactis ΔlmrCD(rho12)* and *ΔlmrCD(rho22)* cells, respectively. However, this was observed only when the strains were exposed to daunomycin. In most cases, daunomycin exposure resulted in a decreased expression of putative MDR genes. These data indicate that the *llmg_0631* and *lmrP* gene contribute to the resistance of the rhodamine adapted cells, with the pronounced daunomycin-dependent expression of the *llmg_0631* gene.

Transport activity of rhodamine 6G adapted strains. To determine the relevance of membrane associated transport proteins in rhodamine 6G resistance, some of the known LmrCD substrates (10) were tested for transport activity in the *L. lactis ΔlmrCD(rho12)* and *ΔlmrCD(rho22)* strains. The anthracycline drug daunomycin acts on DNA by intercalation between the base pairs (21). Under these conditions, the fluorescence of daunomycin is quenched (12), a phenomenon that can be used to monitor the cellular influx.

When daunomycin is added to *L. lactis* cells, a rapid increase in fluorescence is observed followed by a gradual decrease, which signifies the entry of the drug into the cell and its binding to the DNA. Upon energization with glucose, wild type cells show the typical profile of LmrCD-mediated daunomycin efflux evident as an increase in fluorescence (Fig. 3A). On the other hand, the parental strain and both rhodamine mutants that all lack the LmrCD transporter pump showed a reduced transport activity (Fig. 3A), which suggests that the increased daunomycin resistance cannot be attributed to transport.

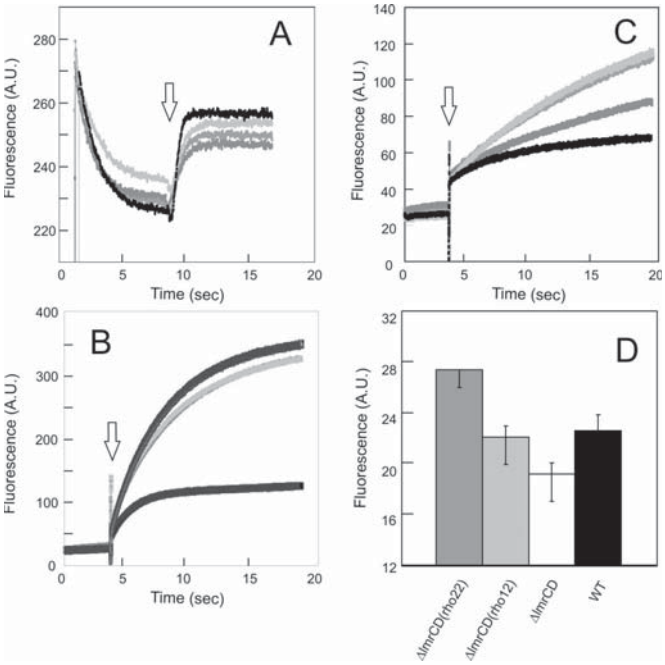

FIG. 3. Transport of various drugs by selected *L. lactis* strains. (A) Daunomycin transport. To a washed de-energized cell suspension (OD_{650nm} ~ 0.5), 2 µM daunomycin was added and after the initial fluorescent spike, the influx of daunomycin was monitored by the quenching of the fluorescence. Next, glucose was added (arrow) and the secretion of daunomycin was monitored as an increase in fluorescence. Wild type (black trace), Δ*LmrCD* (light gray trace), *ΔlmrCD(rho12)* (medium gray trace) and *ΔlmrCD(rho22)* (dark gray trace) cells. (B) Hoechst 33342 and (C) Hoechst 33258 transport. At the arrow, 2 µM of the Hoechst was added to glucose energized cells and the increase of Hoechst fluorescence was taken as a measure of transport. (D) Rhodamine 6G transport. Rhodamine 6G was added to a final concentration of 2 µM to glucose energized wild type (black bar), Δ*lmrCD* (white bar), Δ*lmrCD(rho12)* (light grey bar) and Δ*lmrCD(rho22)* (dark grey bar) cells. After 30 mins, the cells were removed by centrifugation and the free rhodamine 6G concentration was determined fluorimetrically in the supernatant fraction.

