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Isolation of *Lactococcus lactis* Mutants Simultaneously Resistant to the Cell Wall-Active Bacteriocin Lcn972, Lysozyme, Nisin, and Bacteriophage c2

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Lactococcin 972 (Lcn972) is a nonlantibiotic bacteriocin that inhibits cell wall biosynthesis by binding to lipid II. In this work, two mutants resistant to Lcn972, *Lactococcus lactis* D1 and D1-20, with high (>320 arbitrary units [AU]/ml) and low (80 AU/ml) susceptibilities, respectively, have been isolated. Resistance to Lcn972 did not impose a burden to growth under laboratory conditions, nor did it substantially alter the physicochemical properties of the cell surface. However, the peptidoglycan of the mutants featured a higher content of muropeptides with tripeptide side chains than the wild-type strain, linking for the first time peptidoglycan remodelling to bacteriocin resistance. Moreover, *L. lactis* lacking a functional D,D-carboxypeptidase DacA (i.e., with a high content of pentapeptide side chain muropeptides) was shown to be more susceptible to Lcn972. Cross-resistance to lysozyme and nisin and enhanced susceptibility to penicillin G and bacitracin was also observed. Intriguingly, the Lcn972-resistant mutants were not infected by the lytic phage c2 and less efficiently infected by phage sk1. Lack of c2 infectivity was linked to a 22.6-kbp chromosomal deletion encompassing the phage receptor protein gene *pip*. The deletion also included maltose metabolic genes and the two-component system (TCS) F. However, a clear correlation between these genes and resistance to Lcn972 could not be clearly established, pointing to the presence of as-yet-unidentified mutations that account for Lcn972 resistance.

Lactococcus lactis is one of the main components of the mesophilic starter cultures used in cheese manufacturing. Thereby, there is a genuine interest in improving robustness to ensure the success of dairy fermentations. *L. lactis* performance may be compromised by the presence of bacteriophages or other inhibitors such as antibiotics, lysozyme, or bacteriocins in raw milk (12, 27). Many of these antibacterial compounds target cell wall components. Bacteriophages recognize bacterial receptors, mostly of polysaccharide nature, prior to infection (37), while lysozyme acts directly on the cell wall peptidoglycan, hydrolyzing the glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine. Moreover, an increasing number of bacteriocins, antimicrobial peptides synthesized by bacteria, including the lactococcal lantibiotics nisin and lacticin 3147 and the nonlantibiotic lactococcin 972 (Lcn972), have been reported to inhibit cell wall biosynthesis by binding to the cell wall precursor lipid II (4, 23, 42).

As a Gram-positive bacterium, the cell envelope of *L. lactis* consists of a cytoplasmic membrane and a thick cell wall. The cell wall is composed mostly of peptidoglycan (PG) made of glycan strands cross-linked by peptides and secondary polymers such as teichoic acids, proteins, and carbohydrates. *L. lactis* has an A4α-type peptidoglycan with an L-Ala-α-D-Glu-L-Lys-D-Ala as the tetrapeptide and D-Asp in the interpeptide bridge (9). Recent microscopy advances have provided a very detailed knowledge on the PG structure in the *L. lactis* cell wall (1, 41).

The cell wall in Gram-positive bacteria protects cells from osmotic pressure and acts as an exoskeleton, maintaining cell shape, and as scaffold for anchoring other cell envelope components (see reference 40 and references therein). Thus, monitoring its integrity is crucial for survival. In *L. lactis*, the response to cell envelope stress is governed by the two-component system CesSR, which has been shown to be triggered by lysozyme, nisin, and Lcn972 (26, 39) and by heterologous protein secretion and phage infection (11, 33). Although the CesR regulon is not fully understood, certain CesR-regulated components are known to contribute positively to *L. lactis* survival under technological relevant stresses (34).

In this work, we have isolated *L. lactis* mutants resistant to Lcn972 (Lcn972^r) which were characterized with a particular emphasis on cell surface properties, PG composition, and resistance to cell wall-active antimicrobials, such as lysozyme and bacteriophages. Lcn972 is an atypical 66-amino-acid bacteriocin that does not meet the widely accepted criteria of small, heat-resistant hydrophobic peptides. Lcn972 is a highly hydrophilic cationic peptide easily inactivated by heat (25). In contrast to other lipid II-binding bacteriocins, Lcn972 does not form pores in the cytoplasmic membrane and is active exclusively against lactococci (23). These features make Lcn972 a unique candidate to shed light on mechanisms that help *L. lactis* to cope better with cell wall stress.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions. *L. lactis* strains (Table 1) were routinely grown in M17 with 0.5% glucose (GM17), statically and at 30°C. When specified, glucose was replaced by 0.5% maltose (MM17) or chemically defined medium (CDM) (29) was used. *Escherichia coli* strains were grown in 2× YT (35) at 37°C with shaking. When needed, antibiotics erythromycin and ampicillin were used at final con-

