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The Putative Lactococcal Extracytoplasmic Function Anti-Sigma Factor Llmg2447 Determines Resistance to the Cell Wall-Active Bacteriocin Lcn972

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Lactococcin 972 (Lcn972) is a cell wall-active bacteriocin that inhibits cell wall biosynthesis in *Lactococcus lactis*. In this work, the transcriptomes of the Lcn972-resistant (Lcn^r) mutant *L. lactis* D1 and its parent strain were compared to identify factors involved in Lcn972 resistance. Upregulated genes included members of the cell envelope stress (CesSR) regulon, the penicillin-binding protein *pbpX* gene and gene *llmg2447*, which may encode a putative extracytoplasmic function (ECF) anti-sigma factor. The gene *llmg2447* is located downstream of the nonfunctional ECF gene *sigX*$_{pseudo}$. Nisin-controlled expression of *llmg2447* led to high Lcn972 resistance in *L. lactis*, with no cross-resistance to other cell wall-active antimicrobials. Upregulation of *llmg2447* in *L. lactis* D1 (Lcn^r) was linked to the integration of insertion element IS*981* into the *llmg2447* promoter region, replacing the native −35 box and activating the otherwise silent promoter P$_{2447}$. This is the first example of an orphan ECF anti-sigma factor involved in bacteriocin resistance. This new role in neutralizing cell wall-active compounds (e.g., Lcn972) could have evolved from a putative primary function of Llmg2447 in sensing cell envelope stress.

Integrity of the bacterial cell envelope is crucial for the survival of most bacteria. In Gram-positive bacteria, the main component of the cell wall is the peptidoglycan, a polymer made of alternate β-1→4 *N*-acetylglucosamine and *N*-acetylmuramic acid chains covalently cross-linked by short peptides (40). This three-dimensional mesh-like structure surrounds the cytoplasmic membrane and is needed to maintain cell shape and to counteract the inner osmotic pressure. The biosynthesis of peptidoglycan proceeds stepwise at the inner side of the cytoplasmic membrane through the lipid intermediates lipid I and lipid II. The peptidoglycan unit in lipid II is translocated across the membrane, polymerized by transglycosylation, and subsequently cross-linked by transpeptidation to pre-existing peptidoglycan (reviewed in references 1 and 4). The cell wall biosynthesis pathway has been, and still is, a validated target for many antibiotics (36).

Antimicrobial peptides produced by bacteria, the so-called bacteriocins, have been studied largely for their potent antibacterial activity. Their production by bacteria "generally recognized as safe," such as lactic acid bacteria involved in food fermentations, has been the rationale for their use to inhibit undesirable bacteria in food, nisin being the first bacteriocin authorized as a food preservative (11, 15). Under the pressure of the urgent need for new antimicrobials, bacteriocins have been proposed as new anti-infectives, as several of them are also active against multiresistant pathogens (17, 29).

Nisin and other structurally related lantibiotics (lanthionine-containing bacteriocins) have been shown to inhibit cell wall biosynthesis by using the cell wall intermediate lipid II as a docking molecule for effective pore formation (5, 6). However, recognition of lipid II is not restricted solely to pore-forming lantibiotics. We have recently described lactococcin 972 (Lcn972) as the first nonlantibiotic nonpostranslationally modified bacteriocin that binds to lipid II (26). Lcn972 is a plasmid-encoded 7.5-kDa hydrophilic bacteriocin secreted by *Lactococcus lactis* IPLA972; it is highly bactericidal to lactococci and active in the nanomolar range (26, 27). The lack of structural homology to any other lipid II-binding molecule suggests that Lcn972 carries a novel lipid II-binding motif and could lead the way to the improvement of existing antibiotics. It has also been demonstrated that Lcn972 triggers a cell envelope stress response through the two-component system CesSR in *L. lactis*, as do other lipid II-binding molecules, such as bacitracin and vancomycin (28). Besides CesSR, no other cell envelope stress-sensing devices have been characterized so far in lactococci. This is in contrast to other Gram-positive bacteria, such as *Bacillus subtilis*, in which several two-component systems and alternative sigma factors have been implicated in cell envelope stress-sensing and regulatory mechanisms (21).

We have recently characterized *L. lactis* mutants with reduced susceptibility to Lcn972 to better understand the mode of action of this bacteriocin. *L. lactis* strain D1 was firstly isolated upon adaptation to increasing Lcn972 concentrations, and a derivative of it, *L. lactis* D1-20, was selected after *L. lactis* D1 was grown for 200 generations in the absence of Lcn972 (34). Remarkably, both mutants had an altered peptidoglycan composition with a high content of muropeptides with tripeptide side chains. Moreover, a large chromosomal deletion was identified, although a clear correlation of this mutation with the loss of susceptibility to Lcn972 could not be established and the genetic basis of the resistance remained elusive (34). In this work, genome-wide transcriptomics was carried out in order to identify the mechanism of *L. lactis* resistance to Lcn972. From the analysis, a particular gene

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TABLE 1 Bacterial strains and plasmids used in this work

