Applied and Environmental Microbiology, Apr. 2011, p. 2602–2610 Vol. 77, No. 8
0099-2240/11/$12.00 doi:10.1128/AEM.01709-10

Cholate-Stimulated Biofilm Formation by *Lactococcus lactis* Cells▽†

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Received 16 July 2010/Accepted 10 February 2011

Bile acid resistance by *Lactococcus lactis* depends on the ABC-type multidrug transporter LmrCD. Upon deletion of the *lmrCD* genes, cells can reacquire bile acid resistance upon prolonged exposure to cholate, yielding the Δ*lmrCD*^{r} strain. The resistance mechanism in this strain is non-transporter based. Instead, cells show a high tendency to flocculate, suggesting cell surface alterations. Contact angle measurements demonstrate that the Δ*lmrCD*^{r} cells are equipped with an increased cell surface hydrophilicity compared to those of the parental and wild-type strains, while the surface hydrophilicity is reduced in the presence of cholate. Δ*lmrCD*^{r} cells are poor in biofilm formation on a hydrophobic polystyrene surface, but in the presence of subinhibitory concentrations of cholate, biofilm formation is strongly stimulated. Biofilm cells show an enhanced extracellular polymeric substance production and are highly resistant to bile acids. These data suggest that non-transporter-based cholate resistance in *L. lactis* is due to alterations in the cell surface that stimulate cells to form resistant biofilms.

Lactococcus lactis, like other lactic acid bacteria, survives, albeit weakly, in the human gastrointestinal tract (23). Importantly, bacteria that colonize the gastrointestinal tract need to be resistant to bile salts. When they successfully colonize such a niche, they must transit from a planktonic phase to a biofilm phase. A biofilm community is classically characterized by a sessile mode of existence, in which cells are encased in a secreted extracellular matrix while attached or exposed to a surface (21). However, the condition of surface attachment is flexible, since free-floating flocs with minimal surface attachment are also regarded as biofilms (24). Bacteria growing in biofilms exhibit a specific phenotype and are often but not always more resistant (38) to antimicrobial agents than their planktonic counterparts. Such enhanced resistance appears to be caused by numerous mechanisms (14). Several studies have implicated efflux pumps in the antimicrobial resistance of biofilms (14, 25). Although in Gram-positive bacteria ABC-type drug transporters are known to contribute to drug resistance of planktonic cells (25), so far none have been implicated in biofilm-specific antimicrobial resistance. A recent report suggests the involvement of a putative ABC transporter in biofilm-specific resistance in Gram-negative bacteria (42).

The ABC-type multidrug resistance (MDR) transporter LmrCD is a major determinant of drug resistance in *L. lactis*, but it is also involved in bile acid resistance (41). Consequently, *L. lactis* cells that lack the *lmrCD* genes are sensitive to bile acids, such as cholate. When these cells are exposed to stepwise-increasing sublethal concentrations of cholate, they develop an improved resistance to cholate and several other bile acids (41). The resultant strain was termed the *L. lactis* Δ*lmrCD*^{r} strain. Transcriptome and functional analysis suggests that the resistance by this strain is no longer transporter based but is due to cell envelope- and metabolism-related alterations (41). These cells show a decreased growth rate and an increased ability to flocculate, but it is unclear how they perform in biofilms. Biofilm development is a complex multistep process, and both its initiation and consolidation are greatly influenced by environmental stresses, such as bile acids (3).

Here we have investigated the surface and biofilm-forming properties of three different strains of *L. lactis*: (i) the wild type, (ii) the Δ*lmrCD* strain, which lacks the major multidrug transporter LmrCD (27), and (iii) the Δ*lmrCD*^{r} strain, a cholate-resistant strain derived from the *L. lactis* Δ*lmrCD* strain (41). The data indicate a cholate-dependent physicochemical alteration of the Δ*lmrCD*^{r} cell surface and the development of biofilms with a distinctly different matrix structure and composition.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* NZ9000 is a derivative of the plasmid-free *L. lactis* MG1363 strain containing *pepN*::*nisRK* (11, 12), referred to as the wild type. *L. lactis* NZ9000 Δ*lmrCD* (28) and its cholate-resistant derivative, the Δ*lmrCD*^{r} strain (41), 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).

Biofilm formation. The ability of *L. lactis* to form biofilms was assessed by a previously described method with minor modifications (7). Cells were grown for 24 h in GM17 and subsequently diluted to an optical density at 660 nm (OD_{660}) of 1 in fresh GM17 supplemented with selected drugs and detergents at various

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 18 February 2011.

concentrations. Next, aliquots of 200 μl were transferred into the wells of a 96-well flat-bottom microtiter plate. For each drug concentration, 7 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} was measured. For each drug concentration, the biofilm of a single well 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} was used as a measure of the biofilm cell density. The biofilms in the remaining 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% (wt/vol) crystal violet, rinsed with water, and air dried overnight. The crystal violet-stained biofilm was suspended to homogeneity in 200 μl of dimethyl sulfoxide (DMSO), and the OD_{575} 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} to OD_{660} was calculated. Assays were repeated three times with independent cultures.

Growth inhibition bioassays. Growth inhibitions assays were performed with different types of cells that when appropriate were pregrown in the absence or presence of 1.5 mM cholate. All experiments were carried out in triplicate, and data shown are averaged with indicated standard errors of the means.

(i) Planktonic cells. Overnight cultures of the indicated *L. lactis* strains were diluted in fresh GM17 and grown to an OD_{660} of 0.6. Aliquots of 150 μl were transferred to 96-well microtiter plates that contained 50 μl of GM17 medium and, when needed, drugs at a given concentration. Plates were sealed with Parafilm and incubated for 12 h, after which the OD_{660} was measured. Concentrations that inhibited growth by 50% (IC_{50}) and 100% (MIC) were determined.

(ii) Biofilm cells. The drug, EDTA, and bacitracin susceptibilities of *L. lactis* biofilms were determined as described previously (17) with minor modifications. Cells grown for 12 h in GM17 were diluted in fresh GM17 without or with 1.5 mM cholate to an OD_{660} of 0.6. Aliquots of 200 μl were transferred to each well of a 96-well flat-bottom polystyrene microtiter plate covered with a 96-peg lid of polystyrene (Nunc, Denmark). Cells were incubated for 24 h to allow biofilm formation on the pegs. To remove nonadherent cells, the peg lid was dipped into a series of three 96-well plates containing 200 μl of sterile 10 mM phosphate-buffered saline (PBS), pH 7.0, per well. The peg lid was then placed on a fresh plate that contained in each well 200 μl GM17 with the appropriate drug. GM17 medium without drugs served as a positive growth control. Biofilms were incubated overnight at 30°C, rinsed three times in PBS as described above, and placed in a final 96-well plate containing 200 μl of GM17. 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} of the remaining suspension in the wells was determined (0 h). The plate was covered by a normal lid and incubated for 24 h, after which a second OD_{660} reading was taken. The IC_{50} was defined as the drug concentration that showed 50% growth reduction compared to the growth in control wells with no drugs. Wells containing sterile GM17 served as spectrophotometric blanks.