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TABLE 1 Bacterial strains, bacteriophages, and plasmids used in this work

Strain, phage, or plasmid	Description[a]	Reference/source
L. lactis		
MG1363	Plasmid-free and prophage-cured derivative of *L. lactis* NCDO712	13
MG1614	Str^r Rif^r derivative of MG1363, $Lcn972^s$	13
NZ9000	MG1363, carrying *pepN*::*nisRK*	21
D1	MG1614, Lcn972 high-resistant mutant, unstable	This work
D1-20	MG1614, Lcn972 low-resistant mutant derived from D1, stable	This work
Δ*dacA* strain	NZ9000 lacking *dacA* gene	This work
Δ*dacB* strain	MG1363 lacking *dacB* gene	9
Δ*dltD* strain	MG1363 lacking *dltD* gene	10
MGRrF	MG1363 pRV300::*llrF*	30
E. coli		
DH10B	Plasmid free, cloning host	Invitrogen
Phages		
c2	*L. lactis* lytic phage belonging to c2 family	E. Bidnenko
sk1	*L. lactis* lytic phage belonging to 936 family	E. Bidnenko
Plasmids		
pORI280	Em^r $lacZ^+$, integrative plasmid	22
pBL16	600-bp *dacA* flanking regions cloned in pORI280	This work

[a] Str, streptomycin; Rif, rifampin; Em, erythromycin.

centrations of 5 μg ml^{-1} and 100 μg ml^{-1}, respectively. Growth rates (μ) were calculated through linear regressions of the plots of ln(optical density at 600 nm [OD_{600}]) versus time during the exponential growth phase. Bacteriophages c2 and sk1 were propagated on *L. lactis* MG1614. Phage titer was calculated by the standard plate assay. Decimal dilutions in 0.9% NaCl of phage lysates were mixed with 3.5 ml of molten GM17 0.7% agar supplemented with 10 mM $CaCl_2$ and 100 μl of stationary-phase *L. lactis* MG1614 culture. The mixture was spread on GM17 plates and incubated at 30°C for 16 h until clear lytic plaques were visible. Bacterial cultures were stored at −80°C in the appropriate medium and 10% glycerol (vol/vol). Phage lysates were stored at 4°C.

Standard DNA techniques. Standard molecular techniques were followed as described elsewhere (35). Chromosomal DNA was isolated with the GenElute bacterial genomic DNA kit (Sigma-Aldrich, Spain). Restriction enzymes were purchased from TaKaRa (Japan) and T4 ligase from Fisher Scientific (Spain). Oligonucleotides were supplied by Sigma-Aldrich (Spain). Standard PCRs were carried out using Pure *Taq* Ready-to-Go PCR beads (GE Healthcare, United Kingdom). For cloning purposes and PCRs expected to yield long products, the proofreading Phusion high-fidelity DNA polymerase (Fisher Scientific, Spain) was used.

Selection of *L. lactis* resistant to Lcn972. The bacteriocin Lcn972 was purified and quantified as described previously (26). Lcn972 dilutions were done in 50 mM sodium phosphate buffer, pH 6.8. To isolate Lcn972-resistant mutants, *L. lactis* MG1614 was cultivated stepwise in GM17 in the presence of increasing Lcn972 concentrations ranging from 5 AU/ml to up to 400 AU/ml. Overnight cultures grown at the highest Lcn972 concentration were diluted and plated on GM17 to get isolated colonies. A single colony, designated *L. lactis* D1, was randomly selected. *L. lactis* D1-20 was colony-isolated after serial passages of *L. lactis* D1 in GM17 during 200 generations.

Construction of the *L. lactis* Δ*dacA* strain. The flanking 600-bp regions up- and downstream of *dacA* (*llmg2560* in *L. lactis* MG1363; GenBank AM406671) were amplified and fused by splicing by overlap extension PCR (SOE-PCR) using the primers D1 (5′ AACTGCAGTATT GACAAATGCCG 3′), D2 (5′ AAAAACTTTTGGAGCACTGACAGCAA AGCTCG 3′), D3 (5′ AGTGCTCCAAAAGTTTTTTGG 3′), and D4 (5′ GAAGATCTGCTAAACGTGACCC 3′). The SOE-PCR fragment was cloned into the nonreplicating plasmid pORI280 using the engineered restriction sites PstI and BglII in the far ends of primers D1 and D4, respectively, to generate the plasmid pBL16. Transformation of *L. lactis* NZ9000 and selection of deletion mutants proceeded according to Leenhouts et al. (22).