Strain or plasmid	Description[a]	Reference
L. lactis		
MG1363	Plasmid-free derivative of *L. lactis* NCDO712	16
MG1614	Str^r Rif^r derivative of *L. lactis* MG1363, $Lcn972^s$	16
D1	MG1614, highly Lcn972-resistant mutant	34
D1-20	MG1614, low Lcn972 resistance mutant	34
NZ9000	MG1363, *pepN*::*nisRK*; host for nisin-inducible gene expression	12
IL1403	Plasmid free, complete ECF *sigX* gene	2
E. coli DH10B	Plasmid free, cloning host	Invitrogen
Plasmids		
pCR2.1	Cloning of PCR products, Ap^r	Invitrogen
pNZ8020	Nisin-inducible promoter P_{nisA}, Cm^r	12
pPTPL	*lacZ* reporter plasmid, Tet^r	8
pBL51	pNZ8020::*llmg2447*	This work
pBL51Cla	pBL51ΔClaI site, frameshift in *llmg2447*	This work
pBL56	pCR2.1::wild-type *llmg2447* promoter (P_{2447})	This work
pBL57	pCR2.1::hybrid IS*981*-*llmg2447* promoter ($P_{IS981::2447}$)	This work
pBL58	pPTPL::wild-type *llmg2447* promoter (P_{2447})	This work
pBL59	pPTPL::hybrid IS*981*-*llmg2447* promoter ($P_{IS981::2447}$)	This work

[a] Str, streptomycin; Rif, rifampin; Em, erythromycin; Ap, ampicillin; Tet, tetracycline; superscript r, resistant; superscript s, susceptible.

(*llmg2447*) was identified as a key factor and the molecular basis of its transcriptional activation was further investigated. On the basis of the genetic context of this gene, the role of *llmg2447* as an ancient extracytoplasmic function (ECF) anti-sigma factor is discussed.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. The bacterial strains and plasmids used in this work are listed in Table 1. Lactococcal strains were routinely grown as standing cultures in M17 (Oxoid, Basingstoke, United Kingdom) supplemented with glucose at 0.5% (GM17) at 30°C or in a chemically defined medium (CDM) (10). *Escherichia coli* DH10B was used as an intermediate cloning host and was grown in 2×YT medium (35) at 37°C with shaking. When needed, the antibiotic chloramphenicol or tetracycline was used at 5 μg/ml. Ampicillin was used at 100 μg/ml. Antibiotics were purchased from Sigma (St. Louis, MO).

Transcriptome analysis. Genome-wide transcriptional experiments were performed using DNA microarrays covering 2,308 of the 2,435 predicted open reading frames (ORFs) in the genome of *L. lactis* MG1363. DNA microarray experiments were carried out essentially by following the methods for cell disruption, RNA isolation, RNA quality control, cDNA synthesis, indirect labeling, hybridization, and scanning described previously (39). RNA was extracted from four biological replicates of exponentially growing *L. lactis* MG1614 and D1 cultures in GM17 at 30°C (optical density at 600 nm [OD_{600}] of 0.4 at harvesting). Data were processed as described previously (28).

Overexpression of *L. lactis llmg2447*. The gene *llmg2447* was amplified by PCR using 50 ng of genomic DNA of *L. lactis* MG1363, primers X4 and X5 (Table 2), and the high-fidelity *Pwo* polymerase (Roche Applied Science, Penzberg, Germany) in accordance with the manufacturer's recommendations and an annealing temperature of 50°C. The PCR product was purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Buckinghamshire, United Kingdom) restricted with PstI and EcoRI and subsequently cloned into pNZ8020 under the control of the nisin-inducible promoter P_{nisA}, yielding plasmid pBL51 in *L. lactis* NZ9000. A truncated version of *llmg2447* was created by digestion of pBL51 at the unique internal ClaI site, filling of the cohesive ends with the DNA polymerase I Klenow fragment (TaKaRa, Otsu, Japan), and blunt-end ligation to give plasmid pBL51Cla. For induction, purified nisin (kindly supplied by Aplin & Barrett Ltd., Dorset, England) was added to melted GM17 before plating at 0.05, 0.1, and 0.2 ng/ml, as indicated.

TABLE 2 Primers used in this work

Primer	5′–3′ sequence (underlined restriction site)	Position
X1	TTTTGTCTGAATTCCAAAACTCTT (EcoRI)	5′ *llmg2447* promoter
X2	CCGGATCCATTTCCTCCCCTCTC (BamHI)	3′ *llmg2447* promoter
X3	TGTTCGATACATAAAAGCCCG	3′ *llmg2447* promoter
X4	ATCTGCAGTGGCTCCTCTAATC (PstI)	5′ *llmg2447* gene
X5	GCAAAGAATTCAATGACCGCCT (EcoRI)	3′ *llmg2447* gene
X6	ACGAATTCATCAAAGTTTAGGGTATC (EcoRI)	5′ IS*981*::*2447* promoter

Antimicrobial susceptibility assays. Inhibitory activities were determined by the spot-on-the-lawn test (34). Briefly, melted GM17 agar (supplemented or not supplemented with nisin) was inoculated with overnight lactococcal cultures. Once solidified, 5-μl drops of 2-fold serial dilutions of Lcn972 purified as described previously (28) (3,200 to 0 arbitrary units [AU]/ml); nisin (200 to 0 μg/ml); and bacitracin, penicillin G, and vancomycin (64 to 0 μg/ml) were spotted onto the plates. The MIC was defined as the lowest concentration that gave a clear inhibition halo after overnight incubation.

