Isolation of *Lactococcus lactis* mutants simultaneously resistant to the cell wall-active bacteriocin Lcn972, lysozyme, nisin and bacteriophage c2.

Clara Rocesa, Pascal Courtin\(^b,c\), Saulius Kulakauskas\(^b,c\), Ana Rodríguez\(^a\), Marie-Pierre Chapot-Chartier\(^b,c\) and Beatriz Martínez\(^a\)#.

\(^a\)DairySafe group. Department of Technology and Biotechnology of Dairy Products. Instituto de Productos Lácteos de Asturias (IPLA-CSIC). Apdo. 85. 33300 Villaviciosa, Asturias, Spain.

\(^b\)INRA, UMR1319 Micalis, F-78350 Jouy-en-Josas, France.

\(^c\)AgroParisTech, UMR Micalis, F-78350 Jouy-en-Josas, France.

# Corresponding author: Beatriz Martínez

IPLA-CSIC, Apdo. 85. 33300-Villaviciosa, Asturias, Spain.

e-mail: bmf1@ipla.csic.es

Phone: +34 985 89 33 59

Fax: +34 985 89 22 33

Running title: *L. lactis* resistant to Lcn972
**ABSTRACT**

Lactococcin 972 (Lcn972) is a non-lantibiotic 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 have been isolated with a high (>320 arbitrary units (AU)/ml) and low (80 AU/ml) susceptibility, respectively. 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) were 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 by phage sk1. Lack of c2 infectivity was linked to a 22.6 kbp chromosomal deletion encompassing the phage receptor protein *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.

**Keywords** Dairy starter, bacteriocin, resistance, peptidoglycan, surface properties, phage
INTRODUCTION

*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 hydrolysing the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine. Moreover, an increasing number of bacteriocins, antimicrobial peptides synthesized by bacteria, have been reported to inhibit cell wall biosynthesis by binding to the cell wall precursor lipid II, including the lactococcal lantibiotics nisin and lacticin 3147 and the non-lantibiotic lactococcin 972 (Lcn972) (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 mostly composed of peptidoglycan (PG) made of glycan strands crosslinked 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 *L. lactis* cell wall (1, 41).

The cell wall in Gram positive bacteria protect cells from osmotic pressure and acts as an exoskeleton maintaining cell shape and as scaffold for anchoring other cell envelope components (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 (Lcn972R) 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-aminoacid 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.

MATERIAL AND METHODS

Bacterial strains, bacteriophages, and growth conditions. L. lactis strains (Table 1) were routinely grown in M17 with glucose 0.5% (GM17), statically and at 30 °C. When specified, glucose was replaced by maltose 0.5% (MM17) or Chemically Defined Medium (CDM) (29) was used. E. coli strains were grown in 2xYT (35) at 37 °C with shaking. When needed, antibiotics erythromycin and ampicillin were used at a final concentration 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, OD₆₀₀) 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 NaCl 0.9% of phage lysates were mixed with 3.5 ml of molten GM17 0.7% agar supplemented with 10 mM CaCl₂ 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 glycerol 10% (v/v). Phage lysates were stored at 4 °C.

**Standard DNA techniques.** Standard molecular techniques were followed as described elsewhere (35). Chromosomal DNA was isolated with 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 PCR reactions were carried out using PuRe Taq Ready-to-go PCR Beads (GE Healthcare, UK). For cloning purposes and PCRs expected to yield long products, the proof-reading 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 (26). Lcn972 dilutions were done in sodium phosphate buffer 50 mM, pH 6.8. To isolate Lcn972 resistant mutants, *L. lactis* MG1614 was cultivated step-wise 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 *L. lactis* ΔdacA.** The flanking 600 bp regions up- and downstream of *dacA* (*llmg2560* in *L. lactis* MG1363; GenBank AM406671) were amplified and fused by SOE-PCR (Splicing by Overlap Extension-PCR) using the primers D1 (5’ AACTGCAGTATTGACAAATGCCG 3’), D2 (5’ AAAACTTTTGGAGCAGTACGCAAGCTCG 3’), D3 (5’
AGTGCTCCAAAAGTTTTTTGG 3’), and D4 (5’ GAAGATCTGCTAAACGTGACCC 3’). The SOE-PCR fragment was cloned into the non-replicating 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 \textit{L. lactis} NZ9000 and selection of deletion mutants proceeded according to Leenhouts et al. (22).

