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MECHANISMS OF RESISTANCE

Lactococcus lactis Resistance to Aureocin A53- and Enterocin L50-Like Bacteriocins and Membrane-Targeting Peptide Antibiotics Relies on the YsaCB-KinG-LlrG Four-Component System

Aleksandra Tymoszewska,[a] Kirill V. Ovchinnikov,[b] Dzung B. Diep,[b] Małgorzata Słodownik,[a] Edyta Maron,[a] Beatriz Martínez,[c] Tamara Aleksandrzak-Piekarczyk[a]

[a]Institute of Biochemistry and Biophysics, Polish Academy of Sciences (IBB PAS), Warsaw, Poland

[b]Faculty of Chemistry, Biotechnology, and Food Science, Norwegian University of Life Sciences, Ås, Norway

[c]Department of Technology and Biotechnology of Dairy Products, Instituto de Productos Lácteos de Asturias, Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa, Spain

ABSTRACT Resistance to nonribosomally synthesized peptide antibiotics affecting the cell envelope is well studied and mostly associated with the action of peptide-sensing and detoxification (PSD) modules, which consist of a two-component system (TCS) and an ATP-binding cassette (ABC) transporter. In contrast, the mechanisms of resistance to ribosomally synthesized bacterial toxic peptides (bacteriocins), which also affect the cell envelope, are studied to a lesser extent, and the possible cross-resistance between them and antibiotics is still poorly understood. In the present study, we investigated the development of resistance of *Lactococcus lactis* to aureocin A53- and enterocin L50-like bacteriocins and cross-resistance with antibiotics. First, 19 spontaneous mutants resistant to their representatives were selected and also displayed changes in sensitivity to peptide antibiotics acting on the cell envelope (bacitracin, daptomycin, and gramicidin). Sequencing of their genomes revealed mutations in genes encoding the ABC transporter YsaCB and the TCS KinG-LlrG, the emergence of which induced the upregulation of the *dltABCD* and *ysaDCB* operons. The *ysaB* mutations were either nonsense or frameshift mutations and led to the generation of truncated YsaB but with the conserved N-terminal FtsX domain intact. Deletions of *ysaCB* or *llrG* had a minor effect on the resistance of the obtained mutants to the tested bacteriocins, daptomycin, and gramicidin, indicating that the development of resistance is dependent on the modification of the protein rather than its absence. In further corroboration of the above-mentioned conclusion, we show that the FtsX domain, which functions effectively when YsaB is lacking its central and C-terminal parts, is critical for resistance to these antimicrobials.

KEYWORDS aureocin A53-like and enterocin L50-like bacteriocins, class II leaderless bacteriocins, bacteriocin resistance, antibiotic resistance, peptide antibiotics, daptomycin, gramicidin, bacitracin, bacitracin transporter, two-component system

For over half a century, antibiotics have served as the main weapon to combat bacterial infections, but their often-indiscriminate use has led to a rapidly increasing appearance of difficult-to-treat infections caused by antibiotic-resistant pathogens. The simultaneous slowdown of the research on and development of new antibiotics makes alternative solutions a must. The ribosomally synthesized bacterial toxic peptides called bacteriocins as potential novel effective antimicrobial agents have therefore been intensively studied in recent years (1). Numerous bacteriocins produced by Gram-positive bacteria show potential for medical application due to their low toxicity,

Citation Tymoszewska A, Ovchinnikov KV, Diep DB, Słodownik M, Maron E, Martínez B, Aleksandrzak-Piekarczyk T. 2021. *Lactococcus lactis* resistance to aureocin A53- and enterocin L50-like bacteriocins and membrane-targeting peptide antibiotics relies on the YsaCB-KinG-LlrG four-component system. Antimicrob Agents Chemother 65:e00921-21. https://doi.org/10.1128/AAC.00921-21.

Address correspondence to Tamara Aleksandrzak-Piekarczyk, tamara@ibb.waw.pl.

Received 6 May 2021
Returned for modification 6 June 2021
Accepted 29 August 2021
Accepted manuscript posted online 13 September 2021
Published

high potency, and antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis* (VRE) strains (1). Nevertheless, so far, only thiostrepton, a bacteriocin from the thiopeptide family, is commercially used in clinical practice (1), and some bacteriocins are in preclinical and clinical trials (2, 3).

Bacteriocins are classified into class I posttranslationally modified bacteriocins (e.g., the lantibiotic nisin) and class II unmodified bacteriocins (4). Among members of class II, the families of aureocin A53 (AurA53)- and enterocin L50 (EntL50)-like leaderless bacteriocins currently draw more attention due to their abundance and broad antimicrobial activity (5, 6). It is believed that the AurA53- and EntL50-like bacteriocins cause high membrane permeability in the absence of a specific receptor; however, the exact mechanisms of cell killing may differ between individual bacteriocins (7–10). On the other hand, some other bacteriocins such as lantibiotics or most nonribosomally synthesized peptide antibiotics kill sensitive bacteria by targeting critical steps in cell wall biosynthesis. Nisin and nisin-like lantibiotics interact with lipid II, causing inhibition of peptidoglycan synthesis and pore formation (11). Lactococcin 972 (Lnc972) is the only bacteriocin known, apart from lantibiotics, that recognizes lipid II but does not form pores (12). Among peptide antibiotics, lipid II serves as a target for ramoplanin, vancomycin, and teicoplanin (13, 14). A distinct step of cell wall synthesis is inhibited by bacitracin, which binds to the lipid II carrier undecaprenyl-pyrophosphate (UPP) and prevents its dephosphorylation to undecaprenyl phosphate (UP) (15). The mechanism of action of some peptide antibiotics, such as daptomycin and gramicidin, is similar to that of saposin-like bacteriocins. Daptomycin binds to the lipid bilayer and induces membrane permeability in the presence of calcium ions and phosphatidylglycerol (16), while gramicidin forms dimeric channels in the membrane and transports monovalent cations, thereby disrupting ion homeostasis (17).

Resistance to antimicrobials targeting the cell envelope is often mediated by so-called peptide-sensing and detoxification (PSD) modules that usually consist of a two-component system (TCS) and an ATP-binding cassette (ABC) transporter associated with the former by genetic context and function. Because of such compositions, these modules are sometimes referred to as four-component systems (18). The best-known prototype of a PSD module is BceRS-BceAB, first discovered in *Bacillus subtilis* (19). Its activity relies on sensing proteins such as the membrane-bound histidine kinase (HK) BceS and the cognate ABC transporter BceAB, the latter of which not only recognizes the presence of the antimicrobials but also carries out detoxification (20, 21). The ABC transporter comprises the BceB permease and the BceA ATPase, and its expression is regulated via a TCS consisting of the BceS HK and the cytoplasmic response regulator (RR) BceR (19, 22). Other examples of similar four-component systems found in *B. subtilis* include PsdRS-PsdAB and YxdJK-YxdLM, each ensuring robust protection against distinct peptide antimicrobials (23). BceRS-BceAB-type resistance modules are also conserved in many other pathogenic and nonpathogenic low-G+C-content species of the phylum *Firmicutes* (24). Besides ABC transporters, some BceRS homologs orchestrate several other genes whose activity maintains cell integrity in the presence of a stressor, such as *mprF* and those of the *dlt* operon (25). Products of these genes confer some resistance to cationic peptide antimicrobials by lowering the cell surface negative charge through modifications of the membrane and the cell wall, i.e., lysination of phospholipids and D-alanylation of teichoic acids, respectively (25). The role of the BceRS-BceAB-like modules in resistance to cell envelope-acting peptide antibiotics such as bacitracin, vancomycin, or teicoplanin has been extensively studied (25). In contrast, the understanding of BceRS-BceAB-mediated resistance to the bacteriocins targeting the cell envelope is still fragmentary and mainly relies on studies of nisin-resistant mutants (26). Lcn972 is the only nonlantibiotic bacteriocin known to induce a lactococcal BceRS-BceAB homolog, the YsaCB-KinG-LlrG system (27, 28). Moreover, a recent study proposed that this system could also be engaged in *Lactococcus lactis* resistance to AurA53- and EntL50-like bacteriocins since a point mutation in the *ysaB* gene encoding

the ABC transporter permease YsaB decreased the sensitivity to these antimicrobials. However, since the mutation in *ysaB* was accompanied by an additional mutation in the *dxsA* gene connected with lipid metabolism, the involvement of the YsaCB-KinG-LlrG system in resistance to AurA53- and EntL50-like bacteriocins cannot be considered proven (29).

In this study, we examined in detail the genetic basis of *L. lactis* resistance to diverse AurA53- and EntL50-like leaderless bacteriocins, including four functional bacteriocins (BHT-B, Ent7, EntL50, and WelM) and two putative ones identified by homology searches (K411 and salivaricin C [SalC]). First, we show that all AurA53- and EntL50-like bacteriocins exhibit high similarity at the amino acid sequence level within families and very low similarity between families and that K411 belongs to the former family and SalC belongs to the latter. The differences in amino acid sequences notwithstanding, members of both families adopt a similar globular saposin-like fold and have broad-spectrum antimicrobial activity. We then obtained spontaneous resistant mutants by exposing sensitive *L. lactis* strains to selected AurA53- or EntL50-like bacteriocins and identified the mutations responsible for the resistance phenotype. Resistance to bacteriocins from both families turned out to be due to mutations in genes encoding components of the PSD module YsaCB-KinG-LlrG. These mutations also conferred resistance to membrane-targeting peptide antibiotics such as daptomycin and gramicidin and triggered the increased expression of the *dltABCD* and *ysaDCB* operons. We propose that the main determinant of this multiresistance is the activity of the FtsX domain freed from the C-terminal part of the YsaB protein by truncating mutations. Finally, we show that the acquired resistance increased the sensitivity to bacitracin, suggesting that this antibiotic is beyond the control of FtsX.