Promoter cloning and *lacZ* fusions. The wild-type P_{2447} and the hybrid $P_{IS981::2447}$ promoter regions were amplified by PCR from genomic DNA (50 ng) of *L. lactis* MG1363 and *L. lactis* D1, respectively, with PuRe*Taq* Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, United Kingdom). The common reverse primer X2 was used in combination with X1 or X6 to amplify P_{2447} and $P_{IS981::2447}$, respectively (Table 2). The wild-type and hybrid promoter fragments were cloned into pCR2.1 (Invitrogen, Barcelona, Spain) in *E. coli* DH10B, resulting in plasmids pBL56 and pBL57, respectively, and confirmed by DNA sequencing. Both promoter fragments were excised with BamHI and EcoRI and subcloned into reporter plasmid pPTPL upstream of the promoterless *E. coli* *lacZ* gene. The ligation mixtures were used to transform *L. lactis* NZ9000, yielding reporter plasmids pBL58 (P_{2447}-*lacZ*) and pBL59 ($P_{IS981::2447}$-*lacZ*) (Table 1). These plasmids were also introduced into *L. lactis* IL1403.

Enzymatic activity assays. Lactococcal strains were initially screened for β-galactosidase activity on GM17 agar plates supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Apollo Scientific, Stockport, United Kingdom) at 80 μg/ml. Unless indicated otherwise, enzyme activity was quantified in *L. lactis* in the late exponential growth phase (OD_{600} of 2.0). Cells from 1 ml of

TABLE 3 Differential gene expression in Lcn972-resistant *L. lactis* D1 and its parent, *L. lactis* MG1614

Category, locus tag, and gene	Ratio[a]	Bayesian *P*[b]	Function[c]
Carbohydrate/amino acid transport and metabolism			
llmg0187, *celB*	−3.99	1.12E-04	Cellobiose-specific PTS system IIC component
llmg0437, *ptcB*	−2.57	1.06E-08	Cellobiose-specific PTS system IIB component
llmg0454	−3.38	9.67E-11	β-Glucoside-specific PTS system IIABC component
llmg0458	−5.60	1.47E-11	Putative glucosyltransferase
llmg0739, *malE*	−6.35	3.91E-13	Maltose ABC transporter substrate binding protein
llmg0740, *dexC*	−3.91	4.71E-12	Neopullulanase
llmg0741, *dexA*	−3.75	1.61E-04	Oligo-1,6-α-glucosidase
llmg0743, *amyY*	−2.55	2.84E-05	α-Amylase
llmg0745, *mapA*	−6.35	4.58E-09	Maltose phosphorylase
llmg0749, *dxsB*	−6.05	4.94E-14	1-Deoxy-D-xylulose-5-phosphate synthase
llmg0751, *ascB*	−12.72	5.21E-14	6-Phospho-β-glucosidase
llmg1048, *busAA*	2.68	4.54E-14	Glycine betaine/proline ABC transporter
llmg1049, *busAB*	3.00	1.07E-12	Glycine betaine-binding periplasmic protein
llmg1164	2.77	5.74E-04	Putative sugar ABC transporter permease
llmg1869, *apu*	8.42	3.33E-16	Amylopullulanase
llmg1871, *glgP*	6.91	2.22E-16	Glycogen phosphorylase
Signal transduction/regulatory mechanisms			
llmg0746, *malR*	−25.19	3.33E-16	Maltose operon transcriptional repressor
llmg0747, *llrF*	−10.33	5.66E-15	Two-component system regulator LlrF
llmg0748, *kinF*	−5.83	3.03E-12	Sensor protein kinase KinF
llmg2163	2.24	2.73E-11	Hypothetical protein, PspC domain
llmg2164	2.86	1.15E-12	Hypothetical protein
llmgpseudo76, *sigX*	13.15	2.89E-15	ECF sigma factor
llmg2447	37.56	1.47E-14	Putative anti-ECF sigma factor
Miscellaneous			
llmg0619	2.58	7.14E-13	Hypothetical protein
llmg0669	−5.35	8.07E-10	Hypothetical protein
llmg0752, *pip*	−3.08	1.20E-09	Phage infection protein
llmg1271, *gidC*	−2.66	4.94E-09	tRNA (uracil-5-)-methyltransferase Gid
llmg1679, *pbpX*	6.21	1.73E-14	Penicillin-binding protein
llmg2353, *rplQ*	−31.81	4.44E-16	Ribosomal protein L17
llmg2409, *polC*	2.86	5.23E-13	DNA polymerase III PolC