Mapping chromosomal deletion in *L. lactis* D1 and D1-20. A forward primer, 0734F (5′ GAAATGGCCCTGCGACGTGTAG 3′), located within the *llmg0734* locus, was used in combination with the reverse primer, 0752R (5′ CGCTGCATCCAATGTCACAGTC 3′), in *pip* for PCR amplification using *L. lactis* DNA. PCR conditions were 98°C for 30 s; 35 cycles at 98°C for 10 s, 63°C for 30 s, and 72°C for 13 min; and 72°C for 10 min, and Phusion high-fidelity DNA polymerase was used. PCR products were purified with Illustra GFX PCR DNA and the gel band purification kit (GE Healthcare, United Kingdom) and sequenced. Southern hybridization was carried out on 200 ng of total DNA digested with EcoRI and HindIII blotted to a nylon Hybond-N membrane (GE Healthcare, United Kingdom) (35). The DNA probe was labeled by PCR on *L. lactis* MG1614 DNA using primers pipF (5′ CGGATTCATCTATGTTGACC 3′) and pipR2 (5′ AATTGCTTCTCTTTGTCGG 3′), expanding the 5′ end of *pip* (see Fig. 3), and labeled dNTPs from PCR DIG labeling mix (Roche, Spain). PCR conditions were 98°C for 30 s; 35 cycles at 98°C for 10 s, 45°C for 30 s, and 72°C for 2 min; and 72°C for 10 min, using Phusion high-fidelity DNA polymerase. The blots were revealed by immunodetection at 20°C using CDP Star (Roche, Spain) by following the manufacturer's recommendations.

Surface properties. The electrophoretic mobility was measured using stationary-phase cells concentrated to 10^7 CFU/ml in 5 mM NaCl as previously described (15). Hydrophobicity was determined following the microbial-adhesion-to-solvents method (MATS) using hexadecane and stationary-phase cells in 0.15 M NaCl adjusted to a final OD_{600} of 0.8 (2). Each measurement was performed in triplicate, and the assay was carried out twice with independent cultures. Adsorption of Lcn972 to lactococcal cells was performed by mixing 200 μl of exponentially growing cells adjusted to an OD_{600} of 2.0 with 200 μl of Lcn972 (20 μg/ml) in 50 mM sodium phosphate buffer, pH 6.8. Residual inhibitory activity was measured by the agar diffusion test using *L. lactis* MG1614 as an indicator (26).

Cell wall composition. Peptidoglycan (PG) preparations were obtained from 500 ml of exponentially growing (OD_{600} of 0.3) cultures as described previously (9). Briefly, after SDS-lysis, the crude cell wall preparation containing PG was treated with 48% hydrofluoric acid for 16 h at

TABLE 2 Properties of *L. lactis* MG1614 and its derivatives resistant to Lcn972

L. lactis strain	MIC of Lcn972 (AU/ml)	μ (h^{-1})[a]				Lcn972 adsorption (%)	Adhesion to hexadecane[b] (%)	D-Ala (ng/mg dried cells)
		GM17	CDM					
			Glu	Gal	Mal			
MG1614	10	1.07	0.98	0.38	0.39	25.6 ± 3.1	75.75 ± 4.55	17.7 ± 2.7
D1	>320	0.96	0.67	0.2	NG	13.9 ± 0.8	62.90 ± 1.87	14.1 ± 0.6
D1-20	80	0.97	0.68	0.37	NG	14.4 ± 1.6	63.60 ± 1.74	14.3 ± 1.9

[a] Growth was carried out in microtiter plates in GM17 or in tubes in chemically defined medium (CDM) supplemented with glucose (Glu), galactose (Gal), or maltose (Mal) at 0.5%. NG, no growth.
[b] Determined by the MATS method.

4°C to eliminate anionic polymers linked to PG. Two milligrams of purified PG was digested with mutanolysin (Sigma; 2,500 U/ml) for 10 h at 37°C under shaking. Solubilized muropeptides were reduced by sodium borohydride and separated by reverse-phase high-performance liquid chromatography (RP-HPLC) as described previously (9). The percentage of muropeptides with a certain peptide side chain (X = tri, tetra, and penta) with free COOH (donor chain) was calculated according to Glauner et al. (16) as follows: percentage (X) = [Σmonomers(X) + (1/2)Σdimers(X) + (1/3)Σtrimers(X) + (1/4)Σtetramers(X)]/Σall muropeptides. During the isolation of PG, part of the cell wall fraction was kept before the treatment with hydrofluoric acid to determine the sugar composition (6). The cell wall fraction was hydrolyzed with TFA 4 M at 110°C for 3 h and derivatizated with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) for 30 min at room temperature and analyzed by gas chromatography coupled to mass spectrometry (6). The content of D-alanine esterified to teichoic acids was determined after release by alkaline hydrolysis from 10 mg of dried cells from 300 ml of stationary-phase lactococcal cultures in GM17 as previously described (15).

