

A Zn-Dependent Metallopeptidase Is Responsible for Sensitivity to LsbB, a Class II Leaderless Bacteriocin of *Lactococcus lactis* subsp. *lactis* BGMN1-5

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***Lactococcus lactis* subsp. *lactis* BGMN1-5 produces a leaderless class II bacteriocin called LsbB. To identify the receptor for LsbB, a cosmid library of the LsbB-sensitive strain BGMN1-596 was constructed. About 150 cosmid clones were individually isolated and transferred to LsbB-resistant mutants of BGMN1-596. Cosmid pAZILcos/MN2, carrying a 40-kb insert, was found to restore LsbB sensitivity in LsbB-resistant mutants. Further subcloning revealed that a 1.9-kb fragment, containing only one open reading frame, was sufficient to restore sensitivity. The fragment contains the gene *yyjB* coding for a Zn-dependent membrane-bound metallopeptidase, suggesting that this gene may serve as the receptor for LsbB. Further support for this notion derives from several independent experiments: (i) whole-genome sequencing confirmed that all LsbB-resistant mutants contain mutations in *yyjB*; (ii) disruption of *yyjB* by direct gene knockout rendered sensitive strains BGMN1-596 and IL1403 resistant to LsbB; and (iii) most compellingly, heterologous expression of *yyjB* in naturally resistant strains of other species, such as *Lactobacillus paracasei* and *Enterococcus faecalis*, also rendered them sensitive to the bacteriocin. To our knowledge, this is the first time a membrane-bound peptidase gene has been shown to be involved in bacteriocin sensitivity in target cells. We also demonstrated a novel successful approach for identifying bacteriocin receptors.**

Microorganisms inhabit a wide range of habitats, ranging from the depth of the ocean to most surfaces and spaces on Earth, and they are capable of growing on both inorganic and organic matter. In order to defend their own habitats or to invade new territories, they have developed diverse antagonistic strategies to kill or weaken their competitors, one of them being the production of bacteriocins. Bacteriocins comprise a large group of antimicrobial peptides (AMPs) produced by many bacteria (1). They are especially numerous in lactic acid bacteria (LAB), which are common bacteria in diverse food and feed products. LAB are also common inhabitants of the gastrointestinal tracts of both invertebrates and vertebrates. Due to their abundance in our food and in our body, many LAB are generally regarded as safe (GRAS) for use in food applications, and a few bacteriocins are also approved for use as food preservatives (<http://www.fda.gov/ohrms/dockets/dockets/95s0316/95s-0316-rpt0334-02-Ref-01-PLMMDI-vol262.pdf>). There is an increasing interest in developing these AMPs into useful drugs for medical use because of the pressing need for novel sources of antimicrobials, especially important with regard to the alarming emergence of antibiotic-resistant bacteria.

Most bacteriocins have relatively narrow inhibitory spectra, being active mostly against closely related bacteria. However, some have wider inhibitory spectra that include important food spoilage bacteria like *Listeria* and *Bacillus* and pathogens such as some species of *Staphylococcus* and *Enterococcus*. One of the remarkable features of bacteriocins is that they are very potent, being active in nanomolar concentrations (2), thereby surpassing by about 1,000-fold the activity of antimicrobial peptides (e.g., defensins) that humans and other animals produce. One of the major reasons for this extreme potency is that bacteriocins apparently recognize specific receptors on target cells, while the interactions

between eukaryotic peptides and microorganisms are mostly non-specific.