The lipophilic Hoechst 33342 is a cell-permeant, minor groove–binding DNA stains that shows a bright blue fluorescence upon binding to DNA as well as upon partitioning into the hydrophobic membrane lipid bilayer (12). Addition of Hoechst 33342 to the cells results in a rapid increase in fluorescence, which is substantially similar with the Δ*lmrCD* cells as compared to the wild type strain (Fig. 3B). The rhodamine resistant strains that lacks LmrCD followed a fluorescent signal that is slightly below that of the Δ*lmrCD* strain

suggesting a low activity of Hoechst 33342 export (Fig. 3B). On the other hand, the uptake of the more hydrophilic Hoechst 33258 by the *ΔlmrCD(rho12)* strain was similar to that of the *ΔlmrCD* cells, and substantially higher than observed for the wild type cells. The *ΔlmrCD(rho22)* strain, however, showed a much reduced uptake of the Hoechst 33258 (Fig. 3C) suggesting substantial secretion activity for this compound.

To investigate the efflux of the rhodamine 6G, an indirect assay was used that monitors the amount of extracellular rhodamine 6G. Addition of de-energized cells to a buffer with rhodamine 6G resulted in a sharp drop in the measured free extracellular concentrations of rhodamine 6G. Upon addition of glucose, the levels of extracellular rhodamine 6G increased, and this increase was most pronounced with the *ΔlmrCD(rho22)* strain (Fig. 3D) which is consistent with an efflux activity in these cells. With the parental strain, the lowest level of extracellular rhodamine 6G was observed, but still substantial efflux occurred. These data indicate that the *ΔlmrCD(rho22)* strain and to a less extent the *ΔlmrCD(rho12)* strain exhibit an improved ability to secrete rhodamine 6G.

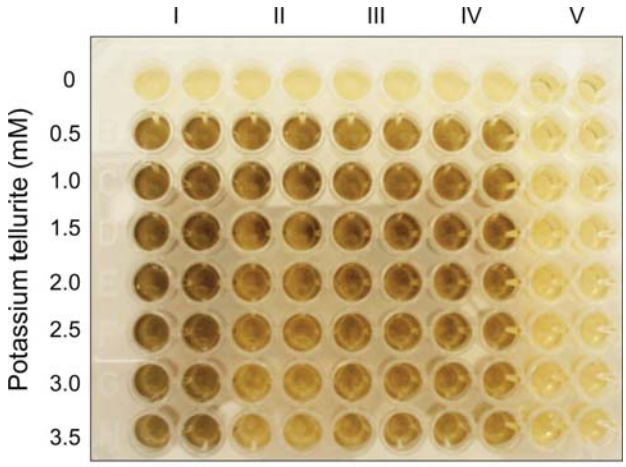

FIG. 4. Reduction of various concentrations of K_2TeO_4 by cell suspensions of the Δ*lmrCD(rho12)* (I), Δ*lmrCD(rho22)* (II), Δ*lmrCD* (III), and wild type (IV) *L. lactis* cells. Assays were performed in microtiter plate wells following 12 hrs of incubation at 30ºC. The reduction activity is evident from the black coloration.

Tellurite reduction. The strains were also tested for tellurite resistance, an indicator of toxicity of oxidative stress. Herein, cells were grown on GM17 plates containing 0.5 to 6 mM K_2TeO_3 (mM) and the reduction to elemental tellurite was followed by the appearance of a black color (24) (Fig. 4). Strain *ΔlmrCD(rho22)* showed a significantly reduced ability to reduce K_2TeO_3 as compared the control strains (Fig. 4), while with the *ΔlmrCD(rho12)* strain, an increase reduction is observed. These data indicate a different phenotype for the *ΔlmrCD(rho22)* and *ΔlmrCD(rho12)* strains, suggesting a decreased and increased resistance to oxidative stress, respectively.