(iii) Biofilm-derived cells. Biofilms were grown essentially as described above. After washing in PBS, cells associated with the biofilms were removed from the pegs by sonication directly in microplate wells and suspended in GM17 containing different concentrations of the drugs. Following a 12-h incubation in the presence of the drug, the OD_{660} was measured.

Determination of CFU. Biofilms of *L. lactis* were prepared as described above in 96-well polystyrene microtiter plates, in which GM17 growth medium was supplemented with 0, 1.5, or 3 mM sodium cholate from a 500 mM stock solution. After 24 h of growth, the planktonic cells were pooled from eight wells per strain for each condition tested. CFU were determined by serial dilution and plating on M17 plates that were incubated at 30°C for 24 h. Biofilm cell populations were determined by adding fresh PBS to the wells of the plate from which the planktonic cells were removed. Cells were detached from the walls with a sterile pipette tip, pooled from three wells, and dispersed by bath sonication for 5 min at 40 kHz (Zenith Ultrasonics, Norwood, NJ). Microscopic inspection of the cells confirmed that the biofilms were dispersed by the treatment. Serial dilutions of the cells were plated on M17 agar, and the number of CFU was determined after growth for 24 h at 30°C. The sonication time was optimized in order to detach the maximum number of adhered cells without cell disruption (assessed by plating the final suspension on GM17 plates).

Contact angle measurements. For cell surface hydrophobicity measurements, contact angles were determined using cells harvested at mid-exponential growth phase. Cells were washed twice with 100 mM potassium phosphate, pH 7.4, and suspended in the same buffer at an OD_{660} of 0.5. Cells (10 ml) were transferred to cellulose acetate membrane filters (pore size, 0.22 nm; Osmonics) to produce an even bacterial lawn. The sheets were washed with Nanopure water and air dried for 30 to 40 min until so-called "plateau contact angles" could be measured using water droplets. Droplets of four liquids (water, formamide, α-bromonaphthalene, and diiodomethane) were applied to each bacterial lawn (using Teflon/glass syringes equipped with 24-gauge stainless steel Luer-tipped hypodermic needles; Gilmont Instruments). The contact angle of the droplet on the bacterial lawn was monitored with an optical microscope equipped with a charge-coupled-device (CCD) camera. For static contact angles, the droplet was allowed to settle for 2 s without needle contact. The mean contact angles for each biological sample were calculated from five droplets, measured on different areas of the membrane surface. The total surface free energies (SFE) and the polar and nonpolar contribution to the total SFE were determined from contact angle measurements as described previously (6). The Van Oss equation was used to calculate the dispersive (γ^{LW}) and polar acid/base (γ^{AB}) components, with the latter subdivided into an acidic (γ^{+}) and a basic (γ^{-}) term. Mean values were calculated. Based on these parameters, the free energy of adhesion to polystyrene was also calculated.

Confocal laser scanning microscopy. Confocal laser scanning microscopy (CLSM) was used to visualize biofilms as described before (30). Biofilms were grown in 24-well tissue culture polystyrene plates in the presence of 1.5 to 3 mM sodium cholate and 24 to 48 mM sodium taurocholate for 24 h, washed with PBS, and stained with the bacterial Live/Dead stain *Bac*Light (Invitrogen) for 30 min in the dark. Excess stain was removed, and the biofilms were submerged in 2 ml PBS. Images were collected using a Leica TCS SP2 CLSM with a 40× water objective using 488-nm excitation and 500- to 523-nm (green, alive) and 622- to 722-nm (red, dead) emission filter settings. Extracellular polymeric substances (EPS) were stained using calcofluor (Sigma).

RESULTS

Cell surface properties of bile acid-resistant *L. lactis*. The multidrug transporter LmrCD of *L. lactis* contributes to bile acid resistance (27). When the *lmrCD* genes are deleted from the chromosome, cells can regain bile acid resistance when challenged for a prolonged time with increasing concentrations of cholate. The cholate resistance in this strain, which is termed the Δ*lmrCD*^{r} strain, is not transporter based. Transcriptome analysis suggests a high incidence of genes involved in cell envelope biogenesis that are up- and downregulated (41). To determine if the Δ*lmrCD*^{r} strain is equipped with an altered cell envelope, we analyzed the global physicochemical properties of the cell surface. For this purpose, contact angle measurements were performed on bacterial lawns, partly dehydrated cells. Surface free energies were calculated from the contact angle measurements in the absence and presence of cholate, which is a measure of the hydrophilicity of the cell surface (Table 1). In the analysis, three strains were compared, i.e., the wild-type strain, the derivative that lacks the LmrCD transporter (Δ*lmrCD*), and the cholate-adapted Δ*lmrCD*^{r} strain. In the absence of cholate, the surfaces of all three strains are predictably hydrophilic (19), with water contact angles (θ_W) in the range of 18° to 29°. However, with exposure to cholate, the contact angle of the Δ*lmrCD*^{r} strain of 1-bromonaphthalene (θ_B) and formamide (θ_F) decreased, indicating that the cells became more hydrophilic. On the other hand, the parental and wild-type strains showed similar contact angles in the presence and absence of cholate. For the Δ*lmrCD*^{r} strain, exposure to cholate resulted in an increase of the Lifshitz-Van der Waals component, γ^{LW}, from 37.7 to 39.3 mJ · m^{-2}, while the Lewis acid-base component, γ^{AB}, increased from 13.2 to 16.5 mJ · m^{-2}. This resulted in an overall negative value for the interfacial free energy of adhesion, ΔG_{adh} (Table 2), to polystyrene for the Δ*lmrCD*^{r} strain. The analysis of the polar component of the surface free energy shows that unlike the parental and wild-type cells, Δ*lmrCD*^{r} cells show pronounced

TABLE 1. Contact angle and surface free energy components of *L. lactis* Δ*lmrCD*^{r}, Δ*lmrCD*, and wild-type cells with and without exposure to cholate