RESULTS

AurA53- and EntL50-like bacteriocins reveal structural similarities. Among the 14 AurA53- and EntL50-like bacteriocins studied here, 12 were to some extent previously tested. The first family comprises AurA53 (30), BHT-B (31), lacticin Q (LacQ) (32), lacticin Z (LacZ) (33), epidermicin NI01 (EpiNI01) (34), and lactolisterin BU (LliBU) (35). They are single-peptide, 42- to 53-amino-acid-long bacteriocins and are highly cationic but with a partially hydrophobic character, with high contents of lysine and tryptophan. The EntL50-like family is comprised of 42- to 44-amino-acid-long bacteriocins, the two-peptide EntL50 (EntL50A and EntL50B) (36) and enterocin 7 (Ent7) (Ent7A and Ent7B) (37), and the single-peptide ones weissellicin M (WelM) and weissellicin Y (WelY) (38). Two other bacteriocins (K411 and SalC) have been assigned in GenBank to the AurA53- and EntL50-like families, respectively, based on their amino acid sequences. However, no studies confirming the activity of the purified peptides have been conducted. K411 comprises 52 amino acids and is encoded by the *aucA* gene on the pKW4 plasmid of the clinical *Corynebacterium jeikeium* strain K411 (NCBI RefSeq accession no. WP_010976360.1) (39, 40). SalC is 34 amino acids long and is found in numerous *Lactobacillus salivarius* strains such as JCM1046, UMNPBX2, and DSM 20554 (NCBI RefSeq accession no. WP_089144005.1, WP_089144005.1, and WP_089144005.1, respectively). To determine the similarity between the AurA53-like (AurA53, BHT-B, K411, LacQ, LacZ, EpiNI01, and LliBU) and EntL50-like (EntL50, Ent7, SalC, WelM, and WelY) bacteriocins, their primary and tertiary structures were compared. AurA53- and EntL50-like bacteriocins exhibited high similarity at the amino acid sequence level within their families (Fig. 1A and B) and only weak conservation between the two families (Fig. 1C). Distinct conserved amino acid motifs were found in each family, AkyGxKaV in the AurA53-like family or AklvxkfG and imqfIGeGw in the EntL50-like family, with some deviations from the consensus for EpiNI01 and both weissellicins (Fig. 1A and B). However, bacteriocins from both families showed similar arrangements of basic, acidic, and nonpolar amino acids, with a predominance of the latter (Fig. 1C).

The tertiary structures of the AurA53- and EntL50-like bacteriocins were predicted based on the templates available in the Protein Data Bank (PDB) and compared with

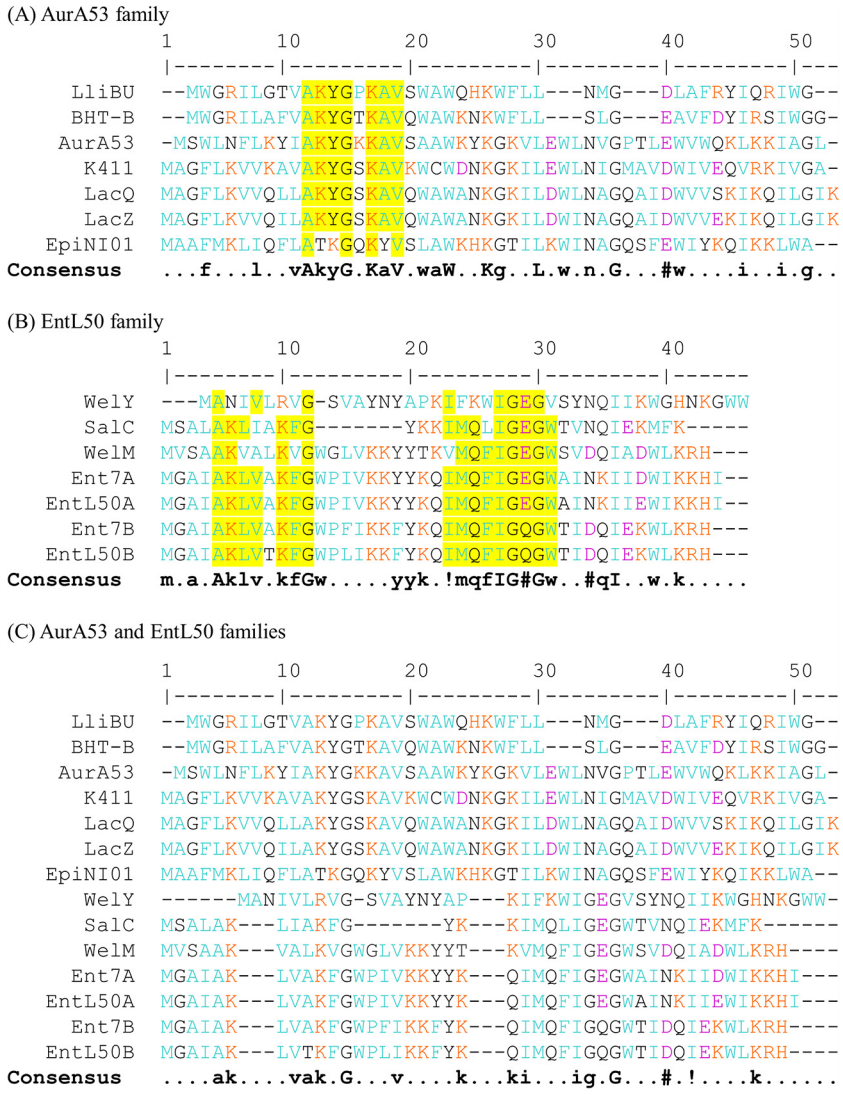


FIG 1 Comparison of amino acid sequences of aureocin A53-like (A), enterocin L50-like (B), and both (C) bacteriocin families. Consensus amino acids are in uppercase, and partial consensus ones are in lowercase. Consensus symbols are as follows: ! is I or V, and # is any of NDQE. Basic, acidic, and nonpolar (hydrophobic) amino acids are indicated with red, blue, and green letters, respectively. Strongly conserved amino acid motifs with a deviation of up to 2 amino acids are highlighted in yellow. The UniProt accession numbers are A0A1K0ITP4 for LliBU, Q3YB73 for BHT-B, Q8GPI4 for AurA53, H9BG66 for EpiNI01, Q576C5 for K411, A4UVR2 for LacQ, A7M6Q0 for LacZ, H1A8I1 for WelY, A0A089RZU8 for SalC, H1A8I6 for WelM, Q1A2D3 for Ent7A, Q1A2D2 for Ent7B, Q7B2P3 for EntL50A, and Q7B2P2 for EntL50B.

the experimental three-dimensional (3D) nuclear magnetic resonance (NMR) structures of AurA53, LacQ, and Ent7 (41, 42), which show a similar globular saposin-like fold. Saposins are human proteins containing four or five α-helices (saposin fold) stabilized by disulfide bridges and interacting with membrane lipids to modify the surrounding structure (43). The saposin-like fold is composed of three or four α-helices with a highly cationic, hydrophilic surface surrounding a hydrophobic core and is stabilized by hydrophobic and electrostatic interactions (44). The saposin-like fold and the mostly solvent-exposed aromatic side chains of several tryptophan residues are believed to be critical for bacteriocin activity and enable peptide-lipid interactions and insertion into the bacterial membrane (6, 44, 45). Despite the low level of amino acid sequence identity, members of both families appear to have a similar globular saposin-like fold comprised of three or four amphipathic α-helices. In all bacteriocin structures, positively

TABLE 1 Spontaneous *L. lactis* mutants resistant to K411 or Ent7[b]

L. lactis mutant(s)[a]	Location of mutation	DNA sequence mutation(s)	Amino acid sequence change(s)
Obtained in the presence of K411			
MUT_29, MUT_40, MUT_49	*ysaC*	623G>T	YsaC, Ser208Ile
MUT_92, MUT_94	*ysaB*	568G>T	YsaB, Glu190X
MUT_125	*ysaB*	574G>T	YsaB, Glu192X
MUT_44, MUT_46, MUT_50	*ysaB*	1293_1297dupTCAAT	YsaB, Trp433PhefsX26
MUT_47	*ysaB*	1696C>T	YsaB, Gln566X
MUT_2, MUT_123	*ysaB*	1915_1933dupTCCTTAGCAACAATTGCAA and 1957_1958insA	YsaB, Ala647SerfsX15; YsaB, Cys653X
MUT_12	*llrG*	529C>A; Δ*ysaCB*	LlrG, Leu177Ile
Obtained in the presence of Ent7			
MUT_314	*ysaB*	1546G>T	YsaB, Glu516X
MUT_318	*ysaB*	1696C>T	YsaB, Gln566X
MUT_321, MUT_322, MUT_324	*ysaB*	553G>T	YsaB, Glu185X
MUT_325	*ysaB*	1744G>T	YsaB, Glu582X

[a]All mutants except MUT_12 were obtained by growing *L. lactis* LMGT 3419 in the presence of bacteriocin. MUT_12 was obtained in the same manner but with MUT_402 (Δ*ysaCB*) as the parental strain.
[b]">," substitution; "dup," duplication; "ins," insertion; "fs," frameshift; X, stop codon. The number after X indicates the length of the frameshift, including the stop codon.

charged basic residues and rarely occurring negatively charged acidic residues were mainly localized on the outer sides (bacteriocin surface), while nonpolar residues were packed inside (bacteriocin core) (see Fig. S2 in the supplemental material). SalC appeared to be less globular and, thus, the least similar to other bacteriocins.

AurA53- and EntL50-like bacteriocins exhibit a broad spectrum of antimicrobial activity. To extend the available information regarding the spectrum of antimicrobial activity of AurA53- and EntL50-like bacteriocins, especially the novel members (K411 and SalC), we assayed six bacteriocins from these two families against more than 100 indicator strains. Overall, bacteriocins from both families were active against a wide range of Gram-positive bacteria from the genera *Bacillus*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Staphylococcus*, and *Streptococcus*, with slight variations for specific strains within species, whereas the activity against Gram-negative bacteria such as *Campylobacter jejuni* and *Pseudomonas aeruginosa* was family specific and involved only EntL50-like bacteriocin representatives (Table S2). Notably, all the bacteriocins were active against strains of the important human pathogens MRSA, *E. faecalis*, and *Enterococcus faecium*, except SalC, which showed no activity toward MRSA. They also showed activity against different serotypes of *Listeria monocytogenes*, a food pathogen. Among Gram-negative bacteria, Ent7, EntL50, WelM, and SalC inhibited the growth of enteropathogenic *C. jejuni* 81176 and, to a lesser extent, *P. aeruginosa* ATCC 9027 (Table S2). Ent7 and EntL50 had the broadest antimicrobial spectrum, showing high activity against almost all Gram-positive bacteria tested. The other bacteriocins showed only a slightly narrower range of antimicrobial activity, being weakly or not active against some species of staphylococci and streptococci and having variable activity against lactobacilli and *Weissella confusa* (Table S2).