DISCUSSION

L. lactis can manifest a variety of drug resistance mechanisms, including drug export, and altered metabolism, membrane composition, and/or cell wall structure. These mechanisms may confer different levels of resistance to drugs, but also vary in specificity. To understand these processes, we have examined a *L. lactis* strain that lacks the major drug transporter LmrCD and subjected this strain to an evolution process by re-challenged these cells with a toxic drug, i.e., rhodamine 6G. Rhodamine 6G is not only a substrate for LmrCD but it has been shown to be a good substrate for other types of multidrug transporters (23). It is known that the MIC values of drugs can be significantly increased by extending the duration of sub lethal exposure as well as by gradually increasing the drug concentration (9). Therefore, the strain was gradually adapted to increasing concentrations of rhodamine 6G. Such adaptation is not transient but the result of the accumulation of multiple mutations, possibly causing the deregulation of MDR transporter expression and other processes. The adaptation process resulted in the isolation of two resistant strains with distinct phenotypes in their resistance to rhodamine 6G. Examination of the expression of putative MDR transporter genes in the two *L. lactis ΔlmrCD(rho22)* and *ΔlmrCD(rho12)* strains relative to the parental strain and the wild-type, suggests the up-regulation of LmrP, a known multidrug transporter that belongs to the MFS. Several other putative MDR transporters were altered in their expression, but this did not correlate with the observed drug resistance phenotypes. However, in the presence of the drug daunomycin, both strains show the significant upregulation of

llmg_0631, a sofar uncharacterized MDR-like transporter belonging to the MFS. Likely, the latter transporter is involved in the resistance phenotype of the two selected drug resistant strains. Transport assays with intact cells indeed demonstrate that the resistant strains have regained the ability to secrete Rhodamine 6G and in the case of the highly resistant *ΔlmrCD(rho22)* strain also of Hoechst33258. However, export of daunomycin in unchallenged cells seemed not to be restored. Unfortunately, the fluorescent assay yielded inconclusive results with daunomycin challenged cells likely because of carry over of the daunomycin from the challenge stage. We therefore conclude that at least for rhodamine 6G, an improved export activity contributes to the resistance phenotype.

The apparent role of the MFS type *llmg_0631* transporter in the drug resistance is unclear. This gene is up-regulated in the resistant strains, but only when the resistant cells are exposed to daunomycin while rhodamine 6G as inducer was not tested. This would be the first example of a MFS transporter involved in daunomycin export as in the producing microbe *S. peucetius,* resistance and secretion are mediated by an ABC type transporter (22). It will be necessary to examine the daunomycin transport activity of a strain in which the *llmg_0631* gene is deleted or overproduced in order to firmly demonstrate a role of this transporter in daunomycin (and possibly rhodamine) transport. The observation that the *llmg_0631* expression in the drug resistant strains is dependent on the presence of daunomycin in the medium suggests the involvement of a regulator. In wild-type strain this phenomenon was not apparent suggesting that the evolution experiment has led to the activation of a cryptic regulatory process. It will be a challenge to identify this regulator in order to reveal the nature of the cryptic expression of the *llmg_0631* transporter.

The high level of resistance of the planktonic cells to daunomycin is an intriguing phenomenon. The drug is a potent chemotherapeutic agent and is widely used for the treatment of hematopoietic and solid tumors. In addition, anthracylines are also known to have antibacterial activity, particularly against Gram positive bacteria (16). In mammals, several mechanisms of action have been proposed for daunomycin-mediated cell death: (i) DNA

damage through doxorubicin-induced DNA intercalation; (ii) ROS generation by redox cycling of the quinone structure; and (iii) inhibition of DNA synthesis by stabilizing the topoisomerase II–DNA complex (15,17). An increase in the intracellular GSH pool has been shown to correlate with reduced doxorubicin sensitivity of tumor cells. It has been suggested that cancer cells can exert an adaptive activation of the antioxidant defense systems thus gradually raising its level in response to daunomycin exposure, leading to chemoresistance (19). This suggests a link between daunomycin resistance and oxidative stress, but it is unclear if the same mechanism may exist in bacteria. Interestingly, the *ΔlmrCD(rho12)* strain, which shows an increased daunomycin resistance, also is more resistant to tellurite toxicity that has been associated with oxidative stress. However, this correlation does not seem to exist for the *ΔlmrCD(rho22)* strain which is even more sensitive to tellurite while maintaining a high daunomycin resistance. Tellurite toxicity although still no fully understood is believed to function through the generation of ROS and O_2^- during intracellular reduction to elemental tellurium (18,25). Also, tellurite resistance in *L. lactis* has been linked to genes involved in redox functions and oxidative stress (25). Since there is a different response of the two resistant strains to tellurite, we conclude that the resistance mechanisms involved in both strains must be vastly different.