Condition and strain description	Contact angle (°)[a]				Parameter value (mJ m^{-2})[b]				
	θ_W	θ_F	θ_B	θ_D	γ^{LW}	γ^-	γ^+	γ^{AB}	γ^{total}
Without cholate[c]									
Δ*lmrCD*^{r}	18 ± 3	25 ± 3.2	32 ± 3	44 ± 3	37.7	**56.4**	0.8	13.4	50.9
Δ*lmrCD*	29 ± 1.8	26 ± 2.6	29 ± 4.8	47 ± 2.5	37.2	46.9	1.1	13.2	51.6
Wild type	29 ± 0.5	28 ± 1.0	32 ± 0.6	49 ± 7.2	36.3	**48.2**	1.1	14.3	50.7
With cholate[c]									
Δ*lmrCD*^{r}	22 ± 3.2	14 ± 1.2	24 ± 1.0	43 ± 0.6	39.3	**47.8**	1.4	16.5	55.8
Δ*lmrCD*	30 ± 2.9	29 ± 1.5	30 ± 2.4	51 ± 1.8	36.3	47.4	1.0	13.7	50.0
Wild type	24 ± 1.5	29 ± 1.5	28 ± 4.9	52 ± 0.8	36.3	**53.5**	0.8	13.1	49.3

[a] θ_W, θ_F, θ_B, and θ_D, contact angles of water, formamide, 1-bromonaphthalene, and diiodomethane, respectively.
[b] γ^{LW}, Lifshitz-Van der Waals component of interfacial tension; γ^-, γ^+, electron donor, and electron acceptor components of interfacial tension; γ^{AB}, Lewis acid-base component of interfacial tension; γ^{total}, total surface tension, which is the additive sum of γ^{LW} and γ^{AB}. The most pronounced changes in comparing cholate-exposed and control cells are highlighted in boldface.
[c] Cells were grown tol stationary phase in GM17 in the presence or absence of 1.5 mM Na-cholate.

changes in both the acidic (or electron acceptor) component (γ^+) and basic (or electron donor) component (γ^-) upon exposure to cholate. This implies that the alteration in the cell surface polarity relates to cholate-dependent changes in the acid-base nature of the cell surface, with only a minor role of the apolar Lifshitz-Van der Waals forces. With the wild-type cells, we noted an increase in the basic component (γ^-) upon exposure to cholate, resulting in a slightly positive ΔG_{adh} and thus a slightly increased hydrophilicity. Overall, these data demonstrate the cholate-dependent alterations in the cell surface properties of the Δ*lmrCD*^{r} cells, rendering these cells more hydrophilic.

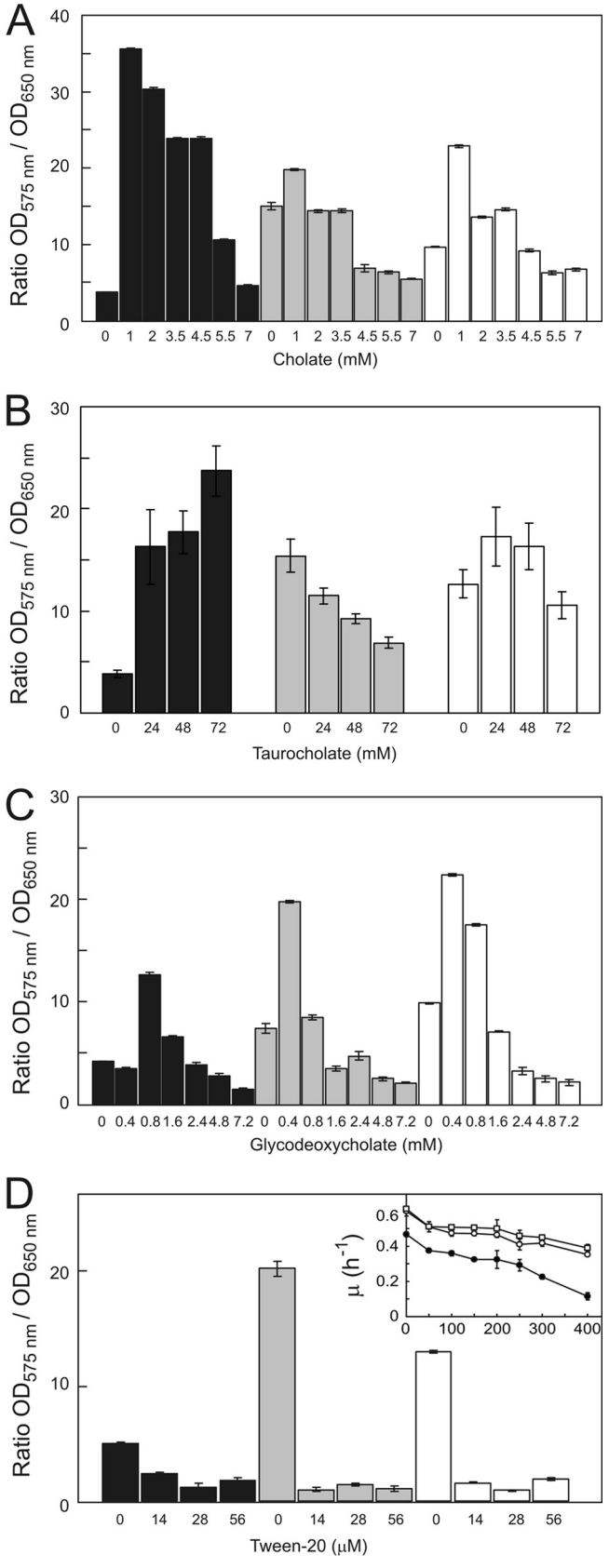
Enhanced biofilm formation by cholate-adapted *L. lactis* Δ*lmrCD* cells. The cholate-adapted Δ*lmrCD*^{r} cells show an enhanced flocculation phenotype in liquid medium compared to the parental strain (41) (see Fig. S1 in the supplemental material). Unlike the physicochemical changes in surface hydrophilicity, the flocculation phenotype was not influenced by the cholate exposure (see Fig. S1 in the supplemental material). Therefore, we examined the effect of cholate on the ability of cells to form biofilms on various surfaces. Herein, we used a crystal violet-based microtiter plate assay. This stain binds to negatively charged molecules, such as peptidoglycan and DNA, and its absorption to cells therefore serves as a measure for biomass at the surface, i.e., biofilms (7). *L. lactis* was grown for 24 h 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. In the absence of cholate, the cholate-adapted *L. lactis* Δ*lmrCD*^{r} strain showed a considerably poorer ability to form biofilms than the wild-type and Δ*lmrCD* cells (Fig. 1A). When cells were exposed to subinhibitory concentrations of cholate, biofilm formation was stimulated for all three strains tested, but the effect was the most pronounced with the Δ*lmrCD*^{r} strain (Fig. 1A). In contrast, the cholate-adapted Δ*lmrCD*^{r} cells showed more biofilm formation on glass, and this was stimulated more strongly by cholate than on polystryrene surfaces (see Fig. S2). Higher cholate concentrations were inhibitory for growth, and consequently the biofilms were inhibited, although the Δ*lmrCD*^{r} strain remained the most effective in biofilm formation even at higher cholate concentrations.