**Mapping chromosomal deletion in \textit{L. lactis} D1 and D1-20.** A forward primer 0734F (5’ GAAATGGCCCTGCGACGTGTAG 3’), located within the \textit{llmg0734} locus, was used in combination with the reverse 0752R (5’ CGCTGCATCCAATGTCACAGTC 3’) in \textit{pip} for PCR amplification using \textit{L. lactis} DNA. PCR conditions were: 98 °C 30 sec; 35 cycles 98 °C 10 sec, 63 °C 30 sec, 72 °C 13 min; 72 °C 10 min and Phusion High-Fidelity DNA Polymerase was used. PCR products were purified with Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) 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, UK) (35). The DNA probe was labelled by PCR on \textit{L. lactis} MG1614 DNA using primers pipF (5’ CGGATTCATCTATGTTGACC 3’) and pipR2 (5’ AATTGCTTCTCTTTGTCGG 3’), expanding the 5’ end of \textit{pip} (see Fig 3) and labelled dNTPs from PCR Dig Labelling Mix (Roche, Spain). PCR conditions were: 98 ºC 30 sec; 35 cycles 98 ºC 10 sec, 45 ºC 30 sec, 72 ºC 2 min; 72 ºC, 10 min, using Phusion High-Fidelity DNA Polymerase. The blots were revealed by immunodetection at 20 ºC using CDP Star (Roche, Spain) following manufacturer’s recommendations.

**Surface properties.** The electrophoretic mobility was measured using stationary phase cells concentrated to $10^7$ cfu/ml in NaCl 5 mM as previously described (15). Hydrophobicity was determined following the microbial-adhesion-to-solvents method (MATS) using
hexadecane and stationary phase cells in NaCl 0.15 M adjusted to a final OD₆₀₀ₙₐ₅ 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 OD₆₀₀ of 2.0 with 200 µl of Lcn972 20 µg/ml in sodium phosphate buffer 50 mM, pH 6.8. Residual inhibitory activity was measured
by the agar diffusion test using L. lactis MG1614 as indicator (26).

**Cell wall composition.** Peptidoglycan (PG) preparations were obtained from 500 ml of
exponentially growing (OD₆₀₀ 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 4 °C to eliminate anionic polymers linked to PG. Two mg of purified PG were
digested with mutanolysin (Sigma, 2500 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) =
\[
\frac{\sum \text{monomers}(X) + \frac{1}{2} \sum \text{dimers}(X) + \frac{1}{3} \sum \text{trimers}(X) + \frac{1}{4} \sum \text{tetramers}(X)}{\sum \text{all muropeptides}}
\]
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 hours and derivatized 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 1:1000 (v/v) in melted GM17 containing 1.2 % agar. Drops (5 µl) of twofold dilutions of Lcn972 were spotted on the plates and incubated at 30 ºC. To determine cross-resistance to cell wall antimicrobials, serial dilutions in GM17 broth of *L. lactis* D1, D1-20 and the wild-type MG1614 overnight cultures were spotted on GM17 plates containing lysozyme 0.5 mg/ml, nisin 10 ng/ml, bacitracin 0.5 µg/ml or penicillin G 0.05 µg/ml. Lcn972 minimal inhibitory concentration (MIC) were carried out in microtiter plates using either GM17 or MM17 essentially as described (5).

Phage assays. The efficiency of plaquing (EOP) was defined as the phage titre on the tested strain divided by that on the reference *L. lactis* MG1614. Each c2 and sk1 phage suspensions were 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 OD_{600nm} of 0.5 with c2 or sk1 phage suspensions to match a ratio phage/bacteria (MOI) of 2x10^{-6} for c2 and 1x10^{-3} for sk1. After 10 min incubation at 30 ºC, samples were centrifuged and the phage titer of the supernatant was determined by the standard plaque assay using *L. lactis* MG1614 as host. The percentage of adsorption was determined as follows: 1-(phage titer of the supernatant/phage titer of a control tube without cells) x 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. Step-wise 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 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 ºC (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 the susceptible *L. lactis* MG1614 (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 \((P < 0.001)\) compared to *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 ultra-structural 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
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 Lcn972R mutants D1 and D1-20 showed a higher content of muropeptides with tripeptide side chains and a reduced content of pentapeptide side chains compared to the wild-type (Fig. 1). This observation suggested an alteration in the Lcn972R mutants of activities involved in PG maturation.