[a] Values for genes whose expression changes >2.5-fold are shown. Negative values indicate downregulation.
[b] Determined by Cyber-T test (24).
[c] According to GenBank accession number AM406671.1.

culture were washed and resuspended in 1 ml of Z buffer (100 mM sodium phosphate buffer [pH 6.8], 10 mM KCl, 1 mM $MgSO_4$) and permeabilized with 0.06 mg/ml hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature. The enzymatic reaction was conducted in microtiter plates using 170 μl of permeabilized cells (or dilutions thereof) in Z buffer and *o*-nitrophenyl-β-D-galactopyranoside (Sigma-Aldrich, St. Louis, MO) at a 0.5 mM final concentration in a total volume of 200 μl. The reaction mixture was incubated at 37°C, and absorbance at 420 nm (A_{420}) was measured for 20 min in a Benchmark Plus microplate spectrophotometer (Bio-Rad). Specific activity of permeabilized cells was calculated as ΔmA_{420}/min/OD_{600} unit. Assays were carried out at least in triplicate and with two independent cultures. When indicated, Lcn972 was added to the cultures at 10, 20, and 40 AU/ml and incubated for 30 min at 30°C prior to the enzymatic activity assay. Induction of the wild-type promoter P_{2447} in *L. lactis* IL1403 was screened in microtiter plates with cultures growing in CDM-glucose in the presence of subinhibitory concentrations of Lcn972, bacitracin, nisin, and vancomycin.

Bioinformatic tools. Nucleotide and protein BLAST (http://blast.ncbi.nlm.nih.gov/) searches were used to find homologous genes or proteins, respectively. Structural predictions and motif searches were performed with InterProScan (http://www.ebi.ac.uk/InterProScan/) and Pfam (http://pfam.sanger.ac.uk/). σ^{70} promoter sequences were identified using Bprom (Softberry, Inc., Mount Kisco, NY). Protein topology was predicted with TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/). The Microbial Signal Transduction database (Agile Genomics, LLC, Mount Pleasant, SC) was consulted to identify ECF sigma factors in *L. lactis*.

Microarray data accession numbers. The DNA microarray data are available at the Gene Expression Omnibus repository under accession number GSE38349.

RESULTS

Transcriptional analysis of Lcn972-resistant *L. lactis* D1. The genome-wide transcriptomic profile of *L. lactis* D1 was compared to that of its parent, *L. lactis* MG1614, to identify mutations involved in loss of susceptibility to the bacteriocin Lcn972 (Table 3). Several genes were differentially expressed. Downregulation of genes already identified as playing a role in tolerance to Lcn972 (*celB*, *ptcC*) was seen (9). Furthermore, a number of genes puta-