TABLE 2. Interfacial free energy of adhesion between polystyrene surface and *L. lactis* cells with and without exposure to cholate

Condition	Strain description	Interfacial free energy of adhesion, ΔG_{adh} (mJ m^{-2})[a]		
		$\Delta G_{adh}{}^{LW}$	$\Delta G_{adh}{}^{AB}$	$\Delta G_{adh}{}^{total}$[b]
Without cholate	Δ*lmrCD*^{r}	−5.1	7.9	2.7
	Δ*lmrCD*	−5.0	1.8	−3.2
	Wild type	−4.7	2.8	−2.0
With cholate	Δ*lmrCD*^{r}	−5.6	3.1	−2.6
	Δ*lmrCD*	−4.7	2.0	2.7
	Wild type	−4.7	5.9	1.2

[a] Cells were grown till stationary phase in GM17 in the presence of 1.5 mM cholate.
[b] The interaction with the surface is thermodynamically unfavorable when the total interfacial free energy of adhesion is positive. Surface free energy (in mJ m^{-2}) values of polystyrene are as follows: γ^{LW}, 41.2; γ^{AB}, 0; γ^+, 0; and γ^-, 9.06 (used in the calculation of the ΔG_{adh} values).

To assess the specificity of the cholate-promoting effect on biofilm formation by the Δ*lmrCD*^{r} strain, several other bile acids and detergents were tested. Unconjugated bile salts containing one or two hydroxyl groups, such as lithocholate, chenodeoxycholate, and deoxycholate, also stimulated biofilm formation. The steroidal anionic detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) showed a weaker stimulatory effect, whereas dehydrocholate, a cholate molecule devoid of the three hydroxyl groups, did not affect biofilm formation (data not shown). The bile acid conjugate taurocholate had a strong effect on biofilm formation by the Δ*lmrCD*^{r} strain (Fig. 1B). On the other hand, glycoconjugates, such as glycodeoxycholate (Fig. 1C) and glycocholate, showed an overall weak stimulatory effect. Nonionic detergents like Tween 20 (Fig. 1D), Triton X-100, and Brij58, the anionic detergents sodium dodecyl sulfate (SDS) and sodium lauroyl sulfate, and the cationic detergent benzalkonium chloride strongly inhibited biofilm formation at concentrations that only marginally inhibited growth of planktonic cells (for Tween 20, see the inset of Fig. 1D). These data demonstrate that the stimulatory effect of cholate on biofilm formation by the Δ*lmrCD*^{r} strain is not due to a general detergent-like phenomenon but is



specific for unconjugated or hydrophilic conjugated bile acids, in particular.

To distinguish between a stimulatory effect of cholate on the cell number and biofilm matrix, an independent assay was performed based on the viable cell count in biofilms. Herein, biofilms were dissolved in growth medium, followed by plating and assessment of the number of CFU. Since *L. lactis* cells grow in short chains, the plating method provides an underestimate of the number of viable cells. However, the chain length differences among the strains (41) could be minimized by sonication (data not shown). Biofilms formed in the absence of cholate showed no major differences in CFU for the wild-type and Δ*lmrCD* strains. However, much lower CFU numbers were found for the Δ*lmrCD*^{r} strain (Fig. 2), consistent with the crystal violet staining data. Exposure to a cholate (1.5 mM) concentration that was inhibitory to the planktonic cells also resulted in lower CFU numbers for both the wild-type and Δ*lmrCD* cells extracted from the biofilms. However, the CFU for the Δ*lmrCD*^{r} biofilms increased significantly (Fig. 2). Higher concentrations of cholate (3 mM) were inhibitory to biofilms of all cell types. These data demonstrate that cholate strongly stimulates biofilm formation by the Δ*lmrCD*^{r} cells.

***L. lactis* biofilms exhibit an increased resistance to both bile acids and drugs.** Biofilm-associated cells typically exhibit an increased resistance to toxic compounds compared to planktonic cells (9). The 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 (36). Interestingly, upon cholate exposure, biofilms of the wild-type strain yielded higher CFU numbers than the Δ*lmrCD* strain (Fig. 2), suggesting that the MDR transporter LmrCD plays a role in biofilm survival. To examine this phenomenon in more detail, the bile acid and drug resistance profiles of the *L. lactis* wild type, Δ*lmrCD* strain, and Δ*lmrCD*^{r} strain were compared. *L. lactis* biofilms were allowed to develop on a polystyrene surface, whereupon they were exposed to different toxic compounds for 18 h. Next, the biofilm cells were harvested and grown in liquid medium for 24 h. Growth was used as a general measure of survival. Likewise, planktonic cells were harvested from the liquid medium, serving as controls. Typically, the biofilm-associated cells showed a greater resistance to both conjugated and unconjugated bile acids than the planktonic cells. Even in the biofilm state, Δ*lmrCD* cells are more sensitive to cholate than wild-type cells, with IC_{50}s of 2.3 and 7.4 mM, respectively (Table 3). The Δ*lmrCD*^{r} biofilm cells showed a remarkably high resis-

FIG. 1. Biofilm formation by *L. lactis* wild-type (white bars), Δ*lmrCD* (gray bars), or cholate-adapted Δ*lmrCD*^{r} (black bars) cells. Cultures were grown for 24 h in GM17 medium containing various concentrations of cholate (A), taurocholate (B), glycodeoxycholate (C), or Tween 20 (D) in 96-well microtiter plates. The OD_{660} of the resuspended biofilm was measured, and cells were stained with crystal violet, whereupon the OD_{575} was measured. Inset: Tween 20 resistance of *L. lactis* wild type (□), Δ*lmrCD* (○), and Δ*lmrCD*^{r} (●) cells. Cells were grown for 8 h in GM17 medium in the absence or presence of various concentrations of Tween 20, and the maximum specific growth rate, μ, was determined. Data presented are averages of five replicates, and error bars represent calculated standard deviations.