Antimicrobial susceptibility assays. The susceptibility of *L. lactis* cell envelope mutants to Lcn972 was tested by the spot-on-the-lawn method. Overnight cultures were diluted 10-fold in Ringer saline solution (Merck, Germany) and inoculated 1:1,000 (vol/vol) in melted GM17 containing 1.2% agar. Drops (5 μl) of 2-fold dilutions of Lcn972 were spotted on the plates and incubated at 30°C. To determine cross-resistance to cell wall antimicrobials, serial dilutions in GM17 broth of *L. lactis* D1 and D1-20 and the wild-type MG1614 overnight cultures were spotted on GM17 plates containing 0.5 mg/ml lysozyme, 10 ng/ml nisin, 0.5 μg/ml bacitracin, or 0.05 μg/ml penicillin G. Determinations of Lcn972 MICs were carried out in microtiter plates using either GM17 or MM17 essentially as described previously (5).

Phage assays. The efficiency of plaquing (EOP) was defined as the phage titer on the tested strain divided by that on the reference *L. lactis* MG1614. Each c2 and sk1 phage suspension was plated in triplicate. Phage adsorption was determined by mixing exponentially growing lactococcal cultures *L. lactis* MG1614, *L. lactis* D1, and *L. lactis* D1-20 at an OD_{600} of 0.5 with c2 or sk1 phage suspensions to match a phage/bacteria ratio (multiplicity of infection [MOI]) of 2×10^{-6} for c2 and 1×10^{-3} for ski1 (i.e., 1 ski1 phage per 1,000 bacterial cells). After 10 min of incubation at 30°C, samples were centrifuged, and the phage titer of the supernatant was determined by the standard plaque assay using *L. lactis* MG1614 as the host. The percentage of adsorption was determined as follows: [1 − (phage titer of the supernatant/phage titer of a control tube without cells)] × 100. The assay was carried out twice with independent cultures, and plating was made in triplicate.

RESULTS

Isolation of *L. lactis* MG1614 resistant to Lcn972. Stepwise exposure of *L. lactis* MG1614 to increasing Lcn972 concentrations resulted in cultures able to multiply in the presence of 400 AU/ml of Lcn972. A single colony, designated *L. lactis* D1, was randomly selected. This mutant was highly resistant to Lcn972 with a MIC of over 320 AU/ml (Table 2). This high-resistant phenotype was lost in the absence of selective pressure for 130 generations, after which a stable phenotype, *L. lactis* D1-20, with a MIC of 80 AU/ml (Table 2), could be maintained at least for 70 generations more.

Resistance to Lcn972 did not clearly impose a burden to *L. lactis*, as similar growth rates were observed in GM17 broth at 30°C (Table 2). However, under more limiting nutritional conditions in CDM-glucose, a decrease of 30% in the growth rate was observed for both $Lcn972^R$ mutants. Growth on galactose was seriously compromised in *L. lactis* D1, and maltose did not support growth (Table 2).

Surface properties and cell wall composition of *L. lactis* MG1614 resistant to Lcn972. Physicochemical properties of the bacterial cell surface may determine the initial interaction of bacteriocins to the target cell and contribute to resistance. Indeed, adsorption of Lcn972 to $Lcn972^r$ cells was reduced by 50% compared to that of the susceptible *L. lactis* MG1614 strain (Table 2). According to their electrophoretic mobility, *L. lactis* MG1614 and the mutants were equally negatively charged (data not shown), whereas the $Lcn972^r$ strains revealed a less hydrophobic character ($P < 0.001$) than *L. lactis* MG1614 (Table 2), anticipating subtle changes in the bacterial surface.

As Lcn972 is active at the cell wall level (23), we looked for alterations in the structure and composition of the cell wall in the $Lcn972^r$ mutants. Macroscopically, cells grew in pairs and short chains irrespectively of their phenotype. Large ultrastructural changes such as a thickened cell wall or absence of the surface polysaccharide layer (6) were not observed by electron microscopy (data not shown). Likewise, the overall content of monosaccharides (glucose, galactose, rhamnose), amino sugars (glucosamine), glycerol, and phosphate of the cell wall fraction of the strains did not reveal substantial differences among the strains (data not shown). Teichoic acids, which can be D-alanylated to modulate the negative charges inside the cell wall, were also analyzed. Although the resistant mutants contained less D-Ala than the wild type, the differences were not statistically significant ($P > 0.05$) (Table 2), suggesting that D-alanylation does not contribute largely to resistance to Lcn972.

On the contrary, remarkable differences among the strains were observed when the soluble peptidoglycan muropeptides were analyzed. While similar percentages of mono-, di-, tri-, and tetramers were found among the strains, both $Lcn972^r$ mutants D1 and D1-20 showed a higher content of muropeptides with tripeptide side chains and a reduced content of pentapeptide side chains than the wild type (Fig. 1). This observation suggested an alteration in the $Lcn972^r$ mutants of activities involved in PG maturation.