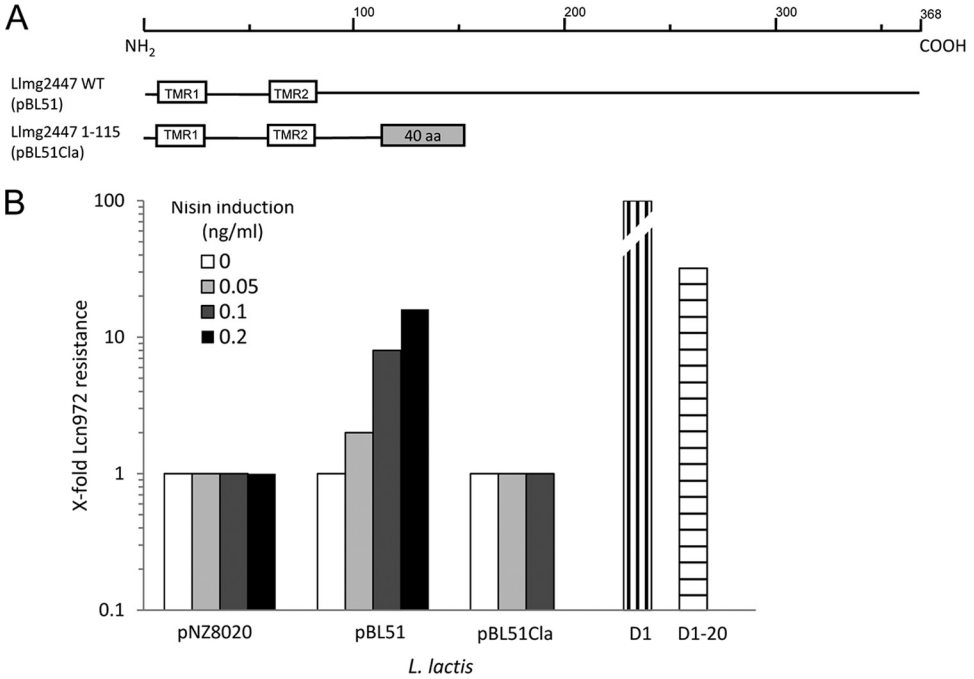

FIG 1 Overexpression of *llmg2447* reduces susceptibility to Lcn972. (A) Domain architecture of Llmg2447 (368 aa) and its truncated version Llmg2447_1-115, which are encoded by pBL51 and pBL51Cla, respectively. TMR, transmembrane region as predicted by TMHMM Server v2.0 and SOSUI (see the text). C-terminal residues that are encoded by vector sequences are shown in the shaded box. WT, wild type. (B) Fold increase in resistance to Lcn972, relative to that under noninducing conditions (0 ng/ml nisin), of *L. lactis* carrying the empty vector (pNZ8020) or *llmg2447*-expressing vector pBL51 or pBL51Cla after induction with several concentrations of nisin (inset). Resistance of the Lcn972-resistant mutants *L. lactis* D1 and D1-20 is given relative to that of their parent, *L. lactis* MG1614. The increase in *L. lactis* D1 resistance was 400-fold and is indicated by the broken column. Results of a representative experiment are shown.

tively involved in carbohydrate metabolism were seen as "down-regulated" because they are absent from *L. lactis* D1 as a consequence of the large deletion, encompassing genes *llmg0736* to *llmg0751*, previously identified in this strain (34). Induction of genes of the cell envelope stress response triggered by Lcn972 in *L. lactis* (*llmg2164-llmg2163*) and of other genes known to be induced in response to Lcn972 and osmotic shock (*busAA*, *busAB*) was observed (28). Thus, the primary response to Lcn972 was still activated in *L. lactis* D1 although Lcn972 was not present in the cultures analyzed. The highest induction level observed (35-fold) was that of *llmg2447*. This gene has not been identified previously as part of the primary response of *L. lactis* MG1614 to Lcn972 or as a member of the CesSR regulon. Therefore, we proceeded to assess its role as a factor in bacteriocin or cell wall-active antimicrobial resistance.

***In silico* analysis of *llmg2447*.** The gene *llmg2447* encodes a 368-amino-acid (aa) protein in *L. lactis* MG1363 (GenBank accession no. AM406671.1). Topology predictions distinguished two N-terminally located transmembrane regions (Fig. 1A) and a 285-aa extracellular C-terminal domain. BLASTp searches identified proteins encoded by other available *L. lactis* genomes with a high degree of identity (85 to 91%), namely, those encoded by *yweB* in *L. lactis* IL1403, KF147, and CV56, and LACR_2469 in *L. lactis* SK11 (GenBank accession no. NP_268324, YP_003354804, ADZ64814, and YP_812019, respectively). No recognizable conserved domains could be identified in order to ascribe a putative function to Llmg2447. However, *llmg2447* and its *L. lactis* homologues are preceded by a gene that codes for an ECF sigma factor (Pfam PF07638) annotated as *sigX* in *L. lactis* IL1403 (GenBank accession no. AAK06264) (see Fig. 2). An ECF sigma factor gene is usually transcribed together with a gene that codes for an anti-sigma factor, a membrane protein involved in regulation of the activity of the cognate ECF sigma factor (19). Considering this genetic context, we postulate that *llmg2447* specifies an anti-sigma factor. In the case of *L. lactis* MG1363, the gene of the cognate ECF sigma factor is a pseudogene by virtue of a single mutation in the Lys5 codon (AAA to TAA), which introduces a premature stop codon into *sigX* (41). The same mutation was confirmed by DNA sequencing to be present in *L. lactis* MG1614 and in the Lcn^r *L. lactis* strains D1 and D1-20 (data not shown).

Overexpression of *llmg2447* reduces susceptibility to Lcn972 but does not protect against other antimicrobials. To ascertain the role of *llmg2447* in resistance to Lcn972, the gene was cloned under the control of nisin-inducible promoter P_{nisA} into pBL51. A truncated version of *llmg2447* was generated by filling in the unique internal ClaI site in *llmg2447*, specifying the protein fragment Llmg2447_1-115. This protein retains the two putative N-terminal transmembrane regions TMR1 and TMR2 of Llmg2447; it contains 40 unrelated amino acid residues derived from the vector in its C terminus (pBL51ClaI; Fig. 1).

Upon induction with increasing concentrations of nisin, *L. lactis* NZ9000/pBL51 became less susceptible to Lcn972 in a concentration-dependent fashion (Fig. 1B), reaching a plateau of 16-fold increased resistance. This is below the resistance level of *L. lactis* D1 and D1-20 (Fig. 1B). It was not possible to use higher concentrations of the inducer (>0.5 ng/ml) because growth of *L. lactis* NZ9000/pBL51 was seriously compromised. This may be due to the intrinsic constraints for production of membrane proteins in