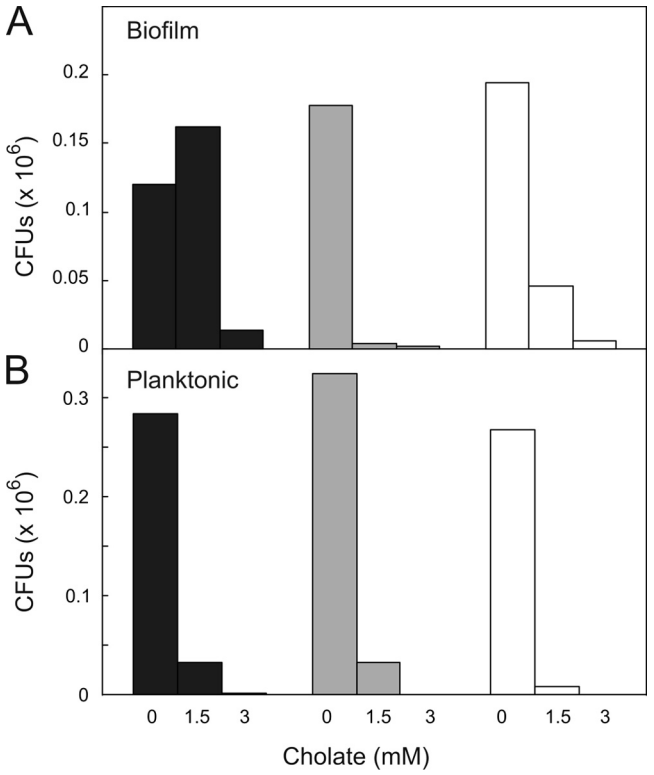


FIG. 2. Planktonic and biofilm growth and cholate-induced killing monitored by CFU. Biofilm formation by *L. lactis* wild-type (open bars), Δ*lmrCD* (gray bars), and cholate-adapted Δ*lmrCD*^{r} (black bars) cells is analyzed. Dilutions (10^{-2}, 10^{-4}, and 10^{-6}) of the liquid culture and resuspended biofilms grown with or without 1.5 mM cholate were spread plated on GM17 agar plates without cholate and incubated at 30°C for 24 h. The experiments were performed in duplicate; two independent experiments show a variation of less than 10%.

tance to cholate (IC_{50} > 14 mM), exceeding that of the wild-type cells. Compared to the planktonic cells, Δ*lmrCD*^{r} biofilms exhibited a substantial increase in resistance to all conjugated and unconjugated bile acids tested (Table 3). *L. lactis* biofilms also exhibited an increased resistance to such toxic drugs as daunomycin, rhodamine 6G, and quinine and several none-bile detergents compared to results for the planktonic cells (Table 3). Interestingly, compared to the wild type, Δ*lmrCD* (and Δ*lmrCD*^{r}) biofilm cells were more susceptible to the zwitterionic detergent CHAPS and the drugs daunomycin and quinine. CHAPS is the zwitterionic derivative (18) of cholate. These compounds are known substrates of LmrCD, indicating that LmrCD contributes to drug resistance of the biofilms.

Next, we determined whether preexposure to cholate affected the drug resistance of the cells. While the bile acid resistance phenotype of planktonic cells was barely altered by preexposure to cholate, cells present in the biofilms gained a large increase in resistance against cholate (Table 3). Δ*lmrCD*^{r} cells were already resistant to the highest concentration of cholate tested. Upon preexposure to cholate, the Δ*lmrCD*^{r} biofilms also showed an increased resistance to the conjugated bile acid glycodeoxycholate, the unconjugated bile acids deoxycholate, chenodeoxycholate, and lithocholate, and some detergents. In contrast, preexposure to cholate rendered wild-type and Δ*lmrCD* biofilms more sensitive to unconjugated bile acids and the detergents TX-100 and Brij-58 (Table 3). Cholate exposure did not alter the resistance of biofilms of wild-type cells toward drugs. Similarly, taurocholate, which promotes strong biofilm formation in Δ*lmrCD*^{r} cells, does not improve the resistance to drugs like daunomycin (see Fig. S3 in the supplemental material). In summary, these data demonstrate that biofilms of *L. lactis* are highly resistant to a range of

TABLE 3. Susceptibility of planktonic and preformed biofilm cells of *L. lactis* wild type, Δ*lmrCD*, and Δ*lmrCD*^{r} strains to various bile acids, detergents, and drugs

Treatment	IC_{50} (mM) of treatment agent for indicated strain with or without preexposure to cholate[a]											
	Planktonic						Biofilm					
	Δ*lmrCD*^{r}		Δ*lmrCD*		Wild type		Δ*lmrCD*^{r}		Δ*lmrCD*		Wild type	
	−	+	−	+	−	+	−	+	−	+	−	+
Bile acid												
Cholate	2.7	2.1	2.1	1.5	2.4	2.5	**>14**	>14	2.3	>14	7.4	>14
Deoxycholate	0.63	0.5	0.58	0.5	0.58	0.6	1.15	2.1	1.18	0.3	1.22	1.0
Chenodeoxycholate	0.31	0.29	0.09	0.24	0.09	0.09	1.12	2.23	1.02	0.26	1.15	0.94
Lithocholate	3.4	>3.6	>3.6	>3.6	>3.6	>3.6	5.5	>7.2	>7.2	>7.2	>7.2	>7.2
Glycocholate	23	>48	>48	>48	>48	>48	>48	>48	>48	>48	>48	>48
Taurocholate	29	5	>48	>48	>48	>48	>48	>48	>48	>48	>48	>48
Glycodeoxycholate	0.58	0.42	0.43	0.60	25	34	37.5	>128	>128	34	>128	>128
Taurodeoxycholate	2	6	51.5	63	72.5	80	114	114	>128	>128	>128	>128
Dehydrocholate	47	40	54	39	>100	50	>160	>160	>160	>160	>160	>160
Detergent												
CHAPS	2.4	0.03	2.5	0.07	3	0.14	0.6	1.26	2.65	2.45	>10	10
Triton X-100	0.086	0.086	0.089	0.089	0.084	0.084	0.02	>1.5	>1.5	0.03	>1.5	0.06
Benzalkonium-Cl	0.004	0.004	0.004	0.004	0.007	0.007	0.007	0.004	0.008	0.007	0.009	0.008
Sodium lauroyl sarcosin	0.7	0.7	0.45	0.45	0.45	0.45	2.1	3.1	2.1	2.9	2.1	3.1
SDS	0.19	0.19	0.29	0.29	0.30	0.30	0.30	0.33	0.30	0.32	0.29	0.31
Brij-58	0.01	0.01	>20	>20	>20	>20	22	1.3	>100	9.5	>100	16
Drug												
Daunamycin	0.0016	0.0015	0.0017	0.0018	0.049	0.088	0.003	0.004	0.013	0.010	0.089	0.083
Rhodamine 6G	0.0031	0.0051	0.0035	0.0052	0.0071	0.009	0.011	0.018	0.017	>0.02	>0.02	>0.02
Quinine	0.40	0.84	0.84	1.24	1.31	1.18	1.3	3.2	1.6	3.3	3.1	3.4

[a] "+" and "−" indicate preexposure to cholate (1.5 mM) or the lack thereof. The most distinct change in biofilm cells of the Δ*lmrCD*^{r} strain compared to the parental strain is highlighted in boldface.