Susceptibility to Lcn972 of cell envelope *L. lactis* mutants. To

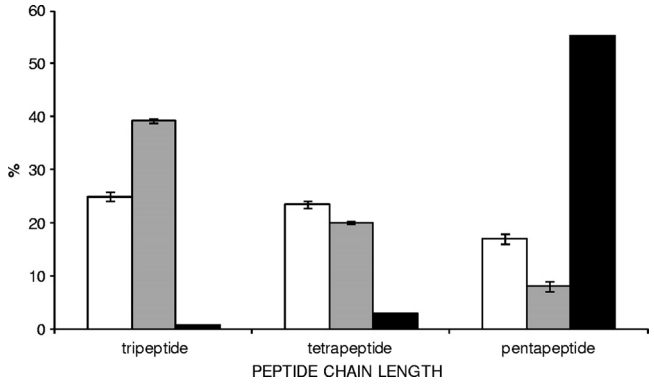

FIG 1 Distribution of muropeptides containing tri-, tetra-, or pentapeptide side chains in the Lcn972-susceptible *L. lactis* MG1614 (white bars), Lcn972-resistant *L. lactis* D1 (gray bars), and *L. lactis dacA* (black bars) strains. Values are the means from two independent peptidoglycan extractions, except for the *dacA* mutant. Error bars indicate standard deviations.

test the putative role of the PG structure on the Lcn972-resistant phenotype, an *L. lactis dacA* cell envelope mutant was generated. *dacA* encodes a D-Ala-D-Ala carboxypeptidase putatively involved in trimming away the last D-Ala residue from the PG pentapeptide. The chromosomal deletion of *dacA* was confirmed by PCR, and absence of the protein was evidenced by the lack of the respective bocillin FL-labeled 45-kDa protein band in membrane protein extracts analyzed by SDS-PAGE (data not shown). *L. lactis dacA* PG analysis revealed a dramatic decrease of muropeptides with tri- and tetrapeptide chains accompanied with a strong increase of muropeptides with pentapeptide side chains (Fig. 1), confirming a major role of DacA in PG maturation in *L. lactis*.

The *L. lactis dacA* mutant and other available *L. lactis* strains defective in cell wall modification enzymes (Table 1) were screened for their susceptibilities to Lcn972. The *L. lactis dltD* strain lacking DltD, a membrane protein involved in LTA D-alanylation, and the *L. lactis dacB* strain lacking the D,L-carboxypeptidase DacB, which cleaves the bond between L-Lys-D-Ala of the pentapeptide side chain, were assayed (Fig. 2). Despite the fact that no significant differences in the D-Ala content were observed among *L. lactis* MG1614 and strains D1 and D1-20, the *L. lactis dltD* strain devoid of D-Ala showed a 4-fold-increased susceptibility to Lcn972. Higher susceptibility was also observed in the case of the *L. lactis dacA* strain, whereas the *L. lactis dacB* strain showed no differences compared to the wild-type strain (Fig. 2). These results indicate that the high content of pentapeptide side chains present in *dacA* PG enhances the antimicrobial activity of Lcn972 and correlated well with their decrease in the Lcn972-resistant mutants (Fig. 1).

Cross-resistance to cell wall-active antimicrobials. In order to ascertain if resistance to Lcn972 in *L. lactis* could interfere with the activity of other cell wall inhibitors, we checked the susceptibility profile of *L. lactis* D1 and D1-20 and the parent MG1614 to lysozyme, which hydrolyzes the PG sugar chains; bacitracin, which inhibits recycling of the PG lipid carrier; penicillin G, which inhibits transpeptidation during the last stage of PG synthesis; and nisin, a pore-forming bacteriocin that also prevents cell wall biosynthesis by binding to lipid II. Both Lcn972^r strains were more resistant to lysozyme and nisin and more susceptible to bacitracin and penicillin G than *L. lactis* MG1614 (Fig. 2). It is worth noting that nisin resistance in *L. lactis* D1 was higher than in D1-20, somewhat correlating with their resistance to Lcn972 (Table 2).