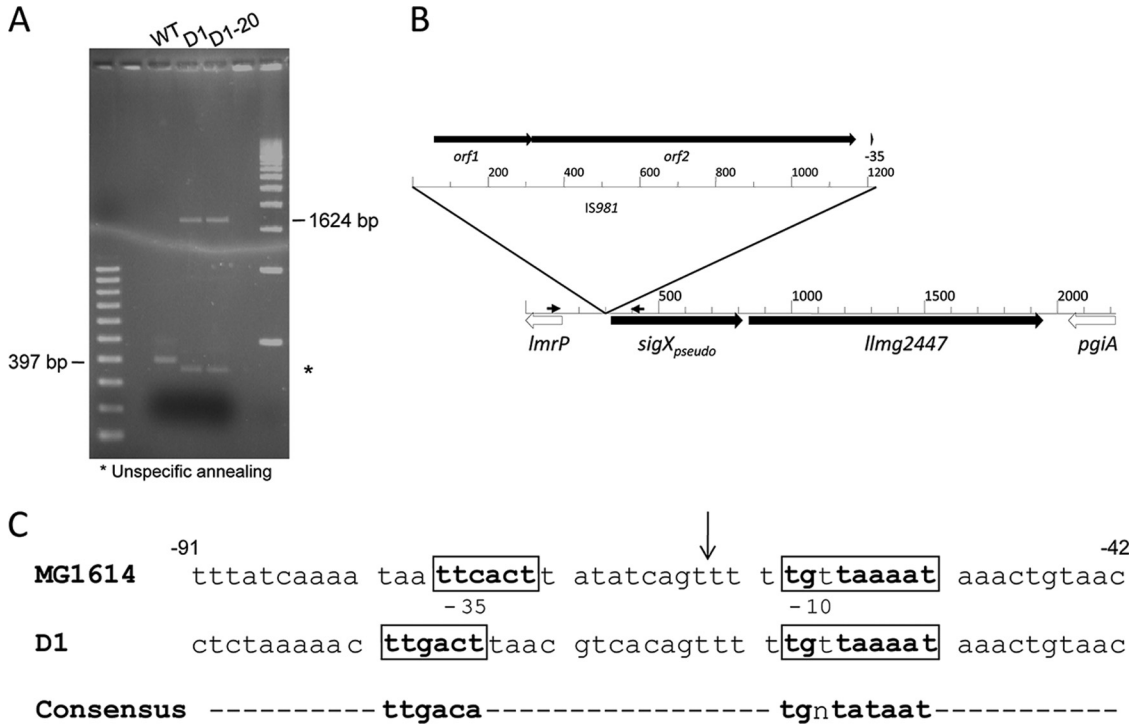

FIG 2 Insertion of IS*981* into the *llmg2447* promoter region (P_{2447}) in Lcn972-resistant (Lcn^r) *L. lactis*. (A) PCR amplification of the *lmrP-sigX*$_{pseudo}$ intergenic region of *L. lactis* MG1614 (wild type [WT]) and Lcn^r *L. lactis* strains D1 and D1-20. Primer positions are shown by small black arrows in panel B. (B) Schematic view of the insertion of IS*981* in *L. lactis* D1. The rulers indicate nucleotides numbers. (C) Nucleotide sequence of wild-type P_{2447} in *L. lactis* MG1614 and that of the hybrid promoter in *L. lactis* D1. The same DNA sequence is present in *L. lactis* MG1363 and D1-20, respectively. Base numbering relative to the translation start site is given. The vertical arrow indicates the position of the IS*981* insertion. Putative −35 and −10 regions are boxed and in bold.

L. lactis (31). On the other hand, overexpression of *llmg2447* did not change the profile of *L. lactis* susceptibility to other cell wall-active antibiotics such as bacitracin, penicillin G, and vancomycin; only the resistance to nisin was increased 2-fold (data not shown). Thus, the protein Llmg2447 appears to be rather specific for Lcn972.

Overall, these results validate the transcriptomic data and confirm the role of *llmg2447* in counteracting the inhibitory activity of Lcn972. Interestingly, the truncated Llmg2447_1-115 protein encoded by pBL1Cla was unable to confer lower susceptibility (Fig. 1), pointing to a possible interference of the extracellular C-terminal domain with Lcn972 activity. However, expression of the truncated *llmg2447* gene was more deleterious than expression of the complete version, and the clones could be induced only with low nisin concentrations (<0.2 ng/ml) (Fig. 1). Consequently, less protein would be synthesized and that might not be enough to confer Lcn972 resistance. Moreover, the stability of the truncated protein could also be compromised.

Mobilization of the insertion sequence IS*981* into the *llmg2447* locus in Lcn^r *L. lactis*. We looked for mutations within the *llmg2447* locus that could explain the high level of *llmg2447* expression detected by the transcriptome analysis of *L. lactis* D1. Only one putative σ^{70} promoter, P_{2447}, could be identified in the *lmrP-sigX*$_{pseudo}$ intergenic region of *L. lactis* MG1363 (Fig. 2). This region was amplified by PCR from the *L. lactis* MG1614 chromosome with primers X1 and X3 (Table 2), and the expected 397-bp PCR product was detected. In contrast, *L. lactis* D1 yielded a 1.6-kbp fragment (Fig. 2). The same enlarged PCR product was detected in *L. lactis* D1-20.