**Susceptibility to Lcn972 of cell envelope L. lactis mutants.** To test the putative role of the PG structure on the Lcn972 resistant phenotype, a cell envelope mutant L. lactis dacA 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-labelled 45 kDa protein band in membrane protein extracts analysed 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.

L. lactis dacA and other available L. lactis defective in cell wall modification enzymes (Table 1) were screened for their susceptibility to Lcn972. L. lactis dltD lacking DltD, a membrane protein involved in LTA D-alanylation and L. lactis dacB lacking the D,L-carboxypeptidase DacB, that 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, *L. lactis* dltD
devoid of D-Ala showed a four-fold increased susceptibility to Lcn972. Higher susceptibility
was also observed in the case of *L. lactis* dacA whereas *L. lactis* dacB 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, D1-20 and the parent MG1614 to lysozyme, that
hydrolyses the PG sugar chains, bacitracin, that inhibits recycling of the PG lipid carrier,
penicillin G that 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<sup>R</sup> 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 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<sup>R</sup> 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
control, *L. lactis* D1 and D1-20 were fully resistant to phage c2 (Table 3). Phage sk1 was still able to infect both Lcn972R mutants, although resistance to sk1 infection 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 Lcn972R 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* Lcn972R 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 pip was used in combination with the reverse 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 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
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 (Table 4). 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* (Table 4), 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 towards bacteriocins may be easily selected under laboratory conditions after exposure to bacteriocins for several generations (3, 17, 19, 38). Mechanisms behind mostly involve changes at the cell envelope that precludes the bacteriocins to reach their target, mainly the plasma membrane. In this work, we have been able to isolate Lcn972<sup>R</sup> *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 gen activation seemed to be involved in the Lcn972 high resistance phenotype. However, stable mutations have also occurred during selection because a stable resistant phenotype (8x 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 electrostatic 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 charges. On the contrary, a remarkable change into 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* lacking the carboxypeptidase DacA and, consequently, with a high content of peptapeptide muropeptides, were more susceptible to Lcn972. Changes at the PG structure level has 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 towards 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 mostly been explained 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 similar gross carbohydrate composition than that of the wild type and also 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\textsuperscript{R} strains could make it less susceptible to the phage endolysin, needed to release the phage progeny. Interestingly, sk1 plaques on the Lcn972\textsuperscript{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 the two component system TCS F. Compelling data has been gathered connecting carbohydrate metabolism with resistance, namely to class IIa bacteriocins that target the mannose phosphotransferase system-PTS\textsuperscript{man} (18, 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 histidin kinase KinF has been reported to be essential (30) and it is likely that counter-mutations have been selected. Transcriptomic analyses are in progress in order to clarify the molecular basis underlying Lcn972 resistance.

**ACKNOWLEDGMENTS**

The work has been funded by grants BIO2007-65061 and BIO2010-17414 of the Ministerio de Ciencia e Innovación (Spain). C.R. is a recipient of a predoctoral JAE-CSIC fellowship. Work of M.P.C.C. and S.K. was supported an INRA Jeune Equipe grant. We thank Christine...
Longin and Sophie Chat for electron microscopy observations with the equipments available on the MIMA2 platform (INRA, Jouy-en-Josas, France) and Romain Briandet (INRA, Micalis, Jouy-en-Josas, France) for his help with electrophoretic mobility experiments. We are also grateful to Verónica Pérez (IPLA-CSIC, Spain) for her technical assistance in performing growth curves, and to Elena Bidnenko (INRA, France) and Mary O’Connell-Motherway (UCC, Ireland) for supplying *L. lactis* phages and *L. lactis* MGRrF, respectively.
REFERENCES


FIGURE LEGENDS

FIGURE 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 (grey bars) and *L. lactis dacA* (black bars). Values are the means of two independent peptidoglycan extractions, except for *dacA* mutant. Error bars indicate standard deviations.

FIGURE 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 sodium phosphate buffer 50 mM, 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).

Ten-fold dilutions of overnight cultures (5 µl) were spotted on GM17 plates containing the indicated concentrations of the antimicrobials.