The distinct differences in susceptibility of the rhodamine adapted mutants between mitoxantrone and daunomycin suggests alternations in the cell envelope composition. Mitoxantrone is an anthracenedione derivative similar to daunomycin, both are known to affect membrane fluidity by binding to ionized phospholipids, daunomycin binding to negatively charged phospholipids whereas the mechanism by which mitotraxone binds is not clear (14). Adherence of cells to surfaces and to each other is critical for biofilm development (8) and any drug interfering with cell adherence will prevent biofilm formation as well. The inhibitory effect of rhodamine 6G and daunomycin on the adhesion and biofilm formation of the rhodamine mutants as well as the control strains is very different from our previous results with strains resistant to cholate (submitted) and related bile acids. Biofilms of the rhodamine mutants were found to be more susceptible to daunomycin and rhodamine 6G while the planktonic cells are highly resistant. This is an

uncommon phenomenon as biofilms are usually more resistant than planktonic cells (7). The apparent discrepancy might be the result of a different gene regulation pattern in planktonic and biofilm cells.

In conclusion, our data demonstrates that even in the absence of the LmrCD transporter, *L. lactis* can regain multidrug resistance when challenged for prolonged periods of time to an increasing concentration of drugs. However, the extensive cross-resistance but also differences in resistance levels in the rhodamine adapted strains suggests that the conferred drug resistance is most likely a multifactorial phenomenon, i.e., the result of a number of resistance mechanisms acting in synergy.

REFERENCES

1. **Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. Driessen, and W. N. Konings**. 1994. Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant Lactococcus lactis. J. Bacteriol. **176**:6957-6964.
2. **Bolhuis, H., G. Poelarends, H. W. van Veen, B. Poolman, A. J. Driessen, and W. N. Konings**. 1995. The Lactococcal lmrP gene encodes a proton motive force-dependent drug transporter. J. Biol. Chem. **270**:26092-26098.
3. **Burmolle, M., J. S. Webb, D. Rao, L. H. Hansen, S. J. Sorensen, and S. Kjelleberg**. 2006. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl. Environ. Microbiol. **72**:3916-3923.
4. **De Kievit, T. R., M. D. Parkins, R. J. Gillis, R. Srikumar, H. Ceri, K. Poole, B. H. Iglewski, and D. G. Storey**. 2001. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in Pseudomonas aeruginosa biofilms. Antimicrob. Agents Chemother. **45**:1761-1770.
5. **de Ruyter, P. G., O. P. Kuipers, and W. M. de Vos**. 1996. Controlled gene expression systems for Lactococcus lactis with the food-grade inducer nisin. Appl. Environ. Microbiol. **62**:3662-3667.
6. **den Hengst, C. D., S. A. van Hijum, J. M. Geurts, A. Nauta, J. Kok, and O. P. Kuipers**. 2005. The Lactococcus lactis CodY regulon: identification of a conserved cis-regulatory element. J. Biol. Chem. **280**:34332-34342.
7. **Frank, K. L., E. J. Reichert, K. E. Piper, and R. Patel**. 2007. In vitro effects of antimicrobial agents on planktonic and biofilm forms of Staphylococcus lugdunensis clinical isolates. Antimicrob. Agents Chemother. **51**:888-895.