AurA53- and EntL50-like bacteriocins induce the emergence of resistant mutants. To search for genetic determinants of resistance to AurA53-like and EntL50-like bacteriocins, we selected 18 spontaneous bacteriocin-resistant mutants by exposing sensitive *L. lactis* LMGT 3419 to K411 or Ent7. Among them, 12 were obtained in the presence of 0.01 to 1 mg/ml K411, and 6 were obtained in the presence of 1 to 2 mg/ml Ent7 (Table 1). Compared to the wild-type strain, all these mutants were equally 16-fold less sensitive to the bacteriocin used for selection and cross-resistant to the other one.

To identify the genetic changes responsible for resistance, the genomes of the resistant mutants were sequenced, revealing significant mutations only in the *ysaCB* operon encoding the ATPase (YsaC) subunit and the permease (YsaB) of the ABC transporter YsaCB. Homology searches revealed that YsaC shares 49% and 45% identities with the ATPases YxdL and BceA, respectively, from the model strain *B. subtilis* 168 (NCBI RefSeq accession no. NP_391843.2 and NP_390916.1, respectively), whereas YsaB is 23% and

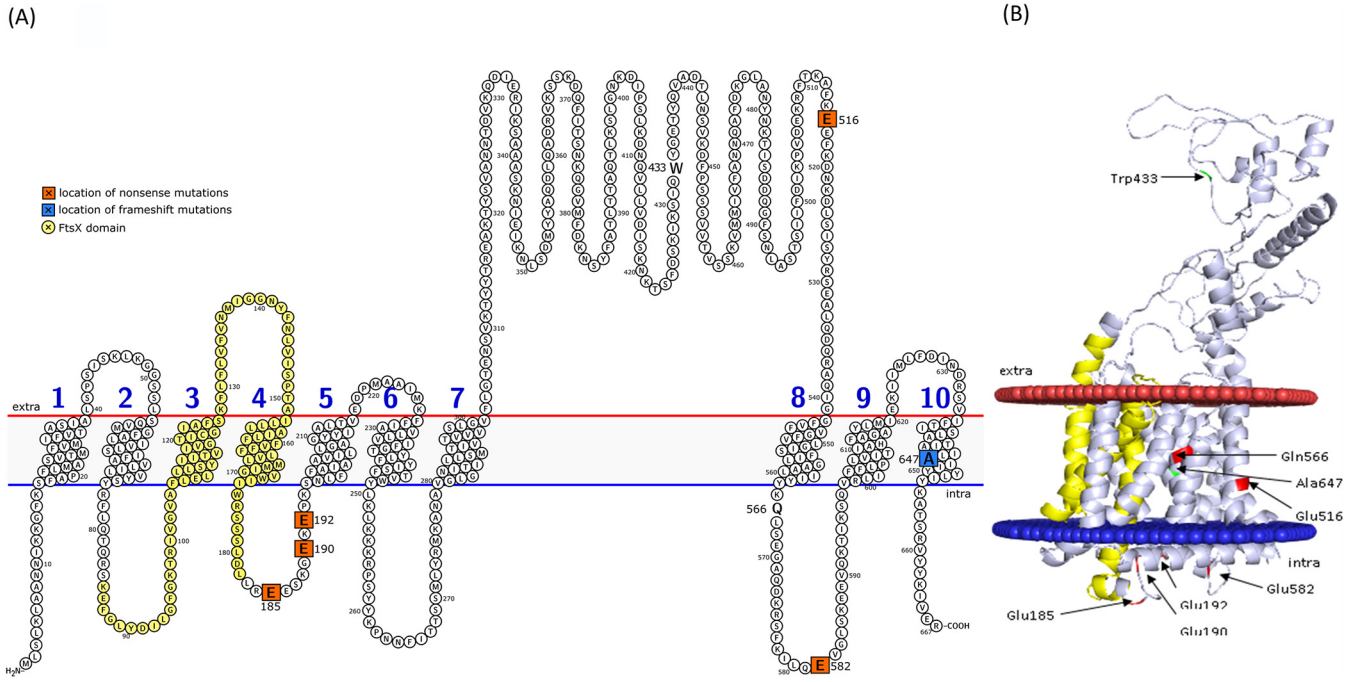


FIG 2 Localization of spontaneous resistance-conferring mutations in the predicted topology (A) and the template-based tertiary structure (B) of *L. lactis* YsaB. Codons of amino acids marked in orange or blue underwent nonsense or frameshift mutations, respectively, in *ysaB* in spontaneous *L. lactis* mutants. The FtsX domain is marked in yellow. The template of the highest significance used by the I-TASSER Web service to predict the tertiary model of YsaB can be found in Table S1 in the supplemental material.

21% identical to the ABC transporter permeases BceB and YxdM, respectively, from *B. subtilis* 168 (NCBI RefSeq accession no. NP_390915.1 and NP_391842.2). In three mutants (MUT_29, MUT_40, and MUT_49), a single identical mutation occurred leading to the Ser208Ile substitution in the C-terminal part of YsaC (Table 1). The other mutants carried nonsense or frameshift mutations in the *ysaB* gene, all resulting in the truncation of the YsaB permease (Table 1 and Fig. 2).

To identify putative *L. lactis* LMGT 3419 resistome genes operating in the absence of the ABC transporter YsaCB, we deleted the *ysaCB* locus to obtain *L. lactis* MUT_402. When exposed to K411, this deletion clone gave rise to the spontaneous mutant *L. lactis* MUT_12 carrying a significant missense mutation in the *llrG* gene, causing the Leu177Ile substitution (Table 1). *llrG* and the downstream *kinG* gene encode a response regulator and a histidine kinase jointly constituting the two-component system KinG-LlrG. These two genes are adjacent to the *ysaCB* operon and together have been proposed to form a four-component YsaCB-KinG-LlrG PSD module (27, 28). LlrG shares 42% and 38% identities with the two-component response regulators YxdJ and PsdR from *B. subtilis* 168, respectively (NCBI RefSeq accession no. QJF42346.1 and QJF42831.1).

Sensitivity of *L. lactis* mutants to bacteriocins. Selected unique representatives of the spontaneous *L. lactis* mutants were precisely assessed for sensitivity to membrane-disrupting AurA53- and EntL50-like bacteriocins (BHT-B, K411, Ent7, EntL50, WelM, and SalC), the cell wall synthesis inhibitor Lcn972, and nisin, which has both mechanisms of action (membrane disruption and inhibition of cell wall biosynthesis). Lcn972 and nisin, the resistance to which has already been shown to involve the lactococcal YsaCB-KinG-LlrG system, were used as controls (28, 46). We assayed wild-type *L. lactis* strain LMGT 3419 and its mutants harboring substitutions in *ysaC* (MUT_40), in different parts of *ysaB* (MUT_2, MUT_94, MUT_314, and MUT_322), or in *llrG* (MUT_12). The parental strain showed different levels of susceptibility to the bacteriocins tested, with the highest level of susceptibility to Ent7 and EntL50 (MIC_{50} = 0.2 μg/ml) and the lowest to BHT-B and SalC (MIC_{50} = 6.3 μg/ml). All the mutants were equally resistant to the two bacteriocins used for mutagenization (K411 and Ent7) and cross-resistant to others.

TABLE 2 Sensitivities of *L. lactis* and its mutants to aureocin A53-like and enterocin L50-like bacteriocins (K411, Ent7, EntL50, WelM, SalC, and BHT-B), nisin, lactococcin 972, and antibiotics (gramicidin, daptomycin, and bacitracin)[a]

Strain	Genotype	K411	Ent7	EntL50	WelM	SalC	BHT-B	nisin	Lcn972	Gramicidin	Daptomycin	Bacitracin
LMGT 3419	wild-type	3.2	0.2	0.2	2.4	6.3	6.3	0.47	0.4	0.023	0.094	12
MUT_40	*ysaC*$^-$	25-(>25)	3.2	1.6	6.3	12.5	25	1.9	3.2	0.023	1.0-1.5	8
MUT_322	*ysaB*$^-$	>25	3.2	1.6	12.5	>25	25	1.9	1.6	0.047	1.5-2	2
MUT_94	*ysaB*$^-$	>25	3.2	0.8	6.3	12.5	25	1.9	1.6	0.094	1.0-1.5	4
MUT_314	*ysaB*$^-$	>25	3.2	0.4	6.3	12.5	25	1.9	1.6	0.047	2	2
MUT_2	*ysaB*$^-$	25-(>25)	3.2	0.8	6.3	12.5	25	1.9	>3.2	0.047	1.5	2
MUT_12	Δ*ysaCBllrG*$^-$	>25	1.6	1.6	6.3	12.5	25	1.9	1.6	0.047	1.5-2	4-6
MUT_400	Δ*ysaC*	2.4	0.15	0.2	2.4	6.3	6.3	0.24	0.4	0.023	0.064	2
MUT_401	Δ*ysaB*	2.4	0.15	0.2	2.4	6.3	6.3	0.24	0.4	0.023	0.064	2
MUT_402	Δ*ysaCB*	2.4	0.15	0.2	2.4	6.3	6.3	0.24	0.4	0.023	0.094	2
MUT_403	Δ*llrG*	1.6	0.15	0.2	1.6	3.2-6.3	6.3	0.24	0.2	0.023	0.064	1.5
MUT_404	Δ*ysaCB*Δ*llrG*	2.4	0.15	0.2	1.6	3.2-6.3	6.3	0.24	0.2	0.023	0.064-0.094	1.5
MUT_405	Δ*ysaCBllrG*$^-$ pIBB-JZK	>25	0.8	1.6	6.3	12.5	25	1.9	1.6	0.047	1.5	3
MUT_406	Δ*ysaCBllrG*$^-$ pIBB-JZK*llrG*	3.2	0.2	0.2	2.4	3.2	6.3	0.47	0.4	0.047	0.5	3
MUT_407	Δ*ysaCB*:pGh (control)	2.4	0.15	0.2	2.4	6.3	6.3	0.24	0.4	0.012	0.75	2
MUT_408	Δ*ysaCB*:pGh*ysaCB*$_{1\text{-}578}$	25	1.2	6.3	9.4	12.5	>25	1.9	3.2	0.047	8	2
MUT_409	Δ*ysaCB*:pGh*ysaB*$_{1\text{-}578}$	2.4	0.15	0.2	2.4	6.3	6.3	0.47	0.4	0.012	0.5	2
MUT_410	Δ*ysaCB*:pGh:P*ptcB*:*llrG*	25	0.6	0.8	9.4	12.5	25	0.94	0.8	0.012	0.75	2
MUT_411	Δ*ysaB*:pGh (control)	2.4	0.15	0.2	2.4	6.3	6.3	0.24	0.4	0.012	0.25	2
MUT_412	Δ*ysaB*:pGh*ysaB*$_{1\text{-}578}$	25	1.2	6.3	4.8	12.5	>25	1.9	0.8	0.023	4	2
MUT_413	Δ*ysaCB*Δ*llrG*:pGh (control)	2.4	0.15	0.2	1.6	3.2-6.3	6.3	0.24	0.2	0.012	0.094	2
MUT_414	Δ*ysaCB*Δ*llrG*:pGh*ysaCB*$_{1\text{-}578}$	2.4	0.15	0.2	1.6	3.2-6.3	6.3	0.24	0.2	0.012	0.094	2
MUT_415	Δ*ysaCBllrG*$^-$:pGh (control)	>25	1.6	1.6	6.3	12.5	25	1.9	0.8	0.023	2	4-6
MUT_416	Δ*ysaCBllrG*$^-$:pGh*ysaCB*$_{1\text{-}578}$	>25	3.2	6.3	12.5	25	>25	3.8	0.8	0.023	8	4