Sequencing of the PCR products confirmed that the promoter region in *L. lactis* MG1614 was indistinguishable from that in MG1363. Moreover, the two Lcn^r mutants were also identical to each other. Subsequent BLASTn/BLASTp searches revealed that both Lcn^r strains carry a copy of the IS*981* element inserted into the *llmg2447* locus. The 1,223-bp insertion was 99% identical at the nucleotide level to IS*981* (GenBank accession no. M33933) and contained the two ORFs, *orf1* and *orf2*, that encode the transposase functions, as described previously (32). Insertion had taken place 63 nucleotides (nt) upstream of the AUG start codon of *sigX*$_{pseudo}$ and resulted in a 4-nt CAGT duplication at either side of the insertion (Fig. 2B).

Closer inspection revealed that IS*981* had been inserted between the putative −35 and −10 regions of P_{2447}, replacing the former with a new −35 region that is present at the 3′ end of the insertion element (Fig. 2C). This new hybrid promoter, $P_{IS981::2447}$, could be responsible for the higher *llmg2447* expression level in the Lcn972-resistant strains. The newly introduced −35 region is located at a proper distance (7) of 18 nt from the extended −10 region (the original distance between the −35 and −10 boxes is only 15 bp) and has only one mismatch with the canonical sequence TTGACA, in contrast to the two mismatches present in the −35 box of P_{2447} (Fig. 2C).

Activation of *llmg2447* in *L. lactis* D1 is mediated by IS*981*. To confirm and compare the activity of both the wild-type and hybrid $P_{IS981::2447}$ promoters, they were cloned upstream of the promoterless *lacZ* gene in plasmid pPTPL, generating pBL58 (P_{2447}-*lacZ*) and pBL59 ($P_{IS981::2447}$-*lacZ*). Visual inspection of the GM17 plates supplemented with X-Gal indicated that the activity

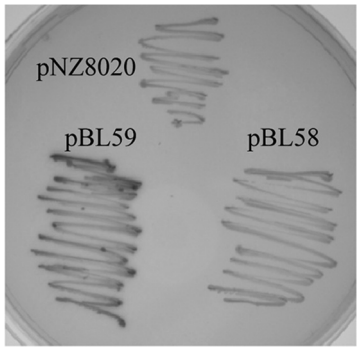

FIG 3 β-Galactosidase activities of reporter plasmids. *L. lactis* NZ9000 carrying pNZ8020 (empty vector), pBL58 (P_{2447}-*lacZ*), or pBL59 ($P_{IS981::2447}$-*lacZ*) was plated onto a GM17 plate containing 80 μg/ml X-Gal. The mean β-galactosidase specific activity ± the standard deviation in six enzymatic activity assays with two independent cultures of exponentially growing *L. lactis* NZ9000 cells carrying these plasmids was calculated. Cells carrying pNZ8020, pBL58 (P_{2447}), and pBL58 (P_{2447}) with Lcn972 added at 10, 20, and 40 AU/ml produced no activity, while those carrying pBL59 ($P_{IS981::2447}$-*lacZ*) produced 294.2 ± 47.2 ΔmA_{420}/min/OD_{600} unit.

of the $P_{IS981::2447}$ promoter was higher than that of P_{2447} (Fig. 3). In fact, β-galactosidase activity was detected only with permeabilized cells of *L. lactis* cells containing the hybrid promoter. Attempts to detect P_{2447} activity at different growth stages or after treatment of the cells with Lcn972 were unsuccessful (Fig. 3). Under the conditions tested, this promoter is inactive or might require active ECF SigX. To test the latter possibility, we introduced plasmid pBL58 (P_{2447}-*lacZ*) into *L. lactis* IL1403, a strain that produces functional ECF SigX (2, 43). However, no β-galactosidase activity was detected during the growth of the strain, even in the presence of Lcn972, bacitracin, nisin, or vancomycin (data not shown).

DISCUSSION

Resistance to bacteriocins may compromise their use as biopreservatives in food or as future anti-infectives. Studies to understand the molecular mechanisms underlying bacteriocin resistance development have been undertaken recently. Genome-wide transcriptomics has been used to analyze resistance to the lantibiotic nisin (22, 25) and the class IIa bacteriocins sakacin P and pediocin PA-1 (30, 38). These reports revealed a rather complex transcriptional response in which more than 100 genes were differentially expressed in the resistant strains relative to their parents. The complexity of these phenotypes is likely due to the dual modes of action of these bacteriocins, i.e., inhibition of cell wall biosynthesis and pore formation by nisin or inactivation of the mannose phosphotransferase (PTS) transporter by the pore-forming class IIa bacteriocins (14, 42).