FIGURE 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 AM406671). Only relevant EcoRI and HindIII sites are shown. Note that genes are not at scale. B. Confirmation of *pip* truncation (white arrow in A) by Southern blot using a PCR probe (black rectangle in A) and *L. lactis* DNA digested with HindIII and EcoRI.
<table>
<thead>
<tr>
<th>Strain, phage, or plasmid</th>
<th>Descriptiona</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. lactis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free and prophage-cured derivative of <em>L. lactis</em> NCDO712</td>
<td>13</td>
</tr>
<tr>
<td>MG1614</td>
<td>Str^R Rif^R derivative of MG1363, Lcn972^S</td>
<td>13</td>
</tr>
<tr>
<td>NZ9000</td>
<td>MG1363, carrying pepN::nisRK</td>
<td>21</td>
</tr>
<tr>
<td>D1</td>
<td>MG1614, Lcn972 high resistant mutant, unstable</td>
<td>This work</td>
</tr>
<tr>
<td>D1-20</td>
<td>MG1614, Lcn972 low resistant mutant derived from D1, stable</td>
<td>This work</td>
</tr>
<tr>
<td>dacA</td>
<td>NZ9000 lacking dacA gene</td>
<td>This work</td>
</tr>
<tr>
<td>dacB</td>
<td>MG1363 lacking dacB gene</td>
<td>9</td>
</tr>
<tr>
<td>dltD</td>
<td>MG1363 lacking dltD gene</td>
<td>10</td>
</tr>
<tr>
<td>MGRrF</td>
<td>MG1363 pRV300::llrF</td>
<td>30</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>Plasmid free, cloning host</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><strong>Phages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c2</td>
<td><em>L. lactis</em> lytic phage belonging to c2 family</td>
<td>E. Bidnenko</td>
</tr>
<tr>
<td>sk1</td>
<td><em>L. lactis</em> lytic phage belonging to 936 family</td>
<td>E. Bidnenko</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pORI280</td>
<td>Em^R, lacZ^+, integrative plasmid</td>
<td>22</td>
</tr>
<tr>
<td>pBL16</td>
<td>600 bp dacA flanking regions cloned in pORI208</td>
<td>This work</td>
</tr>
</tbody>
</table>

---

a Str, streptomycin; Rif, rifampin; Em, erythromycin.
TABLE 2 Properties of *L. lactis* MG1614 and its derivatives resistant to Lcn972 *L. lactis* D1 and *L. lactis* D1-20.

<table>
<thead>
<tr>
<th><em>L. lactis</em></th>
<th>MIC Lcn972 (AU/ml)</th>
<th>$\mu$ (h$^{-1}$)$^a$</th>
<th>Lcn972 adsorption (µ)</th>
<th>Adhesion to hexadecane$^b$ (%)</th>
<th>D-Ala (ng/mg dried cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1614</td>
<td>10</td>
<td>1.07 0.98 0.38 0.39</td>
<td>25.6 ± 3.1</td>
<td>75.75 ± 4.55</td>
<td>17.7 ± 2.7</td>
</tr>
<tr>
<td>D1</td>
<td>&gt;320</td>
<td>0.96 0.67 0.2 –</td>
<td>13.9 ± 0.8</td>
<td>62.90 ± 1.87</td>
<td>14.1 ± 0.6</td>
</tr>
<tr>
<td>D1-20</td>
<td>80</td>
<td>0.97 0.68 0.37 –</td>
<td>14.4 ± 1.6</td>
<td>63.60 ± 1.74</td>
<td>14.3 ± 1.9</td>
</tr>
</tbody>
</table>

$^a$ 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 %.

$^b$ Determined by the MATS method.
**TABLE 3** Phage activity on *L. lactis* MG1614 and the Lcn972 resistant derivatives *L. lactis* D1 and *L. lactis* D1-20.

<table>
<thead>
<tr>
<th>L. lactis</th>
<th>EOP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c2</td>
<td>sk1</td>
</tr>
<tr>
<td>MG1614</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>D1</td>
<td>0.23 ± 0.06</td>
<td>0.23 ± 0.06</td>
</tr>
<tr>
<td>D1-20</td>
<td>0.60 ± 0.06</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> EOP, efficiency of plaquing.

<sup>b</sup> ND, not determined.
TABLE 4 Lcn972 minimum inhibitory concentration (MIC) for *L. lactis* strains

<table>
<thead>
<tr>
<th><em>L. lactis</em></th>
<th>MIC (AU/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Maltose</td>
<td></td>
</tr>
<tr>
<td>MG1614</td>
<td>10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>MGRrF</td>
<td>20</td>
<td>ND²</td>
<td></td>
</tr>
</tbody>
</table>

² ND, not determined