TABLE 4. Effect of *lmrCD* expression on cholate susceptibilities of planktonic and biofilm *L. lactis* cells

Cell configuration	Strain description	Susceptibility to:			
		Cholate (IC_{50}, mM)		Daunomycin (IC_{50}, μM)	
		pIL*lmrCD*	Empty vector	pIL*lmrCD*	Empty vector
Planktonic	Δ*lmrCD*^{r}	4.7	3.8	2.9	1.9
	Δ*lmrCD*	4.1	2.1	49	2.7
	Wild type	4.1	2.6	52	24
Biofilm	Δ*lmrCD*^{r}	16	14.6	50.5	2.8
	Δ*lmrCD*	18.2	2.4	39	3.2
	Wild type	22	4.1	49	19.5

bile acids and show that the resistance of Δ*lmrCD*^{r} biofilms can be enhanced by preexposure of the cells to cholate. The enhancement of sessile cells is restricted to the biofilm phase since the cells that are dislodged from the biofilms behave similarly to the stationary-phase planktonic cells (see Table S1).

Role of LmrCD in drug resistance of *L. lactis* biofilms. The role of LmrCD in biofilm resistance was further examined by providing *L. lactis* wild-type, Δ*lmrCD*, and Δ*lmrCD*^{r} cells with a plasmid carrying the *lmrCD* genes under the control of the endogenous promoter (27). Cells carrying the empty vector served as controls. Both biofilm and planktonic cells harboring plasmid-encoded LmrCD were significantly more resistant to cholate and daunomycin than the control cells (Table 4). With Δ*lmrCD*^{r} biofilm cells, which already have an intrinsically high resistance to cholate, expression of plasmid-encoded LmrCD resulted in a modest increase in cholate resistance, whereas wild-type and Δ*lmrCD* biofilms showed 5- and 8-fold increases in resistance to cholate, respectively (Table 4). Collectively, these results demonstrate that LmrCD provides cholate and drug resistance in the lactococcal biofilms. In addition, other mechanisms persist in the biofilms that render these structures highly resistant to toxic compounds.

Extracellular polymeric substance formation. Extracellular polymeric substances (EPS) are known to play an important role in biofilm formation. EPS formation was visualized by confocal laser scanning microscopy (CLSM) (Fig. 3) using the dye calcofluor (26), which stains the extracellular matrix blue. In addition, the *Bac*Light Live/Dead stain with the dyes SYTO9 (green) and propidium iodide (red) was used to differentiate between viable and nonviable cells, respectively (1). Under normal conditions of growth, the Δ*lmrCD*^{r} cells produced more EPS than the parental and wild-type cells (Fig. 3A to C). Exposure to 3 mM cholate resulted in a partial disruption of biofilms of wild-type and Δ*lmrCD* cells, as evidenced by the high dead cell count (Fig. 3D and E). However, at the same time, EPS formation was stimulated. Cholate stimulated both biofilm and EPS formation by Δ*lmrCD*^{r} cells (Fig. 3F). Remarkably, taurocholate (24 mM) had an even stronger effect on biofilm and EPS formation, in particular with Δ*lmrCD*^{r} (Fig. 3I) and wild-type (Fig. 3G) cells. These data demonstrate that Δ*lmrCD*^{r} biofilm cells produce increased levels of EPS.

Divalent cation dependence of biofilm formation. Since divalent metal cations are known to be involved in the maintenance and stability of biofilms (2), the effect of EDTA on biofilm persistence was determined. While planktonic cells of the different *L. lactis* strains exhibited similar EDTA susceptibilities, with IC_{50}s in the range of 0.7 to 0.8 mM (Fig. 4A), biofilms of wild-type and Δ*lmrCD* cells were entirely insensitive to high concentrations of EDTA (Fig. 4B). Remarkably, under the same set of conditions, the Δ*lmrCD*^{r} biofilms collapsed, showing hypersensitivity to EDTA. This suggests that the integrity of the Δ*lmrCD*^{r} biofilm to a great extent relies on the presence of divalent cations.

The susceptibilities of the biofilms to the antimicrobial peptide bacitracin were determined. Bacitracin is a dodecapeptide that inhibits peptidoglycan synthesis in Gram-positive cocci, a process that is divalent metal cation dependent (32). In the planktonic phase, the Δ*lmrCD*^{r} strain showed a somewhat higher resistance to bacitracin than did wild-type and Δ*lmrCD* cells (Fig. 4C). On the other hand, biofilms of the last two strains appeared rather insensitive to bacitracin, while the Δ*lmrCD*^{r} biofilms were very sensitive to this peptide, showing an IC_{50} of ~15 μg/ml (Fig. 4D). Possibly, the increased sensitivity to bacitracin relates to a requirement for divalent cations in the integrity of Δ*lmrCD*^{r} biofilms. On the other hand, the biofilm cells were about 10-fold more resistant to bacitracin than the planktonic cells, which may reflect the more recalcitrant nature of biofilms to antimicrobial agents.