A general overview of the transcriptomic results presented here indicates that in the case of Lcn972, which inhibits cell wall biosynthesis without perturbing the cytoplasmic membrane, loss of susceptibility involves a discrete number of genes, particularly when those included in the chromosomal deletion (34) are disregarded. This deletion in the Lcn972-resistant strains encompassed maltose catabolic genes (*malEFG*, *mapA*, *malR*) and several genes involved in the degradation of polysaccharides. The upregulation of genes with similar functions in the resistant mutant, such as those for amylopullulanase (*apu*) and glycogen phosphorylase (*glgP*), is presumably done as a response to the absence of the deleted genes rather than to contribute directly to Lcn972 resistance. On the other hand, overexpression of *pbpX*, which codes for the transpeptidase PBP2X, is most likely the reason for the higher content of tripeptide muropeptides in the peptidoglycan of *L. lactis* D1, which has already been linked to resistance to Lcn972 (34).

Overall, of all of the genes with altered expression in *L. lactis* D1, *llmg2447* appears to play a key role in Lcn972 resistance. In fact, resistance levels increased in a concentration-dependent manner relative to *llmg2447* expression up to 16-fold (see Fig. 1). Yet, nisin-induced expression of *llmg2447* never led to a level as high as that of the mutant *L. lactis* D1, which is likely explained by an extra protective effect of other gene products already associated with resistance to Lcn972, such as those of *llmg2164*, *llmg2163*, and *celB* (9, 33).

Activation of *llmg2447* in *L. lactis* D1 is caused by the insertion of IS*981* into the promoter region of *llmg2447* during adaptation of the strain to Lcn972. It is worth noting that this insertion event appears to have been stabilized in the population because it is still present in *L. lactis* D1-20, i.e., after subculturing for 200 generations in the absence of selective pressure. The role of IS*981* in the adaptive evolution of *L. lactis* under stressful conditions is well documented (13). It has been involved, for example, in the activation of an alternative lactate dehydrogenase gene (*ldhB*) in *L. lactis ldhA* mutants (3). As in the case of *llmg2447*, transcriptional activation of *ldhB* is mediated by the site- and orientation-specific insertion of IS*981*, so that a new hybrid and constitutive promoter is created.

Despite the role of *llmg2447* in Lcn972 resistance, this gene was not previously detected in the response of *L. lactis* to Lcn972 (28). This can be explained now by the fact that the wild-type promoter P_{2447} is inactive, even when *L. lactis* is challenged with Lcn972. Moreover, disruption of *llmg2447* in *L. lactis* MG1363 did not change the strain's susceptibility to Lcn972 (our own unpublished results). Therefore, under standard laboratory conditions, *llmg2447* appears to be silent or may require a transcriptional activator or particular sigma factor. In this context, we postulate that *llmg2447* is part of an ancestral cell envelope stress-sensing regulatory device made up of an ECF sigma factor (*sigX* in *L. lactis* IL1403, $sigX_{pseudo}$ in *L. lactis* MG1363) and its cognate anti-sigma factor, *yweB* (in strain IL1403) or *llmg2447* (in strain MG1363). Generally, the anti-sigma factor is a membrane protein (i.e., Llmg2447) that interacts directly with the ECF sigma factor, keeping it inactive under physiological conditions. Upon stress sensed by the anti-sigma factor, the ECF sigma factor is released and is able to direct the transcription of specific genes, that of its own gene and that of the anti-sigma factor gene (19, 37). Absence of activity of the *llmg2447* promoter could thus be due to the lack of a functional cognate sigma factor in *L. lactis* MG1363. However, when the promoter was introduced into *L. lactis* IL1403, a strain with a theoretically functional *sigX* gene, it was still inactive, even in the presence of Lcn972 and other cell wall-active antimicrobials as stimuli. Previous reports have shown that two promoters, one SigA dependent and the other SigX dependent, are involved in *sigX* transcription in *L. lactis* IL1403 (43). Lack of activation of P_{2447} in *L. lactis* IL1403 could be due to the absence of the appropriate promoter signatures recognized by IL1403 SigX or to the inability of the stimuli (i.e., Lcn972, bacitracin, nisin, and vancomycin) to induce the system in *L. lactis* IL1403. ECF sigma factors

usually require external stimuli to activate their own transcription (19, 21).

L. lactis sigX is a member of the ECF110 group established by Starón et al. (37) together with those of *Streptococcus agalactiae*, *S. suis*, and *S. equi*. However, there are no clues to the function of the only ECF sigma factor in lactococci. Considering the role of *llmg2447* in protecting against the cell wall-active bacteriocin Lcn972, it is tempting to speculate that *L. lactis* ECF SigX is part of a cell envelope-sensing regulatory system. Indeed, ECF sigma factors are key elements in the responses of *B. subtilis*, *E. coli*, and *Enterococcus faecalis* to cell wall-active antimicrobials such as lysozyme (18, 20, 23).

In conclusion, the putative membrane protein encoded by *llmg2447* has been identified as a main factor in *L. lactis* resistance to the cell wall-active bacteriocin Lcn972, although the way it protects *L. lactis* remains to be elucidated. The genetic context of *llmg2447* supports the notion that it may have been part of an ancestral ECF sigma factor regulatory system. While this regulative element may not be physiologically relevant in *L. lactis*, the presence of homologous systems in important pathogens such as *S. agalactiae*, a bacterium often involved in mastitis and other infectious diseases, deserves further attention.

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