Sensitivity scale: 8 × | 4 × | 2 × | 2 × | 4 × | 8 × | 16 × | ≥32 ×
increased sensitivity relative to WT← →decreased sensitivity relative to WT

[a]Names of spontaneous mutants (Table 1) are marked in boldface type. MIC_{50} (micrograms per milliliter) (K411, BHT-B, Ent7, EntL50, WelM, SalC, nisin, Lcn972, and gramicidin) or MIC (micrograms per milliliter) (bacitracin and daptomycin) values are shown. The colored sensitivity scale represents the rounded fold changes of the MIC or MIC_{50} relative to wild-type *L. lactis* LMGT 3419. All mutants showed unchanged sensitivity to the other antibiotics tested, including inhibitors of cell wall biosynthesis (amoxicillin, ampicillin, carbenicillin, cefuroxime, cephalothin, ramoplanin, and vancomycin), inhibitors of DNA biosynthesis (ciprofloxacin and norfloxacin), inhibitors of protein biosynthesis (chloramphenicol, chlortetracycline, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, and tetracycline), an inhibitor of folate biosynthesis (trimethoprim), and an inhibitor of DNA, RNA, and protein biosynthesis, respiration, and pyruvate metabolism (nitrofurantoin).

Compared with the wild type, the mutants showed the strongest decrease in sensitivity to K411 and Ent7 (16-fold to both) and a 2- to 8-fold decrease in sensitivity to the other bacteriocins tested, with sensitivity to WelM and SalC being the least affected (Table 2). Thus, the level of resistance did not dependent on the type of mutation (nonsense, frameshift, or missense) or the mutated gene (*ysaC*, *ysaB*, or *llrG*) but rather was bacteriocin specific (Table 2).

We attempted to complement the mutations in *ysaC*, *ysaB*, and *llrG* by cloning a wild-type copy of the respective genes into the expression vector pIBB-JZK under the control of the cellobiose-responsive *ptcB* promoter (P*ptcB*). However, cloned *ysaCB* turned out to be toxic, and we were able to complement only the *llrG* defect carried by the MUT_12 strain. The expression of *llrG* from the pIBB-JZK*llrG* construct fully recovered the sensitivity of the strain to all the bacteriocins tested (Table 2).

In the spontaneous bacteriocin-resistant mutants, the affected proteins could still be expressed, either in a truncated form or carrying single-amino-acid substitutions, which might suggest that they could have retained some activity. To investigate the effects of the complete absence of these proteins, we constructed a series of single, double, and triple *ysaCB* and/or *llrG* deletion mutants (MUT_400, MUT_401, MUT_402, MUT_403, and MUT_404) and assayed them for sensitivity to the selected bacteriocins. All these strains showed unchanged or even slightly increased (2-fold or lower) sensitivity to the agents tested (Table 2), indicating that the resistance of the spontaneous mutants was due to altered functioning of the affected proteins and not a loss of their function (i.e., the spontaneous mutations could be classified as gain-of-function rather than loss-of-function ones).

Antibiotic susceptibility of *L. lactis* mutants. To determine whether the spontaneous mutations conferring resistance to bacteriocins also affected sensitivity to antibiotics, we assayed these mutants and also those with gene deletions or complementations for comparison. For these assays, we used antibiotics acting on different targets such as the membrane (daptomycin and gramicidin), cell wall biosynthesis (amoxicillin, ampicillin,

bacitracin, carbenicillin, cefuroxime, cephalothin, ramoplanin, and vancomycin), DNA replication (ciprofloxacin and norfloxacin) or translation (chloramphenicol, chlortetracycline, clindamycin, erythromycin [Em], gentamicin, kanamycin, streptomycin, and tetracycline [Tet]), folate synthesis (trimethoprim), or respiration and pyruvate metabolism (nitrofurantoin). Only sensitivity to bacitracin, daptomycin, and gramicidin was altered in the mutants compared to the wild-type strain. Sensitivity to bacitracin was increased severalfold (6- to 8-fold in most cases) in all the mutants, the most so (8-fold) in the mutants lacking the *llrG* gene (*llrG* deletion mutants MUT_403 and MUT_404) (Table 2). Notably, the point mutation in this gene present in the MUT_12 strain actually had an opposite effect, as it slightly decreased the sensitivity to bacitracin in comparison to MUT_402 (Table 2), which may suggest a protective function of this mutation in *llrG* with respect to bacitracin resistance. Similarly, the deletion of the *ysaC* gene (MUT_400) increased sensitivity 6-fold, while a point mutation in this gene (MUT_40) had a minor effect only (1.5-fold) (Table 2). In contrast to bacitracin, the sensitivity of some mutants to daptomycin or gramicidin was reduced. Notably, only the spontaneous point mutations showed increased resistance (between 2- and 4-fold to gramicidin and 10- to 20-fold to daptomycin), while the deletions had virtually no effect (Table 2). Complementation of the *llrG* mutation present in MUT_12 with a wild-type copy of the *llrG* gene (MUT_406) partially reversed its effect; i.e., it increased the sensitivity to daptomycin compared with the control strain (MUT_405) (Table 2).

The YsaB region containing the FtsX domain induces resistance to membrane-acting antimicrobials. Prediction of transmembrane helices indicated that the YsaC ATPase is intracellular, whereas the YsaB permease is a transmembrane protein with 10 membrane-spanning helices (TMHs) (Fig. 2). A search of the YsaB amino acid sequence for conserved domains (CDs) revealed the FtsX domain at its N terminus (Pfam accession no. PF02687) spanning 98 amino acids, including TMH3 and -4. It is noteworthy that none of the three mutations in the 5′ portion of *ysaB* affected the FtsX domain. All three mutations were nonsense mutations and immediately followed this region (Fig. 2), thus leading to a significantly truncated YsaB protein comprising only the FtsX domain. To test the functionality of the isolated FtsX domain apparently spared in the mutants and to verify its indirect involvement in the development of resistance, we expressed *ysaC* followed by a 5′ part of *ysaB* encoding only the FtsX domain ($ysaCB_{1-578}$) under the control of its native promoter in the Δ*ysaCB L. lactis* strain MUT_402 (producing strain MUT_408 [Δ*ysaCB*::pGh$ysaCB_{1-578}$]). Compared to the control with the empty pGhost9 vector (MUT_407), MUT_408 showed significantly reduced sensitivity to all bacteriocins tested, as well as to daptomycin and gramicidin, and unchanged sensitivity to bacitracin (Table 2). Thus, it is tempting to speculate that the FtsX domain freed from the C-terminal part of YsaB indirectly confers selective resistance to certain antimicrobials similar in scope and extent to that observed in the spontaneous *ysaB* mutants.

Also, the single spontaneous missense mutation identified in *ysaC* was sufficient to reduce the sensitivity to AurA53- and EntL50-like bacteriocins, Lcn972, nisin, and daptomycin, indicating an important role of YsaC in resistance as well. To test whether the presence of intact YsaC is essential for the protective effect of the FtsX domain, we expressed $ysaB_{1-578}$ without the *ysaC* gene in *L. lactis* MUT_402 (MUT_409). Compared with MUT_407 carrying empty pGhost9, MUT_409 showed unchanged sensitivity to all the bacteriocins and antibiotics tested (Table 2), suggesting that the resistance-conferring properties of FtsX can be developed only in the presence of YsaC. To determine whether the FtsX domain encoded by pGh$ysaB_{1-578}$ was indeed expressed and functional, we introduced it into the Δ*ysaB* strain MUT_401 with the *ysaC* gene intact. Compared to the control strain MUT_411 carrying empty pGhost9, the presence of $ysaB_{1-578}$ (MUT_412) significantly reduced the sensitivity to all bacteriocins, daptomycin, and gramicidin, confirming the functionality of FtsX under these conditions (Table 2).