Resistance to bacteriophages. Although rarely addressed, resistance to phage infection has been observed in nisin-resistant *Staphylococcus aureus* (24) and in a sakacin P-resistant *Listeria monocytogenes* (36). Considering the negative impact of bacteriophages in industrial dairy fermentations, the Lcn972^r strains were challenged with phages c2 and sk1, two lytic phages belonging to the c2 and 936 families, respectively, and commonly found in the dairy environment. As judged by the efficiency of plaquing (EOP) referred to *L. lactis* MG1614 as the control, *L. lactis* D1 and D1-20 were fully resistant to phage c2 (Table 3). Phage sk1 was still able to

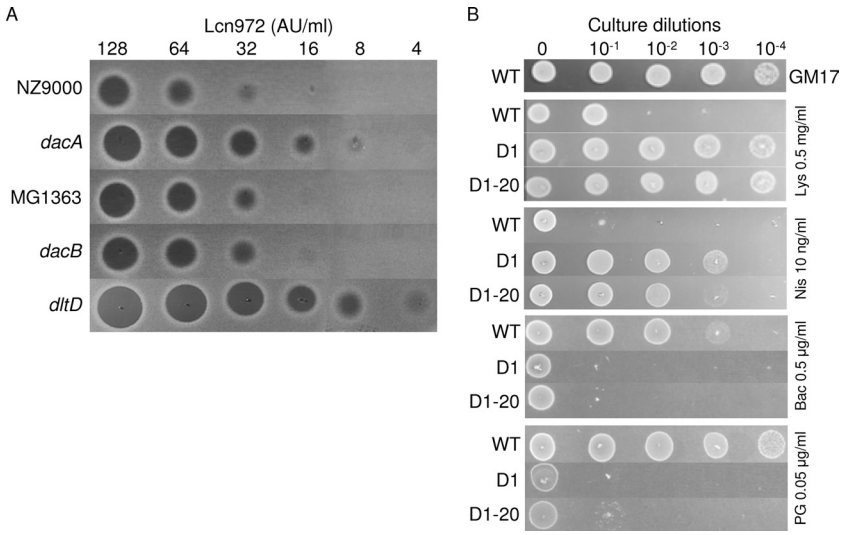

FIG 2 Susceptibility of *L. lactis* strains to cell wall-active antimicrobials. (A) Susceptibility of different *L. lactis* cell envelope mutants to the bacteriocin Lcn972. Twofold dilutions of Lcn972 in 50 mM sodium phosphate buffer, pH 6.8, were spotted (5 μl) on *L. lactis* lawns. (B) Susceptibility of *L. lactis* MG1614 (WT) and the Lcn972-resistant strains *L. lactis* D1 and D1-20 to lysozyme (Lys), nisin (Nis), bacitracin (Bac), and penicillin G (PG). Tenfold dilutions of overnight cultures (5 μl) were spotted on GM17 plates containing the indicated concentrations of the antimicrobials.

TABLE 3 Phage activity on *L. lactis* MG1614 and the Lcn972-resistant derivatives *L. lactis* D1 and *L. lactis* D1-20

L. lactis strain	Phage susceptibility			
	EOP[a]		% adsorption	
	c2	sk1	c2	sk1
MG1614	1.00 ± 0.00	1.00 ± 0.00	92.6 ± 4.8	88.9 ± 4.5
D1	0	0.23 ± 0.06	43.6 ± 8.0	93.7 ± 4.5
D1-20	0	0.60 ± 0.06	ND[b]	94.9 ± 0.7

[a] EOP, efficiency of plaquing.
[b] ND, not determined.

infect both Lcn972^r mutants, although resistance to sk1infection was more pronounced in *L. lactis* D1, somewhat correlating with the Lcn972-resistant phenotype. The distinct phage-resistant phenotype was further confirmed by the low adsorption of phage c2 to *L. lactis* D1, whereas in the case of sk1, adsorption to both Lcn972^r mutants was not affected (Table 3).

Impaired growth on maltose and lack of c2 infection is based on a chromosomal deletion detected in *L. lactis* D1 and D1-20 resistant to Lcn972. Prompted by the extreme resistance to phage c2 displayed by the *L. lactis* Lcn972^r strains, we investigated further the molecular basis underlying this phenomenon. In the case of phage c2, the membrane protein Pip (phage infection protein) is required for phage adsorption and DNA injection (14). Thereby, we aimed at identifying mutations in *pip* which might have occurred upon adaptation to Lcn972. However, attempts to amplify *pip* in *L. lactis* D1 and D1-20 by PCR failed, until a forward primer, 0734F, located 20.6 kbp upstream of *pip* was used in combination with the reverse primer 0752R internal to *pip*. With these two primers, a 2.0-kbp PCR product was obtained on both *L. lactis* D1 and D1-20 DNAs. Sequencing of this PCR product revealed a large