DISCUSSION

This study aimed to resolve the physicochemical basis of bile acid resistance in the lactic acid bacterium *L. lactis*. Our previous work has shown that in planktonic *L. lactis* cells, the ABC-type MDR transporter LmrCD is a major determinant of drug and bile acid resistance (27). Importantly, cells that lack the *lmrCD* genes are able to regain bile acid resistance upon prolonged exposure to increasing levels of sublethal cholate concentrations (41). This Δ*lmrCD*^{r} strain, as used in this study, shows a bile acid resistance which is not efflux based and that, although more effective than LmrCD-mediated cholate resistance, is limited to mostly unconjugated bile acids. Transcriptome analysis and macroscopic visualization of the cells suggest that the cholate resistance of Δ*lmrCD*^{r} cells is related to cell surface changes (41). In addition, Δ*lmrCD*^{r} cells show an enhanced flocculation. Recently it has been reported that such cellular aggregates can be considered free-floating biofilms (34). Therefore, we have investigated the biofilm-forming abilities of Δ*lmrCD*^{r} cells. In the absence of cholate, the cells showed a poor ability to attach to hydrophobic polystyrene surfaces, but attachment was strongly stimulated by the presence of cholate. Contact angle measurements suggest that the cell envelope of the Δ*lmrCD*^{r} cells is hydrophilic, and this likely makes interactions with the hydrophobic polystyrene surface thermodynamically unfavorable. However, in the presence of cholate a shift in the distribution of the cationic and anionic components of the cell envelope properties is observed, and this results in a reduction in the hydrophilicity. Therefore, biofilm formation on a polystyrene surface is more likely to occur. It thus seems that cholate alters the cell surface properties of the Δ*lmrCD*^{r} cells, making these cells more effective in colonizing hydrophobic surfaces. It should be stressed that cholate also stimulates biofilm formation of wild-type and pa-

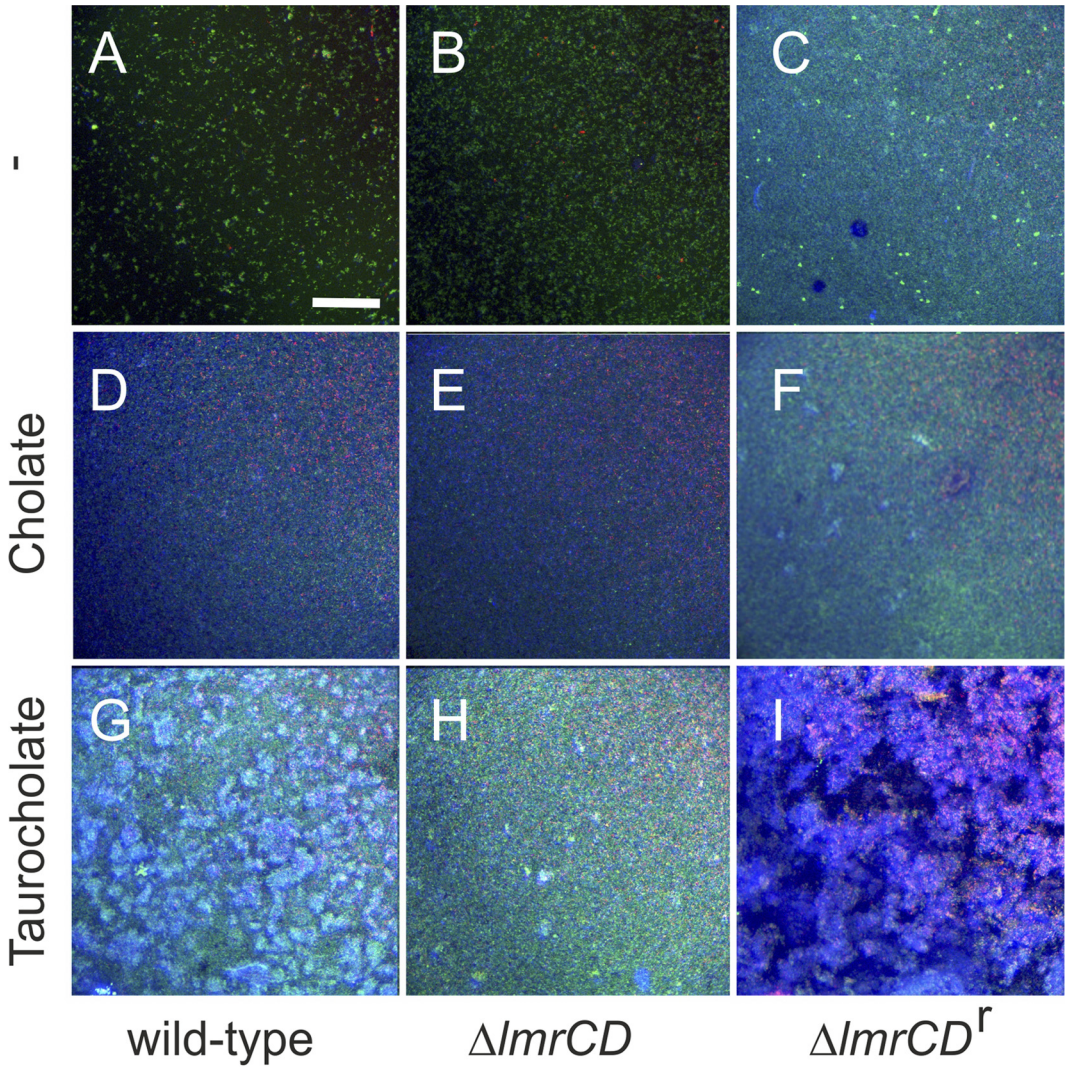


FIG. 3. Confocal laser scanning microscopy of biofilms of the *L. lactis* wild-type (A, D, and G), Δ*lmrCD* (B, E, and H), or Δ*lmrCD*^{r} (C, F, and I) strain formed in the absence or presence of sodium cholate (3 mM) or taurocholate (24 mM). Biofilms were staining with the *Bac*Light viability stain, indicating live cells in green and dead cells in red. Calcofluor stains the EPS in blue. The bar marker corresponds to 75 μm in all images.

rental cells, but the effects with the Δ*lmrCD*^{r} strain are much more pronounced.

In Δ*lmrCD*^{r} cells, several cell envelope proteins are differentially expressed. In particular, the cell wall-anchored protein CluA and the serine protease HtrA are upregulated, and both have been implicated in the clumping phenotype in *L. lactis* (16, 29). The flocculation of the Δ*lmrCD*^{r} cells in aqueous media might be due to increased levels of CluA (41), a cell wall-anchored adhesin. HtrA is involved in the degradation of misfolded proteins (41) but also in protein processing, such as that of the cell wall protein AcmA (autolysin). In *L. lactis*, HtrA has been shown to be involved in cell aggregation due to its role in processing of surface proteins (16). In the Δ*lmrCD*^{r} strain, this may have led to changes in the cell wall protein composition favoring cell adhesion.