The transcriptional regulator LlrG is essential for FtsX-dependent resistance. Prediction of the tertiary structure of the response regulator LlrG revealed that the Leu177Ile substitution found in the spontaneous mutant MUT_12 maps to the winged

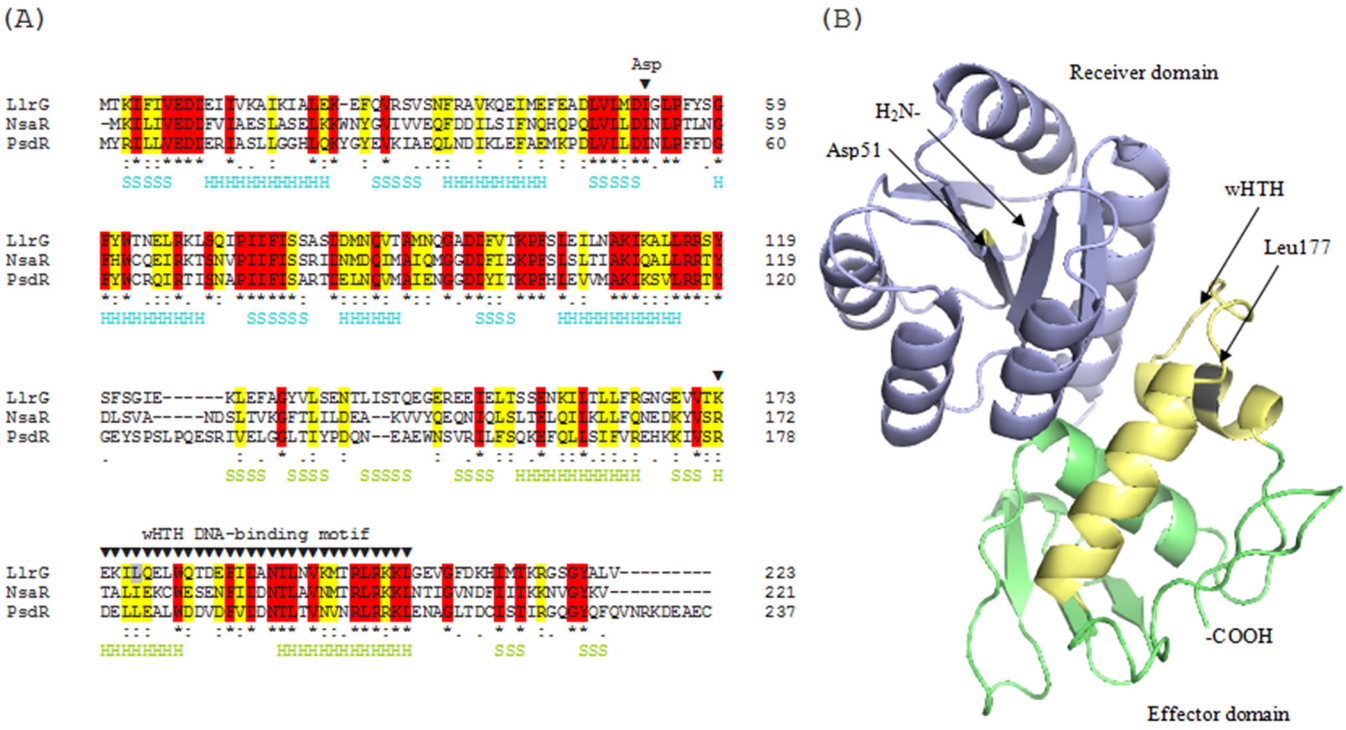


FIG 3 Localization of spontaneous, resistance-conferring mutations in the primary (A) and predicted tertiary (B) structures of *L. lactis* LlrG. In panel A, an alignment of the amino acid sequences of the response regulators LlrG, PsdR, and NsaR from *L. lactis* LMGT 3419, *B. subtilis* 168, and *S. aureus* MOK042, respectively, is shown. Stars mark fully conserved residues, and colons and dots mark partially and weakly conserved ones. Identical and similar amino acids are highlighted in red and yellow, respectively. Predicted LlrG secondary structure elements are indicated with H (α-helix) and S (β-strand). The H- and S-forming receiver and OmpR/PhoB-type effector domains are distinguished with blue and green, respectively. The triangles indicate a residue undergoing phosphorylation (Asp51) and the wHTH DNA-binding motif. The residue changed by a missense mutation (Leu177) is highlighted in gray. The NCBI RefSeq accession numbers are WP_003244535.1 and WP_063644489.1 for PsdR and NsaR, respectively. In panel B, receiver and OmpR/PhoB-type effector domains are distinguished with blue and green, respectively. The wHTH motif is marked in yellow, and Asp51 and Leu177 residues are marked in gray. The template of the highest significance used by the I-TASSER Web service to predict the tertiary model of LlrG can be found in Table S1 in the supplemental material.

helix-turn-helix (wHTH) DNA-binding motif of the C-terminal OmpR/PhoB-type effector domain of LlrG (Fig. 3). Such a localization suggests that it could have a significant effect on LlrG activity. Since the deletion of the *llrG* gene had at most a minor effect on the resistance to bacteriocins, we hypothesized that the Leu177Ile substitution increased LlrG activity rather than abolished it. To test this hypothesis, *llrG* cloned into the high-copy-number pGhost9 vector under the control of the strong P*ptcB* promoter was expressed in *L. lactis* MUT_402. The overexpression of *llrG* significantly decreased the sensitivity of the resulting MUT_410 strain to all bacteriocins tested. In contrast, the sensitivity to bacitracin, daptomycin, and gramicidin was not affected (Table 2).

To test whether LlrG is essential for FtsX domain activity, we examined the sensitivity to antimicrobials of strains with a deletion (*L. lactis* MUT_404 strain) or point mutation (*L. lactis* MUT_12) in the *llrG* gene also carrying $ysaCB_{1-578}$. Compared with the Δ*ysaCB* Δ*llrG* MUT_413 strain harboring empty pGhost9, the presence of $ysaCB_{1-578}$ (MUT_414) did not affect the sensitivity to any of the antimicrobials tested (Table 2). In contrast, in the Δ*ysaCB* with point mutation in *llrG* strain, $ysaCB_{1-578}$ (MUT_416) slightly decreased the sensitivity to BHT-B, Ent7, EntL50, WeiM, SalC, nisin, and daptomycin (Table 2). This decrease was of a substantially lower magnitude than the one caused by $ysaCB_{1-578}$ in the presence of nonmutated *llrG* (Table 2, compare MUT_408 versus MUT_407 with MUT_416 versus MUT_415), indicating that the modes of action of LlrG and FtsX largely overlap. Taken together, these results indicate that in the absence of LlrG, the resistance dependent on the FtsX domain cannot be expressed. Whether this defect is due to a lack of expression of $ysaCB_{1-578}$ or a nonfunctionality of FtsX remains to be established. The nonadditivity of the *llrG* point mutation and $ysaCB_{1-578}$ effects

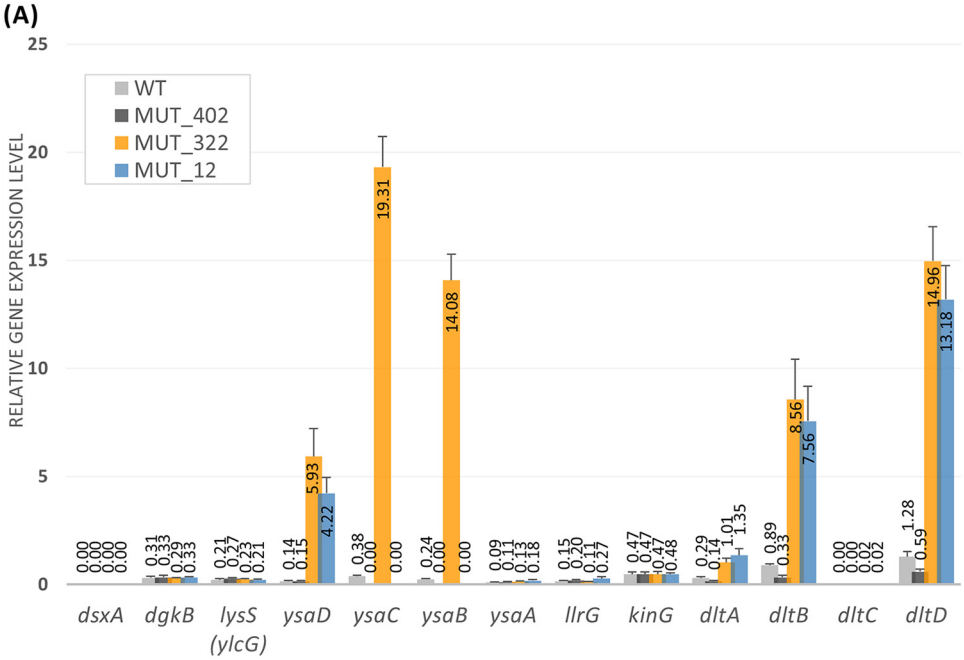
further confirms that LlrG affects the sensitivity to antimicrobials by modulating the expression of genes encoding YsaCB.

Point mutations in *llrG* or *ysaB* result in increased expression of the *ysaDCB* and *dltABCD* operons. To quantitatively test the effect of *llrG* and *ysaB* mutations on the expression of the *L. lactis* resistome, we compared by reverse transcription-quantitative PCR (RT-qPCR) the mRNA levels of individual genes potentially involved in the response to the presence of antimicrobial compounds in the *L. lactis* wild-type strain and its mutants MUT_12 (Δ*ysaCB* and *llrG* point mutation), MUT_322 (*ysaB* deletion), and MUT_402 (Δ*ysaCB*). For these assays, we selected genes from the operons *ysaDCB*, *ysaA-llrG-kinG*, and *dltABCD*; the single genes *dsxA* and *dgkB* previously identified as being involved in conferring AurA53- and EntL50-like bacteriocin resistance (29); and the *lysS* gene (*ylcG*), which was selected as the closest homolog of *mprF* from *B. subtilis*. Comparably low expression levels of most of the genes tested were detected in the *L. lactis* wild type and MUT_402, with *dltC* not being transcribed at all in these two strains. Also, the expression levels of the *ysaA-llrG-kinG*, *dgkB*, and *lysS* (*ylcG*) genes were similarly low in all strains tested, while *dxsA* was not expressed in any strain (Fig. 4A). In contrast, *ysaDCB* and *dltABCD* were upregulated in the MUT_322 mutant. Increased transcription of these genes was also found in MUT_12, with the exception of *ysaBC*, which were deleted in this mutant, and therefore, their expression was undetectable (Fig. 4A). The mutation in *ysaB* had the strongest impact on *ysaDCB* gene expression, as >40-fold activation of their transcription was detected in MUT_322 compared to wild-type *L. lactis* (Fig. 4B). The strong activation of *ysaDCB* in MUT_322 was followed by up to 12-fold induction of the *dlt* operon genes, among which the *dltA* expression level was lower but still 3.5-fold compared to that in the wild-type strain. A similar effect of a nearly 30-fold increase in the *ysaD* transcript level and ca. 10- to 20-fold upregulation of the *dlt* operon genes was induced by the *llrG* point mutation in the absence of *ysaCB* (MUT_12) compared to MUT_402 (Fig. 4B).