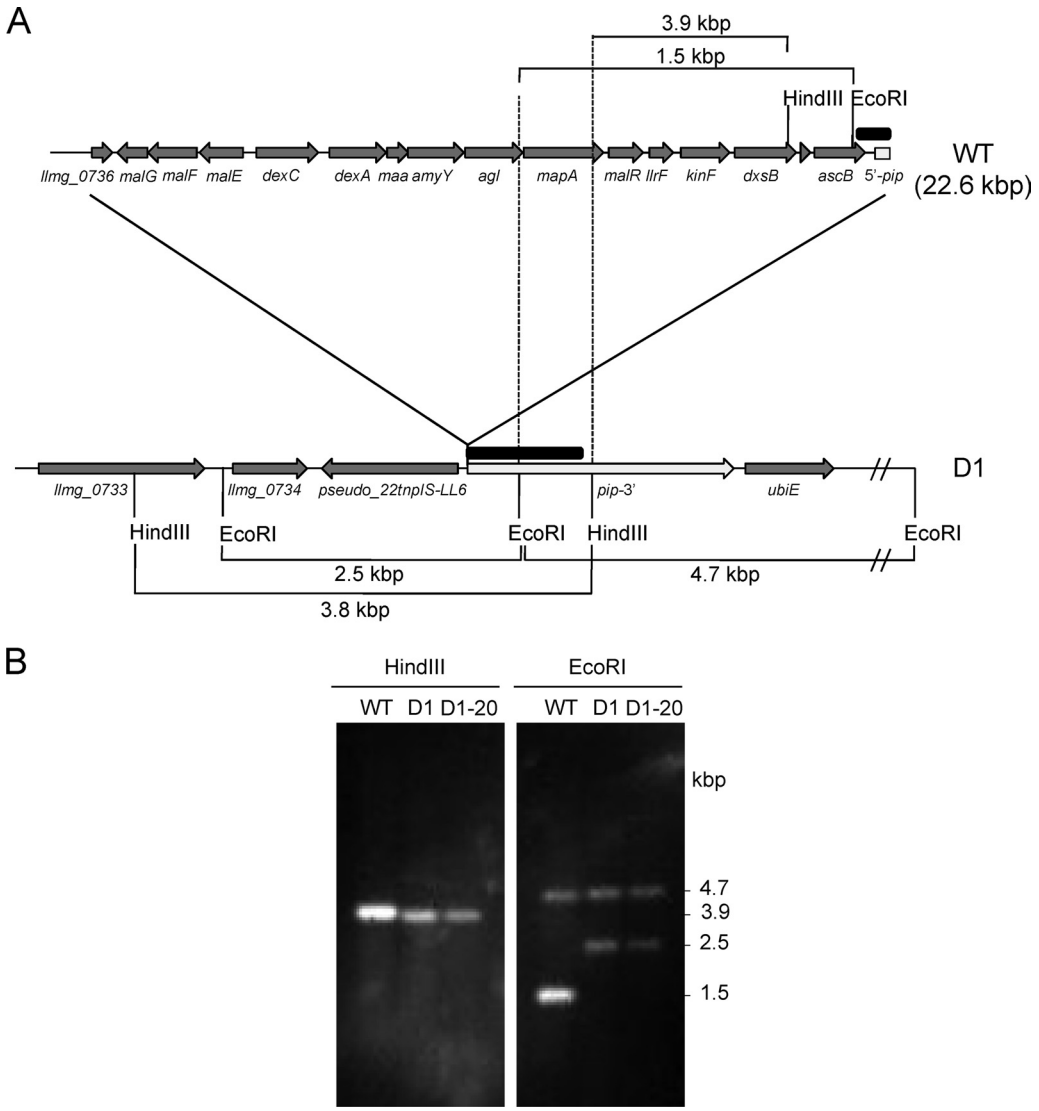

FIG 3 Overview of the 22.6-kbp deletion found in *L. lactis* D1 and D1-20 resistant to Lcn972. (A) Schematic drawing of *L. lactis* D1 deleted genes compared to *L. lactis* MG1614 (WT). Gene annotation according to *L. lactis* MG1363 (GenBank accession no. AM406671). Only relevant EcoRI and HindIII sites are shown. Note that genes are not at scale. (B) Confirmation of *pip* truncation (white arrow in panel A) by Southern blot analysis using a PCR probe (black rectangle in panel A) and *L. lactis* DNA digested with HindIII and EcoRI.

22.6-kbp deletion expanding from *llmg0736* to *pip*, which was further confirmed by Southern hybridization (Fig. 3). This deletion encompassed genes involved in maltose metabolism, the two-component system (TCS) F, and the 5′ end of *pip* and explained both the impaired growth on maltose and the phage-resistant phenotype based on the absence of a functional Pip protein.

Contribution of deleted genes to Lcn972 antimicrobial activity. Experiments were carried out to confirm whether or not the genes included in the deleted region were directly involved in Lcn972 resistance. When *L. lactis* MG1614 was growing on maltose, i.e., inducing maltose metabolic genes, the Lcn972 MIC was 20 AU/ml, differing in only one dilution step from the MIC in glucose (10 AU/ml). Thus, activating maltose metabolism seems not to increase susceptibility to Lcn972, and a correlation to Lcn972 activity could not be established. On the other hand, the MIC of Lcn972 for *L. lactis* MGRrF, which lacks the response regulator LlrF of TCS F, was also two times more resistant than the wild-type *L. lactis* strain (20 AU/ml), pointing to a marginal role of this TCS in Lcn972 resistance.