Biofilm formation is an intricate multistep phenomenon which begins with an initially weak and reversible adhesion of cells to the surface, gradually leading to a firm and irreversible attachment involving production of exopolymeric material (20). This encompasses polysaccharides, proteins, nucleic acids, and other materials and constitutes the major portion of the total volume of a biofilm (31). The changes in overall physicochemical properties of the cell surface of the Δ*lmrCD*^{r} strain most likely relate to a change in the composition and/or amount of cell wall-associated glycopolymers (15) and polysaccharides (5). The lactococcal cell wall consists of a thick peptidoglycan layer with interspersed teichoic and lipoteichoic acids, surface-exposed proteins, and polysaccharides (10). We focused on EPS in the biofilms because of their role in maintaining biofilm integrity (22) and promoting cell adhesion (8). Even in the absence of cholate, biofilms of the Δ*lmrCD*^{r} strain produced greater amounts of EPS than wild-type and Δ*lmrCD* biofilms. EPS formation is further stimulated by cholate and taurocholate. The increase in EPS likely contributes to the observed transition from a hydrophilic to a slightly more hydrophobic cell surface of the Δ*lmrCD*^{r} cells when they are exposed

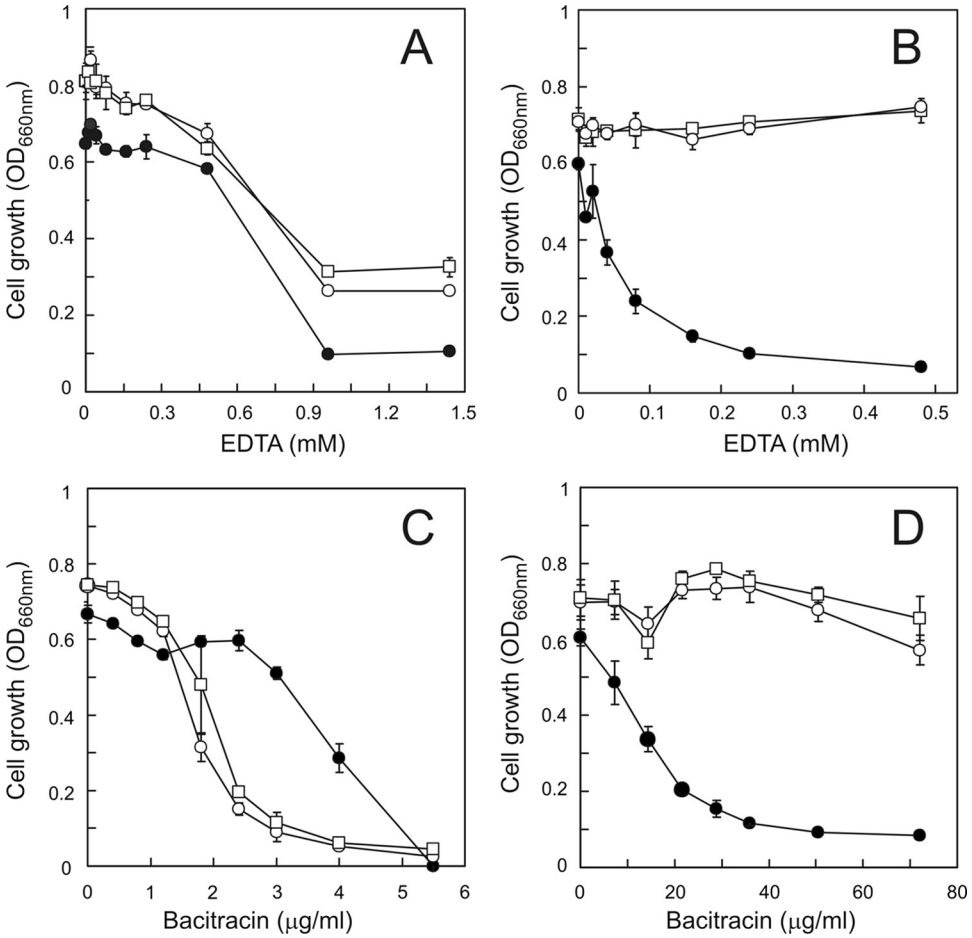


FIG. 4. Influence of EDTA (A and B) and bacitracin (C and D) on the growth of *L. lactis* wild-type (□), Δ*lmrCD* (○), and Δ*lmrCD*^{r} (●) cells. Planktonic (A and C) cells were grown for 12 h in 96-well microtiter plates in GM17 medium containing 0 to 50 mM EDTA or 0 to 80 μg/ml bacitracin. Growth was determined at OD_{660}. Biofilm cells (B and D) were grown for 24 h on pegs immersed in GM17 medium containing 0 to 50 mM EDTA or 0 to 80 μg/ml bacitracin. Biofilm cells were collected and regrown for 18 h in GM17 medium, and growth was determined at OD_{660}.

to cholate. In this respect, some other membrane-acting agents, such as ethanol or cinnamon oil (30), have also been reported to stimulate the production of EPS (13) or alter its composition (35). The biofilm EPS can also serve to sequester ions from the environment (39) and thereby make cells more susceptible to antimicrobials, such as bacitracin, that are cation dependent for their activity. Importantly, EPS has also been shown to reduce the penetration of some antibiotics into the biofilm (37), and this may also contribute to the overall bile acid resistance of the biofilms. Another critical event in adhesion leading to biofilm development is the preconditioning of the surface by macromolecules (6). Surfactant-like molecules may, for instance, promote cell adhesion to the surface, as in the case of surfactants with long acyl chains that elicit a maximum adhesive response (33). Cholate may have a similar effect, although our observations suggest that this is not a general detergent-like effect since structurally related (i.e., steroidal) molecules, like CHAPS, are only weakly effective.

L. lactis encodes a large number of (putative) efflux pumps (41). Several of these, such as the MFS-type pump LmrP (4) and the ABC-type transporters LmrA (40) and LmrCD (27), have been well characterized and show drug extrusion activity in planktonic cultures. Wild-type lactococcal biofilms are more resistant to daunomycin and cholate than is the Δ*lmrCD* strain. Strikingly, wild-type *L. lactis* biofilm cells carrying a multicopy plasmid encoding LmrCD exhibit an even higher level of resistance and can tolerate cholate concentrations even up to 20 mM. This demonstrates that the MDR transporter LmrCD also plays a prominent role in drug and bile acid resistance during biofilm formation and persistence, in addition to the general impermeable nature of the biofilm matrix. Future experiments should determine which other transport systems contribute to biofilm resistance and persistence.

ACKNOWLEDGMENT

A.H.Z. was supported by a scholarship from the Higher Education Commission, Government of Pakistan.

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