8. **Itoh, Y., X. Wang, B. J. Hinnebusch, J. F. Preston, III, and T. Romeo**. 2005. Depolymerization of beta-1,6-N-acetyl-D-glucosamine disrupts the integrity of diverse bacterial biofilms. J Bacteriol. **187**:382-387.
9. **Langsrud, S., G. Sundheim, and A. L. Holck**. 2004. Cross-resistance to antibiotics of Escherichia coli adapted to benzalkonium chloride or exposed to stress-inducers. J Appl. Microbiol. **96**:201-208.
10. **Lubelski, J., J. A. de, M. R. van, H. Agustiandari, O. P. Kuipers, J. Kok, and A. J. Driessen**. 2006. LmrCD is a major multidrug resistance transporter in Lactococcus lactis. Mol. Microbiol. **61**:771-781.
11. **Lubelski, J., W. N. Konings, and A. J. Driessen**. 2007. Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiol. Mol. Biol. Rev. **71**:463-476.
12. **Lubelski, J., P. Mazurkiewicz, M. R. van, W. N. Konings, and A. J. Driessen**. 2004. ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. J. Biol. Chem. **279**:34449-34455.
13. **Lubelski, J., P. Mazurkiewicz, M. R. van, W. N. Konings, and A. J. Driessen**. 2004. ydaG and ydbA of Lactococcus lactis encode a heterodimeric ATP-binding cassette-type multidrug transporter. J. Biol. Chem. **279**:34449-34455.
14. **Marczak, A., A. Wrzesien-Kus, E. Krykowski, T. Robak, and Z. Juzwiak**. 2003. The interaction of daunorubicin and mitoxantrone with the red blood cells of acute myeloid leukemia patients. Cell Mol. Biol. Lett. **8**:885-890.
15. **Minotti, G., P. Menna, E. Salvatorelli, G. Cairo, and L. Gianni**. 2004. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol. Rev **56**:185-229.
16. **Moody, M. R., M. J. Morris, V. M. Young, L. A. Moye, III, S. C. Schimpff, and P. H. Wiernik**. 1978. Effect of Two Cancer Chemotherapeutic Agents on the Antibacterial Activity of Three Antimicrobial Agents. Antimicrob. Agents Chemother. **14**:737-742.
17. **Muller, I., D. Niethammer, and G. Bruchelt**. 1998. Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity. Intl. J. Mol. Med. **1**:491-494.
18. **Perez, J. M., I. L. Calderon, F. A. Arenas, D. E. Fuentes, G. A. Pradenas, E. L. Fuentes, J. M. Sandoval, M. E. Castro, A. O. Elias, and C. C. Vasquez**. 2007. Bacterial toxicity of potassium tellurite: unveiling an ancient enigma. PLoS. One. **2**:e211.
19. **Shim, G. S., S. Manandhar, D. H. Shin, T. H. Kim, and M. K. Kwak**. 2009. Acquisition of doxorubicin resistance in ovarian carcinoma cells accompanies

activation of the NRF2 pathway. Free Radical Biology and Medicine **47**:1619-1631.

20. **Shirtliff, M. E., B. P. Krom, R. A. Meijering, B. M. Peters, J. Zhu, M. A. Scheper, M. L. Harris, and M. A. Jabra-Rizk**. 2009. Farnesol-induced apoptosis in Candida albicans. Antimicrob. Agents Chemother. **53**:2392-2401.
21. **Sprigg, L., A. Li, F. Y. M. Choy, and J. Ausio¦ü**. 2010. Interaction of Daunomycin with Acetylated Chromatin. Journal of Medicinal Chemistry null.
22. **Srinivasan, P., S. N. Palani, and R. Prasad**. 2010. Daunorubicin efflux in Streptomyces peucetius modulates biosynthesis by feedback regulation. FEMS Microbiol. Lett. **305**:18-27.
23. **Su, X. Z., J. Chen, T. Mizushima, T. Kuroda, and T. Tsuchiya**. 2005. AbeM, an H+-Coupled Acinetobacter baumannii Multidrug Efflux Pump Belonging to the MATE Family of Transporters. Antimicrob. Agents Chemother. **49**:4362-4364.
24. **Taylor, D. E., E. G. Walter, R. Sherburne, and D. P. Bazett-Jones**. 1988. Structure and location of tellurium deposited in Escherichia coli cells harbouring tellurite resistance plasmids. J Ultrastruct. Mol. Struct. Res **99**:18-26.
25. **Turner, M. S., Y. P. Tan, and P. M. Giffard**. 2007. Inactivation of an Iron Transporter in Lactococcus lactis Results in Resistance to Tellurite and Oxidative Stress. Appl. Environ. Microbiol. **73**:6144-6149.
26. **van Veen, H. W., K. Venema, H. Bolhuis, I. Oussenko, J. Kok, B. Poolman, A. J. Driessen, and W. N. Konings**. 1996. Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1. Proc. Natl. Acad. Sci. U. S. A **93**:10668-10672.
27. **Zaidi, A. H., P. J. Bakkes, J. Lubelski, H. Agustiandari, O. P. Kuipers, and A. J. Driessen**. 2008. The ABC-type multidrug resistance transporter LmrCD is responsible for an extrusion-based mechanism of bile acid resistance in Lactococcus lactis. J. Bacteriol. **190**:7357-7366.