DISCUSSION

The potent antimicrobial activity of bacteriocins has supported research for their application as food biopreservatives and as lead molecules for the design of new antibiotics. As development of resistance may impair their efficacy, subsequent studies have been addressed to understand the molecular basis of bacteriocin resistance and its impact in the physiology of otherwise susceptible bacteria. Mutants with different degrees of resistance toward bacteriocins may be easily selected under laboratory conditions after exposure to bacteriocins for several generations (3, 17, 19, 38). Mechanisms behind bacteriocin resistance mostly involve changes at the cell envelope that preclude the bacteriocins from reaching their target, mainly the plasma membrane. In this work, we have been able to isolate Lcn972^r *L. lactis* mutants with reduced susceptibility to this cell wall-active bacteriocin. As described for nisin (19), the acquired Lcn972 high-resistant phenotype was lost quickly in the absence of selective pressure. Lcn972 is known to activate the two-component system CesSR (26), whose regulon comprises other regulatory protein genes. Thereby, an adaptive response which may include CesR-mediated gene activation seemed to be involved in the Lcn972 high-resistance phenotype. However, stable mutations have also occurred during selection, because a stable resistant phenotype (8× MIC) could be maintained in the absence of Lcn972.

Resistance to Lcn972 did not strongly alter the overall surface properties of the cells. A common mechanism described for many cationic antimicrobial peptides, including bacteriocins, consists of D-alanylation of LTA (15, 20, 32) to decrease the negative charge and reduce the electrostastic interactions. However, in the case of Lcn972, it does not play a major role, as no differences were observed both in the D-Ala content and the cell net charge. On the contrary, a remarkable change in the PG structure was observed. We could establish a direct correlation between the length of the peptide side chain and the susceptibility to Lcn972. Lcn972-resistant mutants had a reduced content of pentapeptide muropeptides. Moreover, *L. lactis* strains lacking the carboxypeptidase DacA and, consequently, with a high content of peptapeptide muropeptides were more susceptible to Lcn972. Changes at the PG structure level have not been approached when studying bacteriocin resistance mutants. Thus, it is not possible to anticipate if this is a common mechanism of resistance among bacteriocins. Nevertheless, it is worth noting that enhanced susceptibility toward beta-lactam antibiotics and bacitracin, both targeting PG biosynthesis, has been previously linked to resistance to lipid II-binding bacteriocins (8, 19). Altered antimicrobial susceptibility has been explained mostly by the role of cell envelope enzymes (e.g., *dlt* operon and penicillin binding protein genes) in the response to cell envelope stress, specialized ABC exporters, and specific and global regulators (7, 8, 19, 39). In the case of Lcn972, cross-protection to nisin and lysozyme might occur through the activation of CesSR or up-mutations in any of the CesR-regulated genes, but the alteration of the PG could also be important.

Another interesting result was the altered phage susceptibility profile of the Lcn972-resistant mutants. Lack of infectivity of phage c2 was undoubtedly linked to the truncation of *pip* by the chromosomal deletion detected in the Lcn972-resistant strains. In fact, mutated versions of this protein have been previously correlated with resistance to c2 (28). On the other hand, the lower infectivity of sk1 remained unexplained. Resistance to sk1 has been correlated with the absence of a polysaccharide pellicle in *L. lactis* MG1363 (6). However, the Lcn972-resistant strains have a gross carbohydrate composition similar to that of the wild type, and the pellicle was visible in the micrographs. Therefore, there must be some other as-yet-unidentified factors that compromise phage infectivity. Tentatively, the altered tripeptide/pentapeptide ratio in the PG of the Lcn972^r strains could make it less susceptible to the phage endolysin, which is needed to release the phage progeny. Interestingly, sk1 plaques on the Lcn972^r strains were consistently smaller (data not shown).

The deletion found in the Lcn972-resistant strains was rather large and encompassed, besides *pip*, maltose metabolic genes and TCS F. Compelling data have been gathered connecting carbohydrate metabolism with resistance, namely, to class IIa bacteriocins that target the mannose phosphotransferase system-PTS^{man} (18, 31, 36). Impaired growth on cellobiose has also been correlated with tolerance to Lcn972 in producing strains (5). However, our results indicated that maltose metabolism does not play a major role, as cells are similarly susceptible, even slightly more resistant, when growing on maltose. On the other hand, TCS F has been reported to be relevant under oxidative stress (30). According to our results, only a minor role in Lcn972 resistance could be recognized. Therefore, there must be some other mutations that account for the Lcn972-resistant phenotype. In fact, the histidine kinase KinF has been reported to be essential (30), and it is likely that countermutations have been selected. Transcriptomic analyses are in progress in order to clarify the molecular basis underlying Lcn972 resistance.

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