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LsbB Bacteriocin Interacts with the Third Transmembrane Domain of the YvjB Receptor

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ABSTRACT

The Zn-dependent membrane-located protease YvjB has previously been shown to serve as a target receptor for LsbB, a class II leaderless lactococcal bacteriocin. Although *yvjB* is highly conserved in the genus *Lactococcus*, the bacteriocin appears to be active only against the subspecies *L. lactis* subsp. *lactis*. Comparative analysis of the YvjB proteins of a sensitive strain ($YvjB_{MN}$) and a resistant strain ($YvjB_{MG}$) showed that they differ from each other in 31 positions. In this study, we applied site-directed mutagenesis and performed directed binding studies to provide biochemical evidence that LsbB interacts with the third transmembrane helix of YvjB in susceptible cells. The site-directed mutagenesis of LsbB and YvjB proteins showed that certain amino acids and the length of LsbB are responsible for the bacteriocin activity, most probably through adequate interaction of these two proteins; the essential amino acids in LsbB responsible for the activity are tryptophan (Trp^{25}) and terminal alanine (Ala^{30}). It was also shown that the distance between Trp^{25} and terminal alanine is crucial for LsbB activity. The crucial region in YvjB for the interaction with LsbB is the beginning of the third transmembrane helix, particularly amino acids tyrosine (Tyr^{356}) and alanine (Ala^{353}). *In vitro* experiments showed that LsbB could interact with both $YvjB_{MN}$ and $YvjB_{MG}$, but the strength of interaction is significantly less with $YvjB_{MG}$. *In vivo* experiments with immunofluorescently labeled antibody demonstrated that LsbB specifically interacts only with cells carrying $YvjB_{MN}$.

IMPORTANCE

The antimicrobial activity of LsbB bacteriocin depends on the correct interaction with the corresponding receptor in the bacterial membrane of sensitive cells. Membrane-located bacteriocin receptors have essential primary functions, such as cell wall synthesis or sugar transport, and it seems that interaction with bacteriocins is suicidal for cells. This study showed that the C-terminal part of LsbB is crucial for the bacteriocin activity, most probably through adequate interaction with the third transmembrane domain of the YvjB receptor. The conserved Tyr^{356} and Ala^{353} residues of YvjB are essential for the function of this Zn-dependent membrane-located protease as a bacteriocin receptor.

Bacteriocins are small, ribosomally synthesized, cationic, and hydrophobic peptides produced by various bacteria, and they are often found to be active against bacteria closely related to the producers. However, some also have broader inhibitory spectra, including pathogens and problematic bacteria. Producer organisms are immune to their own bacteriocin(s), a property that is mediated by specific immunity proteins (1).

Bacteriocins from Gram-positive bacteria are generally classified into two main groups: the class I lantibiotics, containing post-translationally modified peptides with ring-forming lanthionine or methyllanthionine residues, and class II, composed of non-modified or minimally modified peptide bacteriocins (1–3). Class II bacteriocins are further subdivided into pediocin-like bacteriocins (class IIa), two-peptide bacteriocins (class IIb), circular bacteriocins (class IIc), and nonpediocin one-peptide bacteriocins (class IId) (2).

Bacteriocins have been much studied from a fundamental and scientific perspective and also for their potential applications as food preservatives, and in veterinary and human medicine as alternatives to antibiotics or as synergists (1, 4).

Bacteriocins have a number of positive attributes that have made them especially attractive for various applications (5). Some of them exhibit a broad spectrum of activity, inhibiting microorganisms belonging to different genera and species, including many bacterial pathogens that cause human, animal, or plant infections (6). For clinical applications, bacteriocins have been presented as a viable alternative to antibiotics due to the high specificity of certain bacteriocins against clinical pathogens, including multidrug-resistant (MDR) strains (4). Therefore, these substances have various potential applications in the food industry and medicine, either alone or in combination with other chemicals or methods (7).

The multiplicity and diversity of bacteriocins and the resultant effects of their interactions with targeted bacteria on microbial ecology have been thoroughly studied and remain an area of investigation, attracting many researchers (8).

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The mechanisms involved in the inhibitory activity of bacteriocins produced by Gram-positive bacteria toward target cells have been shown to be diverse. Lipid II, along with related cell wall precursors, has been identified as both the receptor and the target for several class I bacteriocins from the lantibiotic subgroup (9–11), as well as for the class II bacteriocin lactococcin 972 (12). The mannose phosphotransferase system (man-PTS) has been found to be involved in the sensitivity to some bacteriocins, but it was not until 2007 that it was finally established that man-PTS serves as a receptor for the class IIa (i.e., pediocin-like) and some class IId bacteriocins (lactococcin A and lactococcin B) (13, 14). In recent years, several other receptors have been identified through a genome sequencing approach. A maltose-ABC transporter was found to be required in target cells for sensitivity to garvicin ML, a circular bacteriocin (class IIc) (15). By screening of a cosmid library and genome sequencing of resistant mutants, a Zn-dependent metallopeptidase was found to be the target for the LsbB (class IId) and related bacteriocins (16). In addition, UppP has been found to be the bacteriocin receptor for lactococcin G and enterocin 1071 (both class IIb) (17). Although all these bacteriocins differ greatly from each other in many aspects, including physicochemical properties, composition, target specificity, width of spectrum, and mode of action, they all share a feature, which is the fact that they all target components of the bacterial membrane.

LsbB is a 30-amino-acid (aa) leaderless class IId bacteriocin produced by *Lactococcus lactis* subsp. *lactis* BGMN1-5 (18). It has a narrow inhibitory spectrum containing mostly lactococcal strains. The target receptor for LsbB is YvjB, a member of the highly conserved Zn-dependent membrane-located protease M50 protein family (16). Some members of this protein family are known to be involved in gene regulation in response to stress in *Escherichia coli* and *Enterococcus faecalis* (19–22). However, the role of YvjB in *Lactococcus* is presently unknown. It seems that LsbB interacts with YvjB with its C-terminal part (23). In this study, we further analyzed the residues of LsbB and its membrane receptor YvjB that are essential for their interaction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains, their derivatives, and plasmids used in this study are listed in Table 1. Lactococcal and enterococcal strains were grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with D-glucose (0.5% [wt/vol]) (GM17) at 30°C. *Escherichia coli* DH5α, TOP10, M15, and BL21(DE3) were used for cloning and propagation of constructs and were grown in Luria-Bertani (LB) broth aerobically at 37°C. To each medium, agar (1.5% [wt/vol]; Torlak, Belgrade, Serbia) was added for use as a solid medium. Transformants of lactococci and enterococci were selected on GM17 plates containing 10 μg/ml erythromycin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) or 7.5 μg/ml chloramphenicol (final concentration). *E. coli* transformants were selected on LB plates containing 300 μg/ml erythromycin, 100 μg/ml ampicillin, or 100 μg/ml kanamycin, depending on the plasmids used. When necessary, 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) (Fermentas, Vilnius, Lithuania) was added to LB or GM17 medium plates at a final concentration of 50 μg/ml, for blue/white screening of colonies carrying vectors with clones.

Test for bacteriocin activity. For detection of bacteriocin activity of lactococci, recombinant strains, and mutants, an agar well diffusion assay was performed as described previously by Kojic et al. (24). To test the level of LsbB production, zones of inhibition were compared by size and intensity with zones formed by synthetic LsbB (known concentrations) and two control strains, *L. lactis* BGMN1-596T and MG7284 carrying pAZIIlsbB, in order to quantify production by different producers.

DNA manipulations. Electrocompetent *L. lactis* subsp. *cremoris* MG7284 and *Enterococcus faecalis* BGZLS10-27 cells were prepared as described by Holo and Nes (25). Transformations were done by electroporation using an Eppendorf electroporator (Eppendorf, Hamburg, Germany), except *E. coli* DH5α, EC101, TOP10, M15, and BL21, which were transformed by heat shock. Appropriate agar plates with antibiotics were used for the selection of transformants.

Plasmid DNA from *E. coli* DH5α, EC101, TOP10, M15, and BL21 was isolated using the QIAprep Spin miniprep kit (Qiagen GmBH, Hilden, Germany). Digestion with restriction enzymes was conducted according to the supplier's instructions (Fermentas). DNA fragments were purified from agarose gels using a QIAquick gel extraction kit, as described by the manufacturer (Qiagen).

DNA was ligated with T4 DNA ligase (Agilent Technologies, USA), according to the manufacturer's recommendations.

The sets of specific primers used in this study are listed in Table 2. Kapa Taq DNA polymerase (Kapa Biosystems, Inc., Boston, MA, USA) was used to amplify DNA fragments by PCR using a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were purified with a QIAquick PCR purification kit (Qiagen), according to the protocol of the supplier, and sequenced by the Macrogen Sequencing Service (Macrogen, The Netherlands). The DNA Strider program was used for open reading frame (ORF) prediction. Commercial pGEM-T-Easy (Promega, Madison, WI, USA) or laboratory vector pBS-TA (26) was used for cloning of PCR products.

β-Galactosidase activity assay. Membrane damage of lactococcal cells caused by LsbB was determined using a modified β-galactosidase assay described by O'Neill and coauthors (27). The activity of β-galactosidase was determined from the logarithmic phase of MG7284, BGMN1-596, and BGMN1-596R2/23 (16) carrying pNZ8150lacZ1PlcnB (28) by assaying the degradation of ortho-nitrophenyl-β-galactoside (ONPG) (Sigma-Aldrich Co., St. Louis, MO) at 30°C using a modified method described by Miller (29). Cells from 1 ml of logarithmic-phase (optical density at 600 nm [OD_{600}], ~0.6) cultures were collected by centrifugation, resuspended in 500 μl of PP buffer (0.5 M sucrose, 40 mM NH_4-acetate, 10 mM Mg-acetate [pH 7]) containing 4 mg/ml lysozyme, and incubated 30 min at 37°C. Protoplasts were harvested by centrifugation (1,500 × *g*) and resuspended in 200 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Suspensions were divided into two parts of 100 μl and to each was added 500 μl of Z buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, 50 mM β-mercaptoethanol). To one mixture, 20 μl of chloroform and 20 μl of 0.1% SDS were added, and to a second mixture, LsbB (100 μg/ml) was added. These mixtures were incubated for 10 min at room temperature (RT) before centrifuging at 16,000 × *g* for 3 min to pellet undisrupted protoplasts. The supernatants were transferred to new tubes, and 200 μl of ONPG (4 mg/ml) was added. After the appearance of yellow color, the reactions were stopped by adding 250 μl of 1 M Na_2CO_3, and A_{420} values were measured for the supernatants. β-Galactosidase activity in Miller units was calculated as $1{,}000 \times A_{420}/(t \times v \times OD_{600})$, where *t* is time in minutes, *v* is the volume of culture used in the assay in milliliters, and OD_{600} is the optical density of the culture at 600 nm. The efficiency of membrane damage caused by LsbB was calculated as the percentage of β-galactosidase activity obtained by treatment of cells with LsbB (100 μg/ml) compared to those obtained by SDS and chloroform (activity obtained by treatment with SDS and chloroform was taken as 100%).

Site-directed mutagenesis. Desired mutations were introduced through the QuikChange site-directed mutagenesis protocol (Agilent Technologies, USA) with the oligonucleotide primers (and their reverse complements) listed in Table 2. After PCR for site-directed mutagenesis, the methylated template strands were digested by 1 μl (10 U) of DpnI restriction enzyme per reaction (at 37°C for 2 h) and purified by Thermo Scientific GeneJET PCR purification kit, as described by the manufacturer (Thermo Scientific, Lithuania). Subsequently, 4 μl of the purified DNA was used to transform 40 μl of *E. coli* TOP10 high-competency cells by

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Lactococcus lactis subsp. *lactis*		
BGMN1-596	Plasmid-free derivative of *L. lactis* subsp. *lactis* BGMN1-5	18
BGMN1-596R2/23	Mutant of BGMN1-596 semiresistant to LsbB up to 625 μg/ml	16
BGMN1-596T	BGMN1-596 transformed with pMN5 plasmid	18
Lactococcus lactis subsp. *cremoris*		
MG7284	MG1363, Prt^- Lac^- Bac^r Fus^r Spc^r	41
MG7284/pAZIL-lsbB	MG7284 transformed with pAZIL-lsbB	16
Enterococcus faecalis		
BGZLS10-27	Natural isolate, Agg^-	42
BGZLS10-27/pAZIL	BGZLS10-27 transformed with pAZIL	This study
BGZLS10-27/pAZIL-YvjB$_{MN}$	BGZLS10-27 transformed with pAZIL-YvjB$_{MN}$	This study
BGZLS10-27/pAZIL-YvjB$_{MG}$	BGZLS10-27 transformed with pAZIL-YvjB$_{MG}$	This study
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	43
EC101	JM101 containing *repA* gene of pWV01 in chromosome	44
TOP10	F^- *mcrA (mrr-hsdRMS-mcrBC)* ϕ80*lacZ*ΔM15 *lacX74 recA1 ara139 (ara-leu)7697 galU galK rpsL* (Str^r) *andA1 nupG*	Invitrogen
M15(pREP4)	Nal^s Str^s Rif^s Thi^- Lac^- Ara^+ Gal^+ Mtl^- F^- $RecA^+$ Uvr^+ Lon^+	Qiagen
BL21(DE3)	B F^- *dcm ampT hsdS* (r_B^- m_B^-) *gal* λ(DE3)	Agilent Technologies
BL21/p-GEX-6P-3/LsbB	p-GEX-6P-3/LsbB transformant of BL21	This study
BL21/p-GEX-6P-3	p-GEX-6P-3 transformant of BL21	This study
Plasmids		
pMN5	Natural plasmid carrying *lsbB* operon	18; accession no. NC_004922.1
pAZIL	7,109 bp, Em^r, shuttle cloning vector	45; accession no. LMBP9596[b]
pAZIL-lsbB	pAZIL carrying *lsbB* gene	16
pAZIL-lsbB-W25F	pAZIL carrying *lsbB* gene with amino acid change $Trp^{25}Phe$	This study
pAZIL-lsbB-A30-STOP	pAZIL carrying *lsbB* gene with deleted Ala^{30} amino acid	This study
pAZIL-lsbB-K29A-STOP	pAZIL carrying *lsbB* gene with amino acid change $Lys^{29}Ala$ and deleted Ala^{30} amino acid	This study
pAZIL-lsbB-A30+A31	pAZIL carrying *lsbB* gene with addition of Ala^{31} amino acid	This study
pAZIL-lsbB-A30V	pAZIL carrying *lsbB* gene with amino acid change $Ala^{30}Val$	This study
pAZIL-lsbB-A30G	pAZIL carrying *lsbB* gene with amino acid change $Ala^{30}Gly$	This study
pAZIL-lsbB-A30S	pAZIL carrying *lsbB* gene with amino acid change $Ala^{30}Ser$	This study
pAZIL-lsbB-A30P	pAZIL carrying *lsbB* gene with amino acid change $Ala^{30}Pro$	This study
pAZIL-lsbB-K2N-T3S	pAZIL carrying *lsbB* gene with amino acid changes Lys^2Asn and Thr^3Ser	This study
pAZIL-lsbB-K17A-K19A	pAZIL carrying *lsbB* gene with amino acid changes $Lys^{17}Ala$ and $Lys^{19}Ala$	This study
pAZIL-YvjB$_{MN}$	pAZIL carrying *yvjB* gene from BGMN1-596	This study
pAZIL-YvjB$_{MG}$	pAZIL carrying *yvjB* gene from MG7284	This study
pAZIL-YvjB$_{MG}$B	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes $Ser^{257}Gly$ and $Asn^{259}Lys$	This study
pAZIL-YvjB$_{MN}$B	pAZIL carrying *yvjB* gene from BGMN1-596 with insertion of BamHI restriction site	This study
pAZIL-YvjB$_{1/2MN-1/2MG}$	pAZIL carrying first half of *yvjB* gene from BGMN1-596 and second half of *yvjB* gene from MG7284 (YvjB$_{MN}$1-259-YvjB$_{MG}$260-428 aa)	This study
pAZIL-YvjB$_{1/2MG-1/2MN}$	pAZIL carrying first half of *yvjB* gene from MG7284 and second half of *yvjB* gene from BGMN1-596 (YvjB$_{MG}$1-256-YvjB$_{MN}$257-428 aa)	This study
pAZIL-YvjB$_{MG}$E	pAZIL carrying *yvjB* gene from MG7284 with insertion of EagI restriction site at position of aa 327 without amino acid changes	This study
pAZIL-YvjB$_{MN}$E	pAZIL carrying *yvjB* gene from BGMN1-596 with insertion of EagI restriction site at position of aa 327 without amino acid changes	This study
pAZIL-YvjB$_{3/4MG-1/4MN}$	pAZIL carrying first 3/4 of *yvjB* gene from MG7284 and 1/4 of *yvjB* gene from BGMN1-596 (YvjB$_{MG}$1-327-YvjB$_{MN}$328-428 aa)	This study
pAZIL-YvjB$_{1/2MG-1/4MN-1/4MG}$	pAZIL carrying first half of *yvjB* gene from MG7284, 1/4 of *yvjB* gene from BGMN1-596, and last and 1/4 of *yvjB* gene from MG7284 (YvjB$_{MG}$1-256-YvjB$_{MN}$257-327-YvjB$_{MG}$328-428 aa)	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Relevant characteristics[a]	Source or reference
pAZIL-YvjB$_{MG}$-L351F	pAZIL carrying *yvjB* gene from MG7284 with amino acid change Leu^{351}Phe	This study
pAZIL-YvjB$_{MG}$-T353A	pAZIL carrying *yvjB* gene from MG7284 with amino acid change Thr^{353}Ala	This study
pAZIL-YvjB$_{MG}$-Q356Y	pAZIL carrying *yvjB* gene from MG7284 with amino acid change Gln^{356}Tyr	This study
pAZIL-YvjB$_{MG}$-P396Q	pAZIL carrying *yvjB* gene from MG7284 with amino acid change Pro^{396}Gln	This study
pAZIL-YvjB$_{MG}$-L351F-T353A	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Leu^{351}Phe and Thr^{353}Ala	This study
pAZIL-YvjB$_{MG}$-L351F-Q356Y	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Leu^{351}Phe and Gln^{356}Tyr	This study
pAZIL-YvjB$_{MG}$-T353A-Q356Y	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Thr^{353}Ala and Gln^{356}Tyr	This study
pAZIL-YvjB$_{MG}$-T353A-P396Q	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Thr^{353}Ala and Pro^{396}Gln	This study
pAZIL-YvjB$_{MG}$-L351F-T353A-Q356Y	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Leu^{351}Phe, Thr^{353}Ala, and Gln^{356}Tyr	This study
pAZIL-YvjB$_{MG}$-L351F-T353A-P396Q	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Leu^{351}Phe, Thr^{353}Ala, and Pro^{396}Gln	This study
pAZIL-YvjB$_{MG}$-L351F-T353A-Q356Y-P396Q	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Leu^{351}Phe, Thr^{353}Ala, Gln^{356}Tyr, and Pro^{396}Gln	This study
pAZIL-YvjB$_{MG}$-L351F-T353A-Q356Y-T314A	pAZIL carrying *yvjB* gene from MG7284 with amino acid changes Leu^{351}Phe, Thr^{353}Ala, Gln^{356}Tyr, and Thr^{314}Ala	This study
pBluescript	2,958 bp, Amp^r, cloning vector	Stratagene
pGEM-T-Easy	3,015 bp, Amp^r, PCR cloning vector	Promega
pGEM-T-YvjBex1	PCR-amplified fragment carrying region of YvjB protein between first and second transmembrane domains cloned into pGEM-T-Easy	This study
pGEM-T-YvjBex2	PCR-amplified fragment carrying region of YvjB protein between second and third transmembrane domains cloned into pGEM-T-Easy	This study
pGEX-6P-3	4,900 bp, Amp^r, GST expression vector	GE Healthcare Life Sciences
pGEX-6P-3/GST-LsbB	Cloned *lsbB* gene into pGEX-6P-3 vector, GST-LsbB fusion protein	This study
pQE$_{30}$	Amp^r, ColE1 replicon, 6×His expression vector	Qiagen
pQE$_{30}$-YvjBex1	PCR-amplified fragment carrying region of YvjB protein between first and second transmembrane domains cloned as SacI/HindIII into pQE$_{30}$	This study
pQE$_{30}$-YvjBex2	PCR-amplified fragment carrying region of YvjB protein between second and third transmembrane domains cloned as BamHI/HindIII into pQE$_{30}$	This study
pNZ8150lacZ1PlcnB	PlcnB promoter cloned into pNZ8150lacZ1 vector	28

[a] Agg$^-$, aggregative protein negative; Prt$^-$, proteinase negative; Lac$^-$, lactose utilization negative; Bac^r, bacteriocin resistant; Fus^r, fusaric acid resistant; Spc^r, spectinomycin resistant; Str^r, streptomycin resistant; Nal^s, nalidixic acid susceptible; Str^s, streptomycin susceptible; Rif^s, rifampin susceptible; Em^r, erythromycin resistant; Amp^r, ampicillin resistant; aa, amino acid.

[b] LMBP9596, BCCM plasmid collection accession number.

heat shock treatment. The Thermo Scientific GeneJET plasmid miniprep kit was used, according to the manufacturer's recommendations (Thermo Scientific), to obtain and purify the plasmids from the selected colonies, after which sequencing (Macrogen Europe, The Netherlands) was done to confirm the introduction of the desired mutations.

Preparation of bacterial cell membrane. The method for extracting cell membrane enzymes from selected lactococcal strains was applied according to Attri et al. (30), with modifications.

Cells from the bacterial log cultures (MG7284, BGMN1-596, and BGMN1-596/23) were harvested by centrifugation at 3,600 × *g* for 10 min and the cell pellets washed in TEN buffer (50 mM Tris, 10 mM EDTA, 50 mM NaCl [pH 8.0]). Cell pellets (~5 g) were suspended in 2 to 3 ml of PP buffer (see above) with addition of lysozyme (5 mg/ml) and stirred for 1 h at 37°C. Lysozyme-treated cells were harvested by centrifugation at 1,000 × *g* at 4°C for 5 min, and pellets were resuspended in A buffer (50 mM sodium phosphate, 100 mM NaCl, 0.5% Triton X-100 supplemented with the protease inhibitor cocktail at a 1:200 [vol/vol] ratio [pH 8.0]). The cells were subjected to sonication for 1 min at a frequency of 8 kHz while being kept in an ice bath. The homogenate was centrifuged at 16,000 × *g* for 30 min to obtain the supernatant. In the next step, the supernatant was centrifuged at 100,000 × *g* for 18 h at 4°C to pellet the cell membranes. Pellets containing cellular membranes were resuspended in B buffer (20 mM Tris, 5 mM $MgCl_2$, 0.01 mM Zn^{2+} supplemented with the protease inhibitor cocktail at a 1:200 [vol/vol] ratio [pH 6.8]).

In order to obtain more pure membrane fractions, homogenates were centrifuged at 45,000 × *g* for 5 h at 4°C. Supernatants representing cell cytoplasm and pellets representing the membrane fraction were stored in an ice bath (up to 7 days).

Production of polyclonal antibody. Synthetic LsbB (ChinaPeptides Co., Ltd., Shanghai, China) was used for production of anti-LsbB polyclonal antibody.

Two regions of YvjB protein (YvjBex1 from 80th to 185th amino acids and YvjBex2 from 225th to 300th amino acids) that do not contain intermembrane domains were expressed using the pQE$_{30}$ 6×His tag (Qiagen) expression system for the production of anti-YvjB polyclonal antibody. First, using total DNA BGMN1-596 and designed primers ex1 SacI/HindIII for YvjBex1, and ex2 BamHI/HindIII for YvjBex2, corresponding fragments were amplified by PCR. The PCR products were purified and cloned first to pGEM-T-Easy vector, confirmed by sequencing, and recloned as SacI/HindIII or BamHI/HindIII in frame with 6×His tag to pQE$_{30}$ expression vector. Fusion His-tagged proteins were expressed in *E. coli* M15 cells.

His tag affinity purification of YvjBex1 and YvjBex2 proteins was conducted under denaturing conditions. The refolding method using urea to

TABLE 2 Primers used in this study

Primer name	Sequence (5′ to 3′)[a]	Template
lsbB-F	CTCCAAGAATTCCTAAAAAAATAGG	pMN5
lsbB-R	TGATATCTTAAGCTTTTCCACGTTCCCATGG	pMN5
lsbB-A30-STOP-R	TGATATCTTA**TTA**TTTTCCACGTTCCCATGG	pMN5
lsbB-K29A-STOP-R	TGATATCTTA**TTATGC**TCCACGTTCCC	pMN5
lsbB-A30+A31-R	TGATATCTTA**AGC**AGCTTTTCCACGTTCCCATGG	pMN5
lsbB-A30V-R	TGATATCTTA**AAC**TTTTCCACGTTCCCATGG	pMN5
lsbB-A30G-R	TGATATCTTA**ACC**TTTTCCACGTTCCCATGG	pMN5
lsbB-A30S-R	TGATATCTTA**AGA**TTTTCCACGTTCCCATGG	pMN5
lsbB-A30P-R	TGATATCTTA**AGG**TTTTCCACGTTCCCATGG	pMN5
lsbB-SN-F	ATG**AATTCAATCCTACGTTTGTTGCTTGC**	pMN5
lsbB-AKA-R	TGATATCTTAAGCTTTTCCACGTTCCCATGGATAGCCGCCAGT **TGC**CTT**TGC**ATGAC	pMN5
LsbB-tag (GST)-F	GGATCCATGAAAACAATCCTACG	pMN5
LsbX-SalI-R	GTTGTCGACTAATCAATATGTTCC	pMN5
ORFF	GGCGTAAAAGATTCAGG	Total DNA BGMN1-596 or MG7284
ORFR	GAAGGGTTGGTATAAGC	Total DNA BGMN1-596 or MG7284
MGBH257G-F	GAAATTTCAGGATCCAATGGAAAAG	pAZIL-YvjB$_{MN}$ pAZIL-YvjB$_{MG}$
MGBH257G-R	CCATTGGATCCTGAAATTTCTGTGACC	pAZIL-YvjB$_{MN}$ pAZIL-YvjB$_{MG}$
MGBH257G-259K-F	GAAATTTCAGGATCCAAAGGAAAAG	pAZIL-YvjB$_{MN}$ pAZIL-YvjB$_{MG}$
MGBH257G-259K-R	CCTTTGGATCCTGAAATTTCTGTGACC	pAZIL-YvjB$_{MN}$ pAZIL-YvjB$_{MG}$
Eag-F	GATTGCACGGCCGAGTCTTG	pAZIL-YvjB$_{MN}$ pAZIL-YvjB$_{MG}$
Eag-R	CAAGACTCGGCCGTGCAATC	pAZIL-YvjB$_{MN}$ pAZIL-YvjB$_{MG}$
YvjBIV-TA-F	GCAGGACAAGCG**GCC**ACAGCAATTTTCAGAGC	pAZIL-YvjB$_{MG}$
YvjBIV-TA-R	GCTCTGAAAATTGCTGT**GGC**CGCTTGTCCTGC	
YvjBIV-LF-F	GGCAAGAGCAGGT**TTT**CCAACAATTATTCAGTTGTTAGC	pAZIL-YvjB$_{MG}$
YvjBIV-LF-R	GCTAACAACTGAATAATTGTTGG**AAA**ACCTGCTCTTGCC	
YvjBIV-TA2-F	GCAGGTTTGCCA**GCA**ATTATTCAGTTGTTAGC	pAZIL-YvjB$_{MG}$
YvjBIV-TA-R	GCTAACAACTGAATAAT**TGC**TGGCAAACCTGC	
YvjBIV-QY-F	GCCAACAATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-QY-R	GCATAGCTAACAA**ATA**AATAATTGTTGGC	
YvjBIV-PQ-F	GGCAAAGCACTTTCG**CAA**GAGAAAGAATC	pAZIL-YvjB$_{MG}$
YvjBIV-PQ-R	GATTCTTTCTC**TTG**CGAAAGTGCTTTGCC	
YvjBIV-LF-TA-QY-F	GCGGCAAGAGCAGGT**TTT**CCA**GCA**ATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-LF-TA-QY-R	GCATAGCTAACAA**ATA**AATAAT**TGC**TGG**AAA**ACCTGCTCTTGCCGC	
YvjBIV-LF-TA-F	GCGGCAAGAGCAGGT**TTT**CCA**GCA**ATTATTCAGTTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-LF-TA-R	GCATAGCTAACAACTGAATAAT**TGC**TGG**AAA**ACCTGCTCTTGCCGC	
YvjBIV-TA-QY-F	GCGGCAAGAGCAGGTTTGCCA**GCA**ATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-TA-QY-R	GCATAGCTAACAA**ATA**AATAAT**TGC**TGGCAAACCTGCTCTTGCCGC	
YvjBIV-LF-QY-F	GCGGCAAGAGCAGGT**TTT**CCAACAATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-LF-QY-R	GCATAGCTAACAA**ATA**AATAATTGTTGG**AAA**ACCTGCTCTTGCCGC	
YvjBIV-LF-TA-QY-F	GCGGCAAGAGCAGGT**TTT**CCA**GCA**ATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-LF-TA-QY-R	GCATAGCTAACAA**ATA**AATAAT**TGC**TGG**AAA**ACCTGCTCTTGCCGC	
YvjBIV-TA-QY-F	GCGGCAAGAGCAGGTTTGCCA**GCA**ATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-TA-QY-R	GCATAGCTAACAA**ATA**AATAAT**TGC**TGGCAAACCTGCTCTTGCCGC	
YvjBIV-LF-QY-F	GCGGCAAGAGCAGGT**TTT**CCAACAATTATT**TAT**TTGTTAGCTATGC	pAZIL-YvjB$_{MG}$
YvjBIV-LF-QY-R	GCATAGCTAACAA**ATA**AATAATTGTTGG**AAA**ACCTGCTCTTGCCGC	
Ex1/SacIF	GACTGAGCTCAAAAAAGGACAAGC	pAZIL-YvjB$_{MN}$
Ex1/HindIIIR	GGACCACCGAAGCTTGTCAAC	pAZIL-YvjB$_{MN}$
Ex2/BamHIF	GGATCCCCCGCTTACAATGCAGGC	pAZIL-YvjB$_{MN}$
Ex2/HindIIIR	CCTGTAAGCTTATCAAAGAAACC	pAZIL-YvjB$_{MN}$

[a] Restriction sites are underlined; changed amino acid codons are indicated by bold type.

disrupt noncovalent bonds and increase protein solubility was used to solubilize and make the His-tagged YvjBex1 and YvjBex2 more accessible to the nickel-nitrilotriacetic acid (Ni-NTA) resin. The recombinant proteins were purified according to the protocol recommended by The QIAexpressionist (Qiagen). The eluted proteins were dialyzed by ultrafiltration (centrifugal filter units; Amicon Ultra-15 centrifugal filter devices, 3K; Millipore). Polyclonal antibodies were produced by immunization of mice with the synthetic or purified fusion proteins in the animal house of the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy.

Construction of GST-LsbB-tag protein and GST pulldown assay. Specific sets of primers [LsbB-tag(GST)-F and LsbX-Sal-R; Table 2] were designed for amplification of selected a part of the *lsbB* gene from pMN5 plasmid DNA. The PCR product was purified and cloned to pGEM-T-Easy vector and confirmed by sequencing. The *lsbB* gene was transferred from pGEM-T-Easy as a BamHI/SalI fragment into the expression vector pGEX-6P-3 to obtain glutathione *S*-transferase (GST)-LsbB fusion protein. After transforming the expression vector into competent *E. coli* BL21 cells, BL21/p-GEX-6P-3/LsbB and BL21/p-GEX-6P-3 (control strain with empty vector) were grown in 50 ml of LB broth (containing 100 μg/ml

ampicillin) to an OD_{600} (A_{600}) of 0.6, with vigorous agitation at 37°C. Before the next step, 1 ml of the cultures was collected (noninduced samples). Fusion protein expression was induced by 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich) by incubating the cultures for an additional for 2 h at 28°C. One milliliter of the cultures was collected (induced samples) and analyzed. Noninduced and induced samples for both strain BL21/p-GEX-6P-3/LsbB and BL21/p-GEX-6P-3 were boiled for 2 min and checked by protein gel electrophoresis (12.5% gel) prior to Coomassie blue staining. The rest of the BL21/p-GEX-6P-3/LsbB and BL21/p-GEX-6P-3 (control strain) bacterial cultures were harvested by centrifugation at 4,500 × *g* for 10 min, and the cell pellets were suspended in 3 ml of lysis buffer containing 0.5% Triton X-100, 0.2% SDS, 0.5% NP-40, and 0.1% Tween 20. The cells were lysed in the ice for 30 min with gentle agitation. The lysates were subjected to sonication for 1 min (6 times 10 s) at a frequency of 10 kHz while being kept in an ice bath. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4°C to obtain the supernatant.

In order to bind the engineered tagged protein to the matrix, supernatants of BL21/p-GEX-6P-3/LsbB and BL21/p-GEX-6P-3 were incubated with glutathione Sepharose 4 Fast Flow (GE Healthcare, Germany) for 2 h at 4°C with continuous mixing.

Beads of glutathione Sepharose (200 μl) with bound proteins were centrifuged briefly and washed with the same lysis buffer at 4°C. The quantity of GST-LsbB fusion protein and GST protein (20 μl per each) was tested by SDS-PAGE and Western blot analysis using anti-LsbB antibody. Equal amounts of beads with bound GST-LsbB and GST (50 μl each) proteins were homogeneously mixed with the membrane fractions of strains MG7284, BGMN1-596, and BGMN1-596R2/23 (50 μl each) and made up to a volume of 600 μl with supplement buffer (50 mM Tris, 120 mM NaCl [pH 6.8]). The mixture was incubated overnight at 4°C with gentle agitation, washed five times for 30 min each in phosphate-buffered saline (PBS) buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl [pH 7.1]), and protein complexes were pulled down.

Western blotting. Protein(s) associated with the GST-LsbB fusion protein and GST protein was analyzed by SDS-PAGE and Western blot assays using anti-YvjB and anti-LsbB antibodies (independently). Samples were loaded into a 12.5% polyacrylamide gel, subjected to electrophoresis, and transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, Darmstadt, Germany) for 1 h at appropriate voltage (constant voltage). Membranes were incubated with 10% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight at 4°C in order to block nonspecific binding. Following blocking, the membranes were incubated for 1 h at RT with gentle agitation in appropriate dilutions of primary antibodies, as follows: mouse polyclonal antibody anti-YvjB, 1:3,000 dilution, and mouse polyclonal antibody anti-LsbB, 1:5,000 dilution (independently). Primary antibodies were diluted in 5% skim milk in TBST. After washing three times in TBST for 15 min, membranes were incubated for 1 h with horseradish peroxidase-labeled anti-mouse IgG (A9044 anti-mouse; Sigma) at a 1:10,000 dilution in 5% skim milk in TBST. The blots were washed three times in TBST for 15 min. Bands of target proteins were detected using the CN/DAB substrate kit (Thermo Scientific), according to the manufacturer's instructions.

Immunocytochemistry. After plating on coverslips, bacterial cells were fixed in 4% paraformaldehyde (PFA) for 20 min at RT. Cells were blocked in 5% bovine serum albumin (BSA) in PBS for 1 h at RT. Primary antibodies were diluted in PBS containing 1% BSA and incubated overnight at 4°C with mouse polyclonal anti-LsbB (this study, diluted 1:50). Coverslips were washed three times for 10 min in PBS and incubated with biotinylated goat anti-mouse IgG (Vector, Burlingame, CA, USA) for 1 h at RT in 1% BSA, followed by Cy3-streptavidin (diluted 1:5,000; Jackson ImmunoResearch, West Grove, PA, USA) diluted in PBS for 1 h at RT. Bacterial DNA was stained with 0.1 mg/ml 4′,6-diamino-2-phenylindole (DAPI; Sigma-Aldrich). Samples were visualized under an Olympus BX51 fluorescence microscope with appropriate filters and analyzed using the CytoVision 3.1 software (Applied Imaging Corporation, USA).

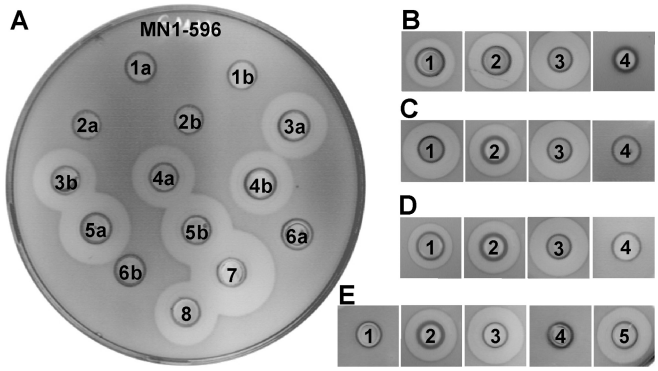

FIG 1 Antimicrobial activity of LsbB-derived mutants. (A) Modifications at or near residue Ala^{30}. 1, ΔAla^{30}; 2, Ala^{30} plus Ala^{31}; 3, $Ala^{30}Val$; 4, $Ala^{30}Gly$; 5, $Ala^{30}Ser$; 6, $Ala^{30}Pro$; 7, BGMN1-596T; 8, MG7284/pAZIL-lsbB (a and b are two colonies from the same transformation). (B) $Trp^{25}Phe$ substitution. (C) $Lys^{17}Ala$ plus $Lys^{19}Ala$ substitution. (D) $Lys^{2}Asn$ plus $Thr^{3}Ser$ substitutions. (E) $Lys^{29}Ala$ plus ΔAla^{30} substitutions. (B to E) 1, mutated LsbB; 2, MG7284/pAZIL-lsbB; 3, BGMN1-596T; 4, BGMN1-596; 5, mutated LsbB with one $Lys^{29}Ala$ substitution. Cultures of transformants were introduced into wells made in soft agar inoculated with indicator strain *L. lactis* BGMN1-596. The size of the zone is referred to the clone containing the wild-type *lsbB* gene (MG7284/pAZIL-lsbB). Inhibition is seen as clear zones around the wells.

RESULTS

LsbB causes membrane damage of sensitive cells. To determine whether LsbB causes membrane damage, different lactococcal strains with different levels of sensitivity to LsbB were exposed to synthetic LsbB. These were the resistant strain MG7284, the sensitive strain BGMN1-596, and the semisensitive mutant BGMN1-596R2/23 (see Table 1). The strains were transformed with pNZ8150lacZ1PlcnB encoding β-galactosidase, which was used as a reporter system for the detection of membrane damage. The results showed that the level of β-galactosidase activity (and thus membrane damage) is directly correlated with the level of sensitivity to LsbB (see Fig. S1 in the supplemental material), i.e., more β-galactosidase activity was found in sensitive cells than in less-sensitive cells. The results provide evidence that LsbB kills target cells by membrane damage.

Identification of essential amino acid residues in LsbB bacteriocin involved in antimicrobial activity. We have previously demonstrated that the last C-terminal 8 amino acids of the LsbB bacteriocin are involved in the interaction with the receptor protein YvjB (23). The residue Trp^{25} plays a particularly important role, as the substitution Trp^{25} to Ala caused a total loss of antimicrobial activity. To further study the importance of Trp^{25} and other amino acids in receptor binding, additional amino acid substitutions were made in LsbB (Fig. 1). It was found that the substitution of Trp^{25} with Phe, an amino acid belonging to the same aromatic group, caused a small reduction in bacteriocin activity, indicating the involvement of specific groups on amino acid 25 in specific interactions with YvjB (Fig. 1B). Previous work indicates that the last residue, Ala^{30}, is important for bacteriocin activity. As expected, the deletion of the terminal Ala^{30}, the addition of another alanine at the end (Ala^{31}), or the substitution of Ala^{30} with Pro caused a total loss of antimicrobial activity of LsbB, whereas substitutions of Ala^{30} with other residues, such as Gly and Val, had no apparent impact on the bacteriocin activity, while substitution with Ser enhanced bacteriocin activity (Fig. 1A). In our previous

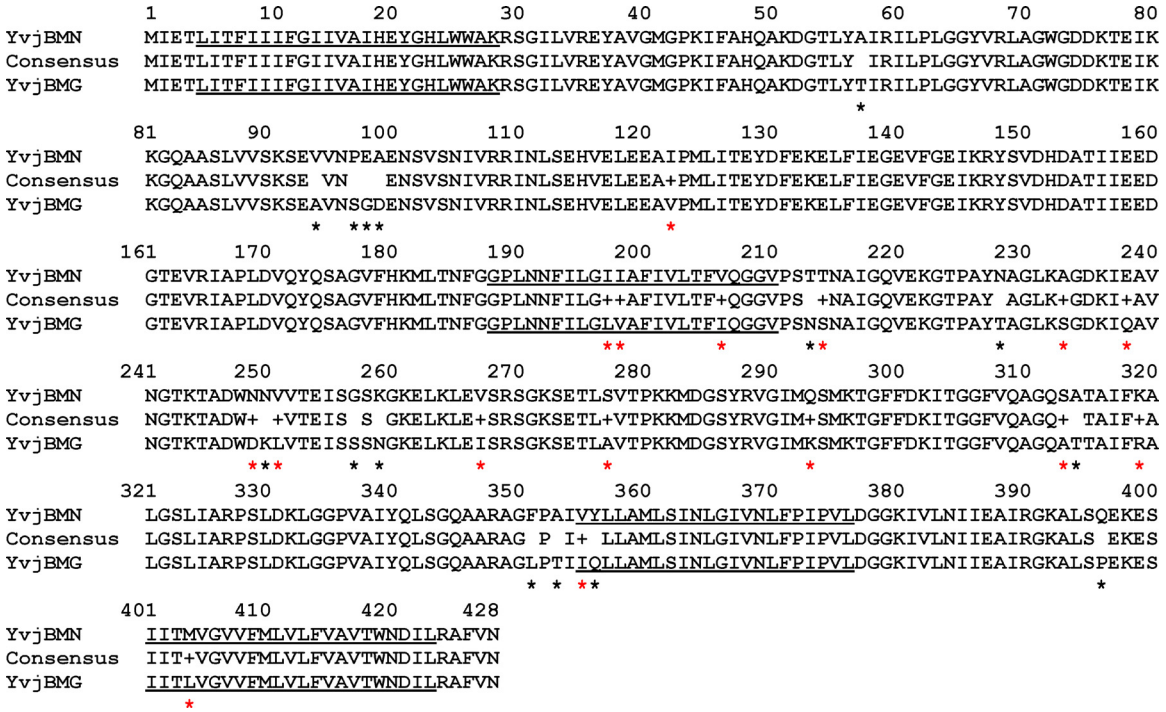

FIG 2 Comparison of the amino acid sequences of $YvjB_{MN}$ and $YvjB_{MG}$ using Strider software. All amino acid differences are indicated by red stars for ones belonging to the same physicochemical group and by black stars for ones belonging to different physicochemical groups. Underlined amino acids represent transmembrane domains.

work (23), we demonstrated that the Lys^{29}Ala mutation increased bacteriocin activity. To assess whether this substitution (Lys^{29}Ala) can replace the importance of the terminal alanine Ala^{30}, we created a double mutant with Lys^{29}Ala and ΔAla^{30}; thus, the resulting peptide was only 29 residues, with alanine as the last residue. As shown in Fig. 1E, this double mutant did not exert any antimicrobial activity at all. These results confirmed that the residues Trp^{25} and Ala^{30} are both important for LsbB's antimicrobial activity, although Ala^{30} appeared to tolerate to some extent a substitution with another aliphatic amino acid or serine. The results might also indicate that the distance between Trp^{25} and the last alanine is crucial, as shortening (termination with Ala^{29} instead of Ala^{30}) or elongation (addition of Ala^{31}) of this distance seriously affected the antimicrobial activity.

To assess the importance of the N-terminal half, we created a double mutant; Lys^{2} and Thr^{3} were changed to Asn^{2} and Ser^{3}, respectively. The activity of the modified peptide did not change drastically (Fig. 1D), indicating that the N-terminal part of LsbB seems to be less sensitive to changes/modifications.

One of the peptides (LcnG-α) of the two-peptide bacteriocin lactococcin G (like LsbB) contains a series of cationic residues ($Arg^{35}Lys^{36}Lys^{37}Lys^{38}His^{39}$) at the C-terminal part, and it has been proposed that this feature functions as a means to force the peptide to the target cell membrane by electrostatic interaction (31). LsbB contains a similar string of basic residues in its C-terminal half ($His^{16}Lys^{17}Lys^{18}Lys^{19}Thr^{20}$). To examine whether this feature has a similar function, the number of cationic residues in this feature was reduced by changing $His^{16}Lys^{17}Lys^{18}Lys^{19}Thr^{20}$ to $His^{16}Ala^{17}Lys^{18}Ala^{19}Thr^{20}$, thus reducing the pI from 10.99 to 10.50. The resulting mutant peptide did not have any apparent adverse effect on bacteriocin activity; in fact, it even slightly enhanced the activity (Fig. 1C).

Based on all these amino acid substitutions, deletions, and additions, it is possible to conclude that the C-terminal part of the LsbB bacteriocin is most important for bacteriocin activity, likely through interaction with the receptor protein YvjB.

Mapping of the region on YvjB involved in LsbB interaction. It has previously been established that among lactococci, there exist both sensitive and resistant strains to LsbB, such as the resistant strain MG7284 and the sensitive strain BGMN1-596, both of lactococcal genotype. The LsbB-resistant strain MG7284 possesses a homolog of YvjB ($YvjB_{MG}$) that differs in 31 residues from the YvjB of the sensitive strain BGMN1-596 ($YvjB_{MN}$). These differences appear to be randomly distributed over the entire protein length (Fig. 2). To search for the regions in $YvjB_{MN}$ and $YvjB_{MG}$ that cause their different sensitivity to LsbB, hybrid proteins containing portions of both proteins were constructed (see Fig. S2 in the supplemental material). A hybrid protein containing the C-terminal half of $YvjB_{MN}$ and the N-terminal half of $YvjB_{MG}$ ($YvjB_{1/2MG\text{-}1/2MN}$) was able to confer sensitivity to LsbB when expressed in strain MG7284 (naturally resistant to LsbB), indicating that the C-terminal half of $YvjB_{MN}$ is involved in target specificity. Further, a hybrid protein ($YvjB_{3/4MG\text{-}1/4MN}$) with an even smaller part of $YvjB_{MN}$ at the C terminus was still capable of conferring sensitivity to LsbB. Different hybrid combinations, as depicted in Fig. S2, further confirmed that the last section of the C terminus of YvjB is most likely involved in direct interaction with LsbB and consequently in sensitivity to the bacteriocin.

Given that the potential LsbB-binding domain is located at the C-terminal end of YvjB, we are interested in finding the mem-

brane topology of this part. Using Phobius (http://phobius.binf.ku.dk/index.html) or TMHMM server version 2.0 for prediction of transmembrane (TM) helices in proteins (http://www.cbs.dtu.dk/services/TMHMM/), YvjB was predicted to possess four transmembrane (TM) domains: TMI from amino acids (aa) 5 to 27, TMII from aa 188 to 210, TMIII from aa 354 to 376, and TMIV from aa 401 to 423 (Fig. 2; see also Fig. S3 in the supplemental material). Based on this prediction, the LsbB-binding domain is likely located within the region containing two transmembrane helices connected by a noncytoplasmic hinge.

Further defining the amino acids involved in receptor function by site-directed mutagenesis. The hybrid molecule $YvjB_{3/4MG\text{-}1/4MN}$ contains the last part of the $YvjB_{MN}$ protein, which differs from $YvjB_{MG}$ in six positions (amino acids Phe^{351}, Ala^{353}, Val^{354}, Tyr^{356}, Gln^{396}, and Met^{404} in $YvjB_{MN}$ are different in comparison to those present at the same positions Leu^{351}, Thr^{353}, Ile^{354}, Gln^{356}, Pro^{396}, and Leu^{404} in $YvjB_{MG}$) (Fig. 2). Some of these differences are likely responsible for the observed difference between $YvjB_{MG}$ and $YvjB_{MN}$ in terms of sensitivity to LsbB. Four of $YvjB_{MG}$'s six amino acids belong to different physicochemical groups from the ones in $YvjB_{MN}$. These were therefore mutated separately and in combination, in both the $YvjB_{MG}$ and $YvjB_{3/4MN\text{-}1/4MG}$ proteins (in constructs pAZILyvjB$_{MG}$ and pAZILyvjB$_{3/4MN\text{-}1/4MG}$, respectively), into the residues present in $YvjB_{MN}$ in order to determine which amino acid(s) is directly responsible for the sensitivity to LsbB. In the first round of mutagenesis (single amino acid changes), it is worth noting that Tyr^{356} was able to transform carrier cells from a resistant to a sensitive phenotype (Fig. 3). This sensitivity occurred, in fact, in both tested YvjB proteins (MG7284/$YvjB_{MG}$ and MG7284/$YvjB_{3/4MN\text{-}1/4MG}$), although the zone of inhibition by BGMN1-596T as an LsbB producer was smaller than for $YvjB_{MN}$. This smaller zone of inhibition indicated a possible additional effect of other amino acids in the interaction with LsbB. In the second round of site-directed mutagenesis (when different combinations of two, three, or four amino acids were changed), some interesting results were obtained, as depicted in Fig. 3. First, only the changes in combination with $Gln^{356}Tyr$ could cause sensitivity of the carriers. However, the zones of inhibition of these mutants were different in size and transparency; for example, adding the change $Leu^{351}Phe$ to $Gln^{356}Tyr$ slightly increased the clear inhibition zone (Fig. 3). Additionally, the change $Gln^{356}Tyr$ plus $Thr^{353}Ala$ drastically increased the zone of inhibition, which was even larger than with $YvjB_{MN}$: however, it contained a high number of revertant-like resistant colonies (Fig. 3; see also Fig. S4 in the supplemental material). Triple and quadruple amino acid changes to $YvjB_{MG}$ and $YvjB_{3/4MN\text{-}1/4MG}$ showed that additional changes reduced the number of revertant-like resistant bacteria in the inhibition zone and restored the size of the zone to the size obtained with $YvjB_{MN}$. In summary, it was concluded that the amino acid in the YvjB receptor most responsible for the interaction with LsbB and the sensitivity of cells to LsbB is Tyr^{356}, whereas the other amino acids are also important but to a lesser extent than Tyr^{356}.

It is worth noting that the residue Tyr^{356} is located at the beginning of the predicted third transmembrane helix (from residue 355 to residue 376), with the C-terminal end pointing toward the cytoplasm. More important, Tyr^{356} is closely flanked by three residues that differentiate $YvjB_{MG}$ from $YvjB_{MN}$ in this region, suggesting that these residues together probably form a site to which LsbB binds to kill target cells.

Number of aa changes	Position and type of aa substitutions	Sensitivity to LsbB
0	$YvjB_{MN}$	+++
0	$YvjB_{MG}$	–
1	$Leu^{351}Phe$	–
1	$Thr^{353}Ala$	–
1	$Gln^{356}Tyr$	+
1	$Pro^{396}Gln$	–
2	$Leu^{351}Phe$, $Thr^{353}Ala$	–
2	$Leu^{351}Phe$, $Gln^{356}Tyr$	+
2	$Thr^{353}Ala$, $Gln^{356}Tyr$	+++
2	$Thr^{353}Ala$, $Pro^{396}Gln$	–
3	$Leu^{351}Phe$,$Thr^{353}Ala$, $Gln^{356}Tyr$	+++
3	$Leu^{351}Phe$, $Thr^{353}Ala$, $Pro^{396}Gln$	–
4	$Leu^{351}Phe$, $Thr^{353}Ala$, $Gln^{356}Tyr$,$Pro^{396}Gln$	+++
4	$Leu^{351}Phe$, $Thr^{353}Ala$, $Gln^{356}Tyr$,$Thr^{314}Ala$	+++

FIG 3 Antimicrobial activity of LsbB on different mutants obtained by site-directed mutagenesis of $YvjB_{MG}$ protein. Cultures of MG7284 transformants carrying different amino acid substitutions of YvjB protein were inoculated as indicator strains into soft agar in which wells were loaded with *L. lactis* BGMN1-596T. The size of the zone is referred to the clone containing the wild-type *yvjB* gene (*L. lactis* BGMN1-596). Inhibition is seen as clear zones around the wells. −, no inhibition; +, inhibition zone up to 3 mm; +++, inhibition zone >5 mm; aa, amino acid.

LsbB interacts directly with YvjB. It was of interest to determine if the LsbB bacteriocin interacts directly with the YvjB receptor.

In a GST pulldown assay, LsbB coupled to GST (it was decided to fuse the GST tag at the N terminus of LsbB, as our previous studies determined that the C terminus was important for the interaction with the target receptor [23]) was able to bind $YvjB_{MN}$ protein from membrane fractions and to retain it during stringent washing (Fig. 4). Similar experiments determined that $YvjB_{MG}$ and $YvjB_{MNR2/23}$ proteins were able to bind LsbB but much less efficiently than $YvjB_{MN}$ (Fig. 4B, lanes 2, 9, and 5, respectively). In the control reaction with GST alone, no unspecific binding was detected (Fig. 4B, lanes 3, 6, and 8, respectively).

The GST pulldown assay is an *in vitro* assay. Thus, to determine the interaction *in vivo*, an analysis with fluorescent antibodies against YvjB and LsbB proteins was performed in live cells. The immunocytochemistry assay was performed with transformants of *E. faecalis* BGZLS10-27 harboring the pAZIL vector carrying different *yvjB* genes ($yvjB_{MN}$ and $yvjB_{MG}$). We used the enterococcal strain as a host, because there was no lactococcal strain without endogenous YvjB protein to serve as a negative control (the neg-

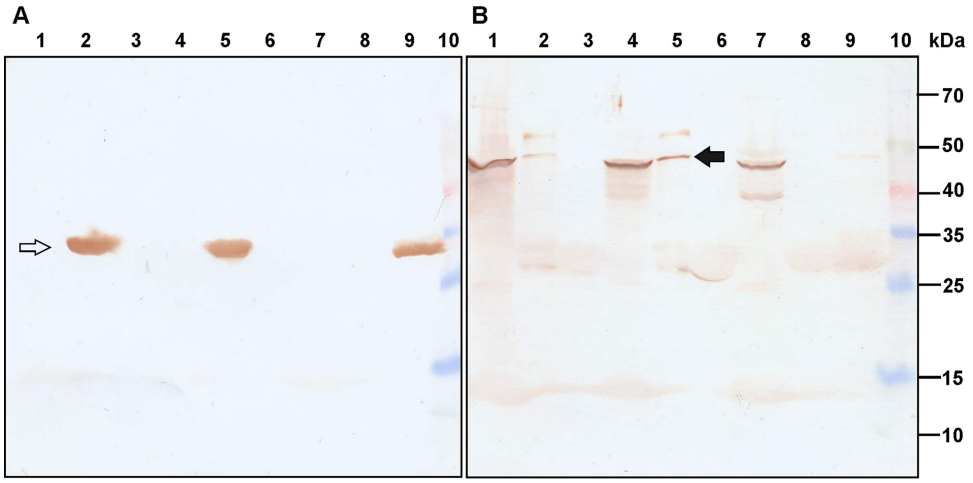

FIG 4 GST pulldown assay with purified cell membranes. (A) Western blot with anti-LsbB antibody. (B) Western blot with anti-YvjB antibody. Lane 1, total cell membrane fraction of MG7284; lane 2, proteins that interacted with GST-LsbB from the cell membrane fraction of MG7284; lane 3, proteins that interacted with GST from the cell membrane fraction of MG7284; lane 4, total cell membrane fraction of BGMN1-596; lane 5, proteins that interacted with GST-LsbB from the cell membrane fraction of BGMN1-596; lane 6, proteins that interacted with GST from the cell membrane fraction of BGMN1-596; lane 7, total cell membrane fraction of BGMN1-596R2/23; lane 8, proteins that interacted with GST from the cell membrane fraction of BGMN1-596R2/23; lane 9, proteins that interacted with GST-LsbB from the cell membrane fraction of BGMN1-596R2/23; lane 10, Spectra multicolor broad range protein ladder (Thermo Fisher Scientific). The quantities of proteins of membrane fractions of MG7284, BGMN1-596, and BGMN1-596R2/23 were normalized to 100 μg per reaction. The white arrow indicates the GST-LsbB fusion protein, while the black arrow indicates $YvjB_{MN}$ protein from the GST pulldown assay.

ative control was strain BGZLS10-27 transformed with empty pAZIL vector). In addition, this particular enterococcal strain was used because it was resistant to LsbB and nonreactive with the antibodies used (data not shown). Before beginning this assay, the sensitivity of BGZLS10-27 transformants carrying different constructs was confirmed by an antimicrobial assay (see Fig. S5 in the supplemental material). The *in vivo* immunocytochemistry assay showed that LsbB interacts specifically with the cells expressing $YvjB_{MN}$ (Fig. 5). Full matching of the color that locates the presence of transformed cells (DAPI) and fluorescent antibody that locates LsbB bacteriocin (LsbB) is obtained only when transformants BGZLS10-27/pAZIL-$YvjB_{MN}$ (merge) were used. In experiments that used the transformants carrying empty plasmid BGZLS10-27/pAZIL (control) or cloned YvjB gene from the strain MG7284 (BGZLS10-27/pAZIL-$YvjB_{MG}$), colocalization of these two signals was not obtained.

DISCUSSION

In recent years, two important needs have emerged: the increasing consumer demand for natural food preservatives, such as bacteriocins produced by lactic acid bacteria, and the demand for new antimicrobial compounds in response to the continuing emergence of antibiotic-resistant bacteria, in the face of declining numbers of new antibiotics (32–34). Consequently, the research and application of bacteriocins are ever-more-attractive propositions. Until now, it has been shown that the antimicrobial activity of bacteriocins is directly related to the presence of specific receptor proteins in the membranes of target bacteria. Thus, information on the receptors to which bacteriocins bind is potentially of great importance (4).

In our previous studies, we identified the Zn-dependent metallopeptidase YvjB as the receptor for the bacteriocin LsbB (16) and further established that the C-terminal part of LsbB is involved in the interaction with the receptor protein YvjB (23). In this work, we combined site-directed mutagenesis with the construction of hybrid protein molecules to define the residues of LsbB and YvjB involved in the interactions that eventually lead to the killing of target cells. We demonstrated that the essential amino acids are Trp^{25} and Ala^{30} in the LsbB C terminus and Tyr^{356} and Ala^{353} in the penultimate transmembrane domain of YvjB receptor.

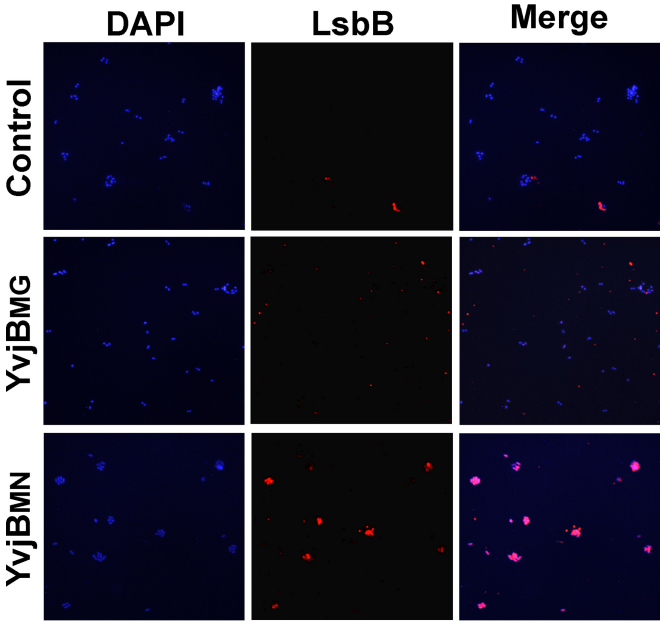

FIG 5 Immunocytochemistry assay with anti-LsbB antibodies was performed on transformants of *Enterococcus faecalis* BGZLS10-27 with pAZIL vector (control), pAZIL-$YvjB_{MG}$ (YyjBMG), and pAZIL-$YvjB_{MN}$ (YvjBMN). Bacterial DNA was stained with 4′,6-diamino-2-phenylindole (DAPI).

Such a site-directed mutagenesis approach has been used for amino acid substitution in different bacteriocins of lactic acid bacteria to analyze function or to improve antimicrobial activity (35–40). The results obtained by site-directed mutagenesis of the LsbB bacteriocin are consistent with data obtained by other laboratories, where most amino acid substitutions cause a reduction in or complete loss of activity. It is interesting, however, that some substitutions, such as alanine to serine ($Ala^{30}Ser$), enhanced bacteriocin activity, most likely due to better interaction and positioning in the YvjB receptor. In addition, we suggest that not only residues Trp^{25} and Ala^{30}, but also the distance between them, play an essential role in the antimicrobial activity of LsbB.

The construction of hybrid molecules and site-directed mutagenesis with structure-function assays enabled us to define essential domains responsible for the interaction of the target receptor YvjB with LsbB. It is interesting that all key amino acids involved in the interaction with LsbB are located in the beginning of the penultimate transmembrane domain of YvjB (Fig. 2; see also Fig. S3 in the supplemental material). The $Gly^{188}Ser$ mutant obtained by random mutagenesis (16) also includes changes in the beginning of the second transmembrane domain and shows a semiresistant phenotype. From the results presented here, it is possible to conclude that these regions are most likely accessible for interaction with LsbB. It is interesting that the beginning of the third transmembrane domain (amino acids Ala^{353}, Val^{354}, and Tyr^{356}) seems to be very close to the outside surface of the membrane, possibly making it accessible for LsbB interaction (see Fig. S3). In contrast, the beginning of the second transmembrane domain is located closer to the cytoplasmic side, indicating that both sides of the membrane are involved in the interaction of YvjB with LsbB, causing membrane damage of cells. It could be postulated that the third transmembrane domain is involved in the first interaction with LsbB, but the second transmembrane domain is also important for complex formation and membrane damage of sensitive cells. Mutants with an amino acid substitution in the second transmembrane domain, such as $Gly^{188}Ser$, are semiresistant, meaning that they can interact with LsbB on the surface but require higher concentrations of LsbB for complex formation and membrane damage of the cells. Given that LsbB *in vitro* was able to interact with all three YvjB proteins (from resistant strain MG7284, semiresistant mutant BGMN1-596R2/23, and sensitive strain BGMN1-596) but not *in vivo* indicates the involvement of other factors present in cell wall in the control of the interaction. Any changes to the essential amino acids in the bacteriocin receptor protein cause partial or complete resistance to certain bacteriocins. For this reason, it is important to identify specific receptors for bacteriocins and the domains involved in the interaction. The identification of new bacteriocin receptors present in potential target bacteria and understanding of their mechanisms of action are of great importance for the design of target-specific engineered antimicrobials.

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