Some bacteriocin receptors have already been identified. Among bacteriocins from Gram-positive bacteria, nisin and several other lantibiotics (class I) specifically bind to the cell wall precursor, lipid II, and kill target cells by inhibiting cell wall synthesis and/or forming lethal pores in the membrane (3). The non-lantibiotic bacteriocin lactococcin 972 also employs lipid II as a binding molecule, but the killing mechanism has not been elucidated (4). The mannose-phosphotransferase system (man-PTS) has been found to serve as a receptor for some nonlantibiotics (class II) (5). This is true for most pediocin-like bacteriocins (subclass IIa), which are related in both sequence and structure (6), and for the unrelated bacteriocins lactococcin A and B (subclass IId non-pediocin-like and linear bacteriocins) (7). Recently, another sugar transporter, a maltose-ABC transporter, was found to be required in target cells for sensitivity to garvicin ML, a circular bacteriocin (subclass IIc) (8). Although these bacteriocins target different receptors, their receptors all have in common that they are components of the bacterial membrane. This is in line with the general view that bacteriocins target the cell membrane, where lethal pores are formed, causing cellular leakage, disruption of membrane integrity, and cell death as the final outcome.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain, plasmid, or cosmid	Relevant characteristic(s) ^a	Source or reference
Strains or mutants		
<i>Lactococcus lactis</i> subsp. <i>lactis</i>		
IL1403	Plasmid-free derivative of IL-596, LsbB ^s	25
B464	<i>ptm</i> deletion mutant of IL1403, LsbB ^s	5
BGMN1-59WT	Plasmid-free derivative of <i>L. lactis</i> subsp. <i>lactis</i> BGMN1-5, LsbB ^s	26
BGMN1-596T	Derivative of BGMN1-596WT with pMN5 plasmid, LsbB ^f , LsbB producer	26
BGMN1-596R2/12, -16, -22, -23, -27	MNNG-induced LsbB ^f mutants of strain BGMN1-596	This study
BGMN1-596R3/11, -17, -19, -21, -25	MNNG-induced LsbB ^f mutants of strain BGMN1-596	This study
BGMN1-596R3/19-pAZILcos/MN2	Complemented mutant with cosmid pAZILcos/MN2, LsbB ^s	This study
BGMN1-596R3/19-pAZIL/ZnMP	Complemented mutant with plasmid pAZIL/ZnMP, LsbB ^s	This study
BGMN1-596SR1	Spontaneous LsbB ^f mutant	This study
BGMN1-596SR2	Spontaneous LsbB ^f mutant	This study
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>		
MG7284	Prt ⁻ Lac ⁻ Bac ^s Fus ^r Spc ^r LsbB ^f	
MG7284/pAZIL-lsbB	Derivative of MG7284 with pAZIL-lsbB, LsbB ^f , LsbB producer	This study
MG7284/pAZIL/ZnMP	Derivative of MG7284 with pAZIL/ZnMP, LsbB ^s	This study
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>		
BGHN14	LsbB ^f	27
BGHN14/pAZILSJ/ZnMP	Derivative of BGHN14 with pAZILSJ/ZnMP, LsbB ^s	This study
<i>Enterococcus faecalis</i>		
BGZLS10-27	LsbB ^f	Laboratory collection
BGZLS10-27/pAZIL/ZnMP	Derivative of BGZLS10-27 with pAZIL/ZnMP, LsbB ^s	This study
<i>Escherichia coli</i>		
DH5 α	$\lambda^- \phi 80dlac\Delta M15 \Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^- m_K^-)$ <i>supE44 thi-1 gyrA relA1</i>	16
HB101	F ⁻ <i>hsdS20 (r_B^- m_B^-) supE44 recA13 ara-14 proA2 rpsL20(Str^r) xyl-5 mtl-1 galK2</i> <i>lacY1</i> λ^-	28
EC101	JM101 containing <i>repA</i> gene of pWV01 in chromosome	29
Plasmids		
pMN5		10
pAZIL	7,109 bp; Em ^r , shuttle cloning vector	18
pAZIL/ZnMP	pAZIL carrying <i>yvjB</i> gene	This study
pAZILSJ	pAZIL with <i>ori</i> and <i>repA</i> sequence of pSJ2-8	Laboratory collection
pAZILSJ/ZnMP	pAZILSJ carrying <i>yvjB</i> gene	This study
pGhost9	Em ^r , thermosensitive vector	19
pGhost9/ES	pGhost9 carrying part of <i>yvjB</i> gene	This study
p-GEM-T-Easy	3015 bp; Amp ^r , PCR cloning vector	Promega
Cosmids		
pAZILcos	8,194 bp; Em ^r , shuttle cosmid vector	18
pAZILcos/MN2	Complemented cosmid pAZILcos carrying 40-kb chromosomal DNA fragment of BGMN1-596	This study
pAZILcos/MN2-SI2	Cosmid pAZILcos/MN2 deleted with Sall restriction enzyme	This study
pAZILcos/MN2-Ps2	Cosmid pAZILcos/MN2 deleted with PstI restriction enzyme	This study
pAZILcos/MN2-Nc2	Cosmid pAZILcos/MN2 deleted with NcoII restriction enzyme	This study
pAZILcos/MN2-Sp9	Cosmid pAZILcos/MN2 deleted with SpeI restriction enzyme	This study

^a Amp^r, resistance to ampicillin; Em^r, resistance to erythromycin; Bac⁻, bacteriocin nonproducer; Bac^s, sensitive to bacteriocin; Prt⁻, proteolytically inactive; Lac⁻, lactose-fermenting ability; LsbB^s and