DISCUSSION

In recent years, antibiotic-resistant bacterial strains have been emerging faster than new antimicrobials are being developed and approved for medical use. AurA53- and EntL50-like bacteriocins are saposin-like, broad-spectrum, lipid-interacting antimicrobials that, owing to their unique characteristics, offer a promising alternative to combat infections caused by antibiotic-resistant pathogens (6). In contrast to the numerous studies on their physicochemical properties, structure, activity, and mode of action, mechanisms leading to the development of resistance to these agents have never been investigated. Since such resistance may limit their future application, detailed studies are needed to minimize this potential problem. Here, we studied the *L. lactis* resistome involved in the appearance of resistance to four known (BHT-B, Ent7, EntL50, and WelM) and two newly identified (K411 and SalC) AurA53- and EntL50-like bacteriocins and its relationship with susceptibility to antibiotics targeting the cell envelope. We showed that these bacteriocins have broad-spectrum activity, including strains of *E. faecalis*, *E. faecium*, *S. aureus*, and *Streptococcus* spp., whose antibiotic-resistant mutants are currently the most prevalent causes of severe, hospital-acquired infections (47), as well as strains of *L. monocytogenes* and *C. jejuni* responsible for foodborne infections (48), suggesting their likely effectiveness in clinical use. Moreover, the inhibitory activity of AurA53- and EntL50-like bacteriocins against *Lactococcus garvieae*, the etiological agent of lactococcosis in fish and cow mastitis (49, 50), may be a rationale for their use in veterinary medicine also. Most of the other lactic acid bacteria examined were sensitive to the tested bacteriocins, which may be a limitation for their use as natural preservatives during fermentation processes in the dairy industry. However, since oral lactobacilli are one of the etiological factors in the development of dental caries (51), it is conceivable that AurA53- and EntL50-like bacteriocins may find application in dental care products.

Our results indicate that resistance to AurA53- and EntL50-like bacteriocins can develop as an indirect result of point mutations in the *ysaCB* or *llrG* gene encoding the YsaCB-KinG-



(B)

	MUT_402	MUT_322	MUT_12
dsxA	-	-	-
dgkB	⇨ 1.1	⇨ 0.9	⇨ 1.0
lysS (ylcG)	⇨ 1.3	⇨ 1.1	⇨ 0.8
ysaD	⇨ 1.0	⇧ 41.0	⇧ 28.4
ysaC	-	⇧ 51.0	-
ysaB	-	⇧ 57.8	-
ysaA	⇨ 1.2	⇨ 1.3	⇨ 1.6
llrG	⇨ 1.3	⇨ 0.7	⇨ 1.4
kinG	⇨ 1.0	⇨ 1.0	⇨ 1.0
dltA	⇘ 0.5	⇗ 3.5	⇗ 9.4
dltB	⇘ 0.4	⇗ 9.6	⇧ 22.8
dltC	-	-	-
dltD	⇘ 0.5	⇧ 11.7	⇧ 22.2

FIG 4 Relative gene expression levels in the *L. lactis* wild type (WT) and MUT_12, MUT_322, and MUT_402 mutants. (A) mRNA levels determined by RT-qPCR. (B) Fold induction of gene expression in the MUT_402, MUT_322, and MUT_12 mutants compared with the wild-type strain and the MUT_402 mutant. "−" indicates the inability to determine the induction rate due to undetectable gene expression in at least one of the strains used in the comparison. Means from at least three biological repeats and standard deviations (SDs) (error bars) are presented.

LlrG peptide-sensing and detoxification (PSD) module (Fig. 5). This is one of the most important systems regulating the stress response in the cell envelope and was speculated to protect *L. lactis* from AurA53- and EntL50-like bacteriocins, but no studies have been performed to confirm those speculations (29). It is believed to be a homolog of the prototypical BceAB-BceRS PSD module from *B. subtilis* (27, 28), which mediates resistance to peptide antimicrobials such as bacitracin, mersacidin, actagardine, and plectasin (23). Nonetheless, since YsaCB-KinG-LlrG also shares comparable amino acid sequence similarity with two other *B. subtilis* Bce-like modules, PsdAB-PsdRS and YxdLM-YxdJK, which respectively protect against nisin, subtilin, and gallidermin and the cationic antimicrobial peptide LL-37 (23), it therefore cannot be considered a direct functional equivalent of any of the three. So far, the lactococcal YsaCB-KinG-LlrG module has been shown to protect against only nisin and Lcn972 (28, 46). Here, in spontaneous *L. lactis* mutants resistant to AurA53- and EntL50-like bacteriocins, we identified single point mutations in three genes encoding the

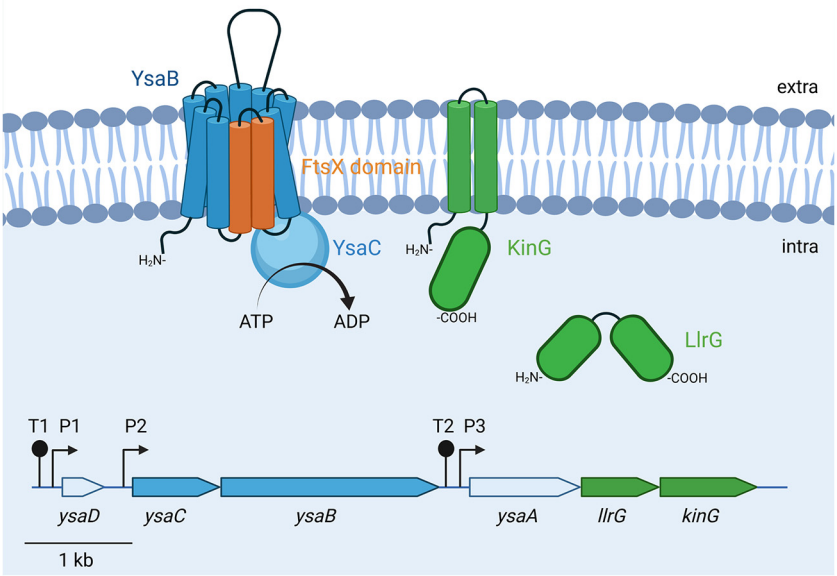


FIG 5 Schematic representation of the YsaCB-KinG-LlrG stress response-regulatory system and the DNA regions encoding the respective proteins. The components of the system are the ATP-binding domain ABC transporter YsaC encoded by *ysaC*, the ABC transporter permease YsaB encoded by *ysaB*, the histidine kinase KinG encoded by *kinG*, and the response regulator LlrG encoded by *llrG*. The *ysaD* and *ysaA* genes encode a bitopic membrane protein of the YxeA family and a polytopic membrane protein containing a VanZ domain (Pfam accession no. PF04892) that is found in glycopeptide antibiotic resistance proteins, respectively. The T1 and T2 transcription terminators and the P1 and P2 promoters were identified previously (28), whereas the putative P2 promoter (5′-ATGAAG/20 nt/TAAAAT-3′) was identified in the present study. (The figure was created with BioRender.)

lactococcal YsaBC-KinG-LlrG system. Mutations in *ysaB* were the most common, but those in *ysaC* and *llrG*, although less frequent, were also sufficient to confer the same level of resistance to the bacteriocins tested, indicating that the components of the YsaBC-KinG-LlrG system more or less equally participate in the mechanism of resistance development through the accumulation of mutations. Notably, the mutation in *llrG* conferred resistance to bacteriocins in the absence of *ysaCB*, pointing to its independence from this ABC transporter. In previously obtained *L. lactis* mutants resistant to Lcn972, the causative mutations caused amino acid substitutions in the middle and C-terminal parts of the YsaB protein, specifically in the intracellular loops between TMH4 and -5 or TMH8 and -9 or within TMH7 and TMH9 (27, 28). Here, we observed a similar localization of mutations, but they were wholly different, nonsense or frameshift rather than missense, and they all led to the truncation of YsaB, sparing its N-terminal FtsX domain. Since none of the single, double, or triple deletion mutants of *ysaB*, *ysaC*, and/or *llrG* that we constructed were resistant to any of the bacteriocins tested, we concluded that the resistance of the spontaneous mutants was not due to an inactivation of the encoded proteins but instead relied on specific changes in their functionality. Also, previously reported point mutations in *ysaCB*, *kinG*, or *llrG* have been proposed to decrease *L. lactis* sensitivity to Lcn972 and nisin, and those localized in *ysaB* were shown to cause the constitutive expression of *ysaDCB* (27, 28). Similar gain-of-function mutations in the *nsaS* and *nsaR* genes encoding the two-component system (TCS) in *S. aureus* have also been identified as being responsible for nisin resistance. It was shown that nisin by itself could not sufficiently induce NsaSR to trigger resistance, whereas the mutations in *nsaS*, *nsaR*, or their promoter led to the constitutive activation of NsaSR and the upregulation of its regulon, resulting in nisin resistance (52, 53). Such mutation-driven activation of the TCS can lead to a modulation of the expression of various genes that protect cells from cell envelope stress via diverse mechanisms. For example, in *B. subtilis*, BceRS and PsdRS regulate the expression of the cognate ABC transporters only, while YxdJK mediates resistance to LL-37 through likely indirect control of

dltABCD operon expression as well (54, 55). Similarly, the GraRS TCS in *S. aureus* mediates resistance to nisin (56), vancomycin, and polymyxin B (57) by regulating the expression of the cognate ABC transporter as well as of the *mprF* gene and the *dltABCD* operon (58). Here, it was tempting to speculate that the KinG-LlrG TCS of *L. lactis* could regulate the expression of the *lysS* (*ylcG*) and/or *dlt* genes to introduce positive charges into the cell envelope, thereby preventing its interaction with the positively charged bacteriocins. This hypothesis is supported by the fact that a putative BceR-like binding box was detected in the promoter of the *dltABCD* and *ysaDCB* genes in *L. lactis* (24, 28). Indeed, in this study, both operons responded with upregulation to a point mutation in *llrG*, suggesting the functionality of both operators and the role of LlrG modification in conferring resistance to the bacteriocins tested, most probably by activating Dlt-dependent cell wall modification. On the contrary, modified LlrG does not appear to be involved in the regulation of the gene expression of *lysS* (*ylcG*), the closest lactococcal homolog of *mprF* from *B. subtilis* involved in protection against antimicrobials through cell membrane modification. One could also assume that KinG-LlrG upregulates the *dgkB* and *dxsA* genes for lipid metabolism, previously proposed to be involved in membrane remodeling for protection against AurA53- and EntL50-like bacteriocins (29); however, this study showed that both genes are beyond LlrG control. The role of the point mutation in *ysaB* in the mechanism of resistance to the bacteriocins and peptide antibiotics tested remains unclear. This mutation, like that in *llrG*, also leads to the upregulation of *dltABCD* but without an increase in *llrG* transcription, suggesting that the activation of LlrG action occurs at the protein rather than the gene level.

Bacteriocins are often thought to have different mechanisms of action, and thereby also to require distinct resistance mechanisms, than commonly used antibiotics and consequently should be active against antibiotic-resistant pathogens (59). However, we show here that bacteriocins targeting the cell envelope, such as the AurA53- and EntL50-like ones, nisin, and Lcn972, evoke a cell response similar to those of some antibiotics, i.e., engaging the same PSD module, and therefore can be subject to the same resistance mechanisms, which are activated after specific point mutations in genes encoding module components. So far, only a few studies have shown a correlation between resistance to nisin on the one hand and resistance to bacitracin, gramicidin, and some β-lactams on the other (60–62) or between Lcn972 and bacitracin, vancomycin, and penicillin G (28, 63). Here, of the wide range of antibiotics tested against the AurA53- and EntL50-like bacteriocin-resistant mutants, significant correlations were observed only for membrane-acting peptide antibiotics such as daptomycin and bacitracin targeting the lipid II cycle UPP intermediate. The changes in resistance levels of spontaneous mutants observed for another membrane-acting antibiotic, gramicidin, were of a lesser degree than those for daptomycin, which suggests that the resistance mechanisms for these two antibiotics may not be identical. Notably, we found no changes in resistance to antibiotics acting on intracellular or nonmembrane targets of the cell envelope, such as various lipid II moieties. Daptomycin is currently the frontline treatment for infections caused by antibiotic-resistant *S. aureus* or *Enterococcus* spp., and daptomycin resistance is most commonly associated with mutations in genes involved in the cell wall and membrane modifications (*mprF*, *dltABCD*, and *pgsA*) and the stress response (*liaFSR* and *vraSR*) (16, 64). Gramicidin, because of its toxicity, is used less extensively, and the mechanism of resistance is still poorly understood. It has previously been linked to an increased content of etherized D-alanine in teichoic acid (65), while increased sensitivity to gramicidin was observed in a *Streptococcus pneumoniae* mutant lacking the YsaCB homolog (61). Here, we propose the activation of the YsaCB-KinG-LlrG module in *L. lactis* through diverse gain-of-function mutations in the *ysaCB* or *llrG* gene as an indirect mechanism of resistance to these two membrane-targeting peptide antibiotics. In turn, the upregulation of the *dltABCD* genes induced by the modified LlrG leading to a reduction in the negative charge of the cell envelope seems the most likely direct resistance mechanism responsible for the repulsion of cationic calcium-daptomycin complexes.

Bacitracin resistance has previously been linked with YsaCB in *L. lactis* (28). While the mechanism of lactococcal YsaCB-dependent resistance is unknown, it has been elucidated for its homolog in *B. subtilis*, the BceAB transporter, which forms a sensory complex in the cytoplasmic membrane through a direct interaction between the permease BceB and the histidine kinase BceS (22). BceS monitors the ability of BceAB to detoxify through a flux-sensing mechanism and activates BceAB expression by phosphorylating the response regulator BceR (20). Subsequently, BceAB binds to the bacitracin-UPP complex through the action of a large extracellular loop of BceB and releases this cell wall precursor from the grip of the antibiotic, thereby allowing uninterrupted murein synthesis (21, 66). Here, we show that the mechanism of bacitracin resistance, although based on the same YsaCB-KinG-LlrG module, differs from that acting on the bacteriocins studied here or membrane-acting antibiotics. Since most of the mutations affecting this module, be it the spontaneous point mutations or deletions of individual genes, increased the sensitivity to bacitracin, it can be assumed that this system works against it most efficiently in its native form, whereas resistance to the other antimicrobials tested here requires specific mutations. An exception to this rule was a point mutation in *llrG*, which resulted in decreased sensitivity to bacitracin compared to the sensitive parental strain MUT_402. Hence, extrapolating the available data from *B. subtilis*, we propose that mutations in *L. lactis* that shortened or deprived the cell of YsaB thereby prevented its UPP-releasing activity or inhibited flux sensing between YsaCB and KinG, leading to the hypersensitivity of the mutants to bacitracin. On the other hand, the protective effect against bacitracin of the point mutation in *llrG* may be due to the enhancement of the activity of the encoded response regulator and the resulting activation of *dltABCD* and *ysaD* mediated by the modified LlrG, as we demonstrated by quantitative analysis of the expression of these genes.

In addition to the large extracellular loop of the YsaB permease, we identified an FtsX domain at its N terminus and propose it to be an indirect, critical driver of resistance against the envelope-targeting bacteriocins, daptomycin, and gramicidin but not bacitracin. No information is available on the determinants of the contribution of FtsX to resistance, but over 260 transporters containing this domain have been identified in genomes of *Firmicutes* and classified as BceAB-type transporters (67). Here, using Pfamseq data, we identified several proteins with a YsaB-like architecture containing the FtsX domain at their N termini and a large extracellular loop, such as ABC transporters from three Bce-like modules in *B. subtilis* or the VraDE transporter form *S. aureus*. The FtsX domain is present at the C termini of the FtsX, MacB, and LolC proteins of *Escherichia coli*, members of the MacB transporter superfamily that are involved in cell division, resistance to macrolide antibiotics, and the transport of lipoproteins, respectively (68). Nevertheless, it has not been confirmed that the functions of the proteins containing the FtsX domain are due to its presence and not to their other domains. However, our present observation that all *ysaB* point mutations increasing the resistance to bacteriocins were downstream of this domain strongly indicated its critical role. This conclusion was confirmed by expressing the FtsX domain alone in *trans*, which also caused resistance. The mechanism by which the FtsX domain confers resistance remains unknown, but its action is clearly dependent on both LlrG and YsaC since in the absence of either of them, FtsX failed to cause resistance. On the other hand, the overexpression in *trans* of *llrG* or a specific amino acid substitution affecting its ability to activate the transcription of *dltABCD* was sufficient to confer resistance even in the absence of FtsX. This suggests that the modified or overproduced LlrG confers resistance to diverse antimicrobials by activating Dlt-mediated cell wall remodeling independently of the FtsX domain or, for that matter, even the whole YsaCB transporter.

Altogether, our results show that *L. lactis* readily acquires resistance to AurA53- and EntL50-like bacteriocins and cross-resistance to membrane-active peptide antibiotics through gain-of-function point mutations in genes encoding components of the YsaCB-KinG-LlrG PSD module. In the case of the YsaB permease, the critical driver of resistance is the trimming off of the N-terminal FtsX domain from the central and C-

terminal parts. The function of the FtsX domain requires the presence of the ATPase YsaC and the transcriptional regulator LlrG, suggesting a cascade of events from activating mutations in the ABC transporter YsaCB, through the LlrG-dependent activation of the *dltABCD* genes involved in protective cell wall remodeling. In contrast, genes involved in cell membrane modifications in response to cellular stress appear to be independent of LlrG-mediated control. A shortcut to resistance bypassing the YsaCB system is also possible through the direct activation of LlrG by mutation. Owing to the distinct resistance mechanisms resulting from the two-domain structure of YsaB, the acquisition of resistance to AurA53- and EntL50-like bacteriocins and membrane-active peptide antibiotics concomitantly increases the susceptibility to bacitracin, which provides a rationale for designing multicomponent formulations with an appropriately selected composition allowing the risk of the development of resistance to be minimized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The microorganisms and plasmids used are listed in Table S1 in the supplemental material. Lcn972-producing *L. lactis* IPLA 972 and nisin-producing *L. lactis* LMGT 4215 were grown in M17 medium (Oxoid, UK) supplemented with 0.5% glucose (GM17). All indicator strains were grown in brain heart infusion (BHI) medium (Oxoid, UK) except for *Campylobacter jejuni*, which was grown in blood agar base no. 2 (Oxoid, UK). *L. lactis* LMGT 3419-derived strains with spontaneous mutations or deletions and their derivates carrying pGhost9 with the *ysaCB* genes cloned were grown in BHI medium. Strains carrying pIBB-JZK or pGhost9 with the *llrG* gene cloned under the control of the cellobiose-responsive P*ptcB* promoter were grown in GM17 or M17 medium supplemented with 1% cellobiose (CM17) to induce *llrG* expression. *Enterobacter* sp. and *Weissella confusa* were incubated at 30°C under aerobic conditions without shaking, *Clostridium beijerinckii* was grown at 37°C under anaerobic conditions without shaking, and all other strains were incubated as described previously (69). When appropriate, erythromycin (Em) or tetracycline (Tet) was added to 5 μg/ml or 10 μg/ml, respectively. Soft agar and agar plates were prepared by adding agar (Merck, Germany) to 0.75% and 1.5%, respectively.

Bacteriocin preparation. Lyophilized K411, BHT-B, Ent7 (peptides Ent7A and Ent7B), EntL50 (peptides EntL50A and EntL50B), WelM, and SalC bacteriocins with a purity of over 95% were synthesized by a commercial service (PepMic, People's Republic of China). Stock solutions (1 mg/ml) were prepared by dissolving the bacteriocins in 0.1% trifluoroacetic acid (TFA; Sigma, Germany). Nisin Z was purified from *L. lactis* LMGT 4215 by ammonium sulfate precipitation followed by cation exchange and reverse-phase chromatography (RPC) as previously described (70). After RPC, the fractions with the highest antimicrobial activity were pooled, and the presence of nisin Z was confirmed by mass spectroscopy, which showed a distinct peak corresponding to the nisin Z monoisotopic mass (3,329 Da) (data not shown). The purity of nisin Z after RPC was 87% (assessed by analytical RPC–high-performance liquid chromatography [HPLC]). The protein concentration was assessed by a Qubit 3.0 fluorometer (Thermo Fisher Scientific), and the final concentration of nisin Z was 0.45 mg/ml. Lcn972 was purified from *L. lactis* IPLA 972 by consecutive cation exchange and hydrophobic interaction chromatography as previously described (71). Purity was determined by the tricine-SDS-PAGE method (72). No protein bands above 1.8 kDa were detected. Purity was estimated to be above 90%. The protein concentration was 0.12 mg/ml, determined with a bicinchoninic acid (BCA) kit (Pierce, Belgium) using bovine serum albumin as a reference.

Inhibitory spectrum assay and generation of spontaneous resistant mutants. The activity spectrum of bacteriocins was determined using the spot-on-lawn inhibition spectrum assay as described previously (73). Spontaneous resistant mutants were selected by growing *L. lactis* LMGT 3419 or *L. lactis* MUT_402 in the presence of a bacteriocin as described previously (73). Briefly, 100 μl of a culture of the parental strain grown overnight and bacteriocin to the concentration listed below were added to 5 ml of soft BHI agar and poured onto the BHI agar plate. Subsequently, the plate was incubated at 30°C for resistant bacterial colonies to appear. The following mutants were obtained: MUT_40, MUT_44, MUT_46, MUT_47, MUT_49, MUT_50, MUT_92, MUT_94, and MUT_12 at a K411 concentration of 1 mg/ml; MUT_123 and MUT_125 at a K411 concentration of 0.5 mg/ml; MUT_2 and MUT_29 at a K411 concentration of 0.01 mg/ml; MUT_325 at an Ent7 concentration of 2 μg/ml; and MUT_314, MUT_318, MUT_321, MUT_322, and MUT_324 at an Ent7 concentration of 1 μg/ml. The isolation scheme for the bacterial mutants is shown in Fig. S1 in the supplemental material. The sensitivity of the mutants was determined using microtiter plates with serial 2-fold bacteriocin dilutions as described previously (73). The lowest bacteriocin concentration reducing bacterial growth by at least 50% was taken as the MIC_{50}.

DNA isolation and manipulation. Genomic DNA for PCR or sequencing was isolated using a genomic minikit (A&A Biotechnology, Poland) and prepared with a Nextera XT DNA sample preparation kit, a Nextera XT indexing kit, and a PhiX control V3 kit (Illumina, USA) according to the manufacturer's instructions. Samples were sequenced using a MiSeq sequencer (Illumina). PCR products or DNA fragments from agarose gel were purified with a Wizard SV gel and PCR cleanup system (Promega, USA). Plasmid DNA was isolated using a plasmid minikit or a plasmid midi AX kit (A&A Biotechnology). All DNA restriction and modification enzymes were purchased from Thermo Fisher Scientific.

Bioinformatic analysis. Genome sequencing results were analyzed using CLC Genomics Workbench 6.5.2 (Qiagen, Germany). Between 1.5 million and 2.5 million 250-nucleotide (nt) paired-end reads were obtained for each sequenced strain. The average depth coverage was 98.78%, and the breadth of coverage was 99.9995 to 100%. Only mutations that occurred at positions with high coverage (the number of reads ranged from 25× to 101×, and the average number of reads was 62×) and had a high frequency of occurrence (occurring in 90 to 100% of the reads) were considered significant. Nucleotide sequences were translated *in silico* to protein sequences using the translate tool on the ExPasy online server (74). Protein sequences were compared using MultAlin software (75) or Clustal Omega software at the EMBL-EBI online server (76). Conserved domains (CDs) were identified using the CD-search tool of the NCBI Conserved Domain Database (77) or the Pfam Database (78). Transmembrane helices were predicted with the HMMTOP server (79). The YsaB primary structure was visualized with Protter (80). Template-based 3D models were built using the I-TASSER Web service (81). Templates of the highest significance used by I-TASSER and the quality indicators of the predicted models (confidence score [C-score], template-modeling score [TM-score], and root mean square deviation [RMSD]) are summarized in Table S1. The PyMOL molecular graphics system, version 2.0 (Schrödinger, LLC), was used to visualize the 3D models, and the PPM Web server was used to predict the orientation of the YsaB 3D model in the membrane (82).

Construction of deletion mutants. Deletions of the *ysaC*, *ysaB*, *ysaCB*, and/or *llrG* gene were performed using homologous recombination. First, DNA fragments upstream (UP) and downstream (DN) of the target gene(s) were amplified with suitable primer pairs, ATPUPfor/rev and ATPDNfor/rev for *ysaC*, PERUPfor/rev and PERDNfor/rev for *ysaB*, ATPUPfor/rev and PERDNfor/rev for *ysaCB*, and LlrGUPfor/rev and LlrGDNfor/rev for *llrG* (Table S1). After amplification, PCR products were purified, digested with EcoRI, and ligated with the use of T4 DNA ligase. An additional PCR was performed using the UPfor and DNrev primers to amplify the joined flanking fragments. PCR products were purified and cloned into the pGEM-T Easy vector (Promega, USA) by TA cloning, and the correctness of the sequences was verified by sequencing with primer pair 1224/1233 (Table S1). Next, the inserts were cloned into pGhost9 using ApaI and NotI, and the presence of the inserts was confirmed with primer pair pGhfor/rev (Table S1). To force double-crossover recombination, *L. lactis* LMGT 3419 cultures harboring DNA fragments flanking the *ysaC*, *ysaB*, *ysaCB*, or *llrG* gene and an *L. lactis* MUT_402 culture harboring DNA fragments flanking the *llrG* gene were diluted 10^3-fold in BHI medium supplemented with Em (5 μg/ml) and incubated for 1.5 h at 30°C and then for 3.5 h at 37°C. Afterward, the cultures were streaked onto BHI agar plates containing Em (5 μg/ml) and incubated at 37°C to select integrants. To remove the integration vector, the integrants were cultivated in the absence of antibiotics at 30°C. The deletion of the targeted genes was confirmed by colony PCR with primer pairs ABCOUTfor/rev for *ysaCB* and LlrGOUTfor/rev for *llrG* (Table S1). The absence of pGhost9 was confirmed by colony PCR with primer pair pGhfor/rev and susceptibility tests with Em. The isolation scheme for the bacterial mutants is shown in Fig. S1 in the supplemental material.

Construction of expression mutants. The pGhost9 vector was used to express the *ysaC* gene plus the truncated *ysaB* gene encoding the FtsX domain only (*ysaCB*$_{1–578}$) or truncated *ysaB* only (*ysaB*$_{1–578}$). First, *ysaCB*$_{1–578}$ with the native promoter was amplified with primer pair YsaCBfor/FtsXrev (Table S1) using genomic DNA of *L. lactis* MUT_125 as a template. The PCR product was purified and cloned into the pGEM-T Easy vector by TA cloning, and the correctness of the sequence was verified by sequencing with primer pair 1224/1233. Next, the insert was cloned into pGhost9 using ApaI and NotI, and its presence was confirmed with primer pair pGhfor/rev. To obtain pGh*ysaB*$_{1–578}$, *ysaC* was deleted from the pGh*ysaCB*$_{1–578}$ construct. The deletion was performed by PCR with primer pair ΔysaCfor/rev (Table S1) with pGh*ysaCB*$_{1–578}$ as the template and ligation of the PCR product. The recombinant plasmids were expressed in *L. lactis* MUT_402.

To express the *llrG* gene, pIBB-JZK with a cellobiose-inducible promoter, P*ptcB*, and pGhost9 were used. First, *llrG* was amplified using primer pair LlrGfor/rev (Table S1). The PCR product was purified and cloned into pGEM-T Easy, and the correctness of the sequence was verified by sequencing with primer pair 1224/1233. Next, *llrG* was cloned into pIBB-JZK under the control of P*ptcB* using BamHI and XhoI, and the presence of the insert was confirmed by sequencing with primer pair pIBB-JZKfor/rev (Table S1). Finally, *llrG* under the control of P*ptcB* was cloned into pGhost9 using PstI and NotI, and the presence of the insert was confirmed with primer pair pGhfor/rev. To complement a spontaneous mutation, pIBB-JZK*llrG* was expressed in *L. lactis* MUT_12 in CM17 medium with Tet. To overexpress *llrG*, the pGh:P*ptcB*:*llrG* plasmid was expressed in *L. lactis* MUT_402 in CM17 medium with Em. The isolation scheme for the bacterial mutants is shown in Fig. S1 in the supplemental material.

Antibiotic susceptibility tests. The wild type, spontaneous mutants, and deletion mutants of *L. lactis* LMGT 3419 were streaked onto BHI agar plates. *L. lactis* mutants carrying pGhost9 were streaked onto BHI agar plates with Em. The *L. lactis* mutant carrying pJZK-IBB was streaked onto CM17 agar plates with Tet. Susceptibility to antibiotics was determined as described previously (29). Briefly, cell suspensions were spread onto ISO-BHI agar plates (Oxoid), with Em when appropriate, or CM17 agar plates with Tet, and strips containing antibiotics were applied to the plates. Inhibition zones were measured after 48 h of incubation at 30°C. Bacitracin strips were purchased from Liofilchem (Italy), while the remaining antibiotic strips were obtained from bioMérieux (France). Sensitivity to gramicidin (Sigma), ramoplanin (Sigma), carbenicillin (Millipore, Germany), and chlortetracycline (Millipore) was determined using microtiter plates with serial 2-fold antibiotic dilutions (73).

Quantification of gene expression. The expression of the *ysaDCBA*, *llrG*, *kinG*, *dltABCD*, *lysS* (*ylcG*), *dxsA*, and *dgkB* genes was measured by reverse transcription-quantitative PCR (RT-qPCR). First, bacterial pellets were collected from 10-ml cultures of *L. lactis* LMGT 3419, *L. lactis* MUT_322, *L. lactis* MUT_402, and *L. lactis* MUT_12 in the mid-stationary phase (optical density at 600 nm [OD_{600}] of 0.8), and total

RNA was extracted according to the manufacturer's instructions using the GeneMATRIX universal RNA purification kit (EURx, Poland). Bacterial pellets from at least three independent cultures were used for RNA isolation. First-strand cDNA was then obtained using random primers according to the manufacturer's instructions using the RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific). Finally, real-time quantitative PCR was performed on the PikoReal 96 real-time PCR system (Thermo Fisher Scientific). Gene-specific primers (Table S1) were created with the Primer Quest program (Integrated DNA Technologies). Each reaction mixture contained a cDNA template (in three amounts, 108, 36, and 12 ng per well, each in duplicate), 1× concentrated real-time 2× HS PCR master mix SYBR A (A&A Biotechnology), specific forward and reverse primers (100 nM each), and water to a 10-μl final volume. Reactions were performed with an initial denaturation step (95°C for 3 min), followed by 45 to 50 cycles of denaturation (95°C for 12 s) and primer annealing-extension (68°C for 30 s). Fluorescence measurements were performed during the annealing-extension step of each cycle. Melting point temperature analysis was done in the range from 60°C to 95°C with temperature increments of 0.2°C. The quality of the results was assessed by the product melting curves and the expected threshold cycle (C_T) differences between the three cDNA quantities. Rare outlier data were excluded from calculations. All results were normalized to a single common cDNA concentration for all genes by using the three cDNA concentrations to calculate individual yields for each primer pair. The amount of each target gene was calculated using a modified ΔC_T method, taking the geometric mean C_Ts of two common genes as a reference. Results were normalized to the *purM* and *tuf* reference genes encoding phosphoribosylaminoimidazole synthetase and elongation factor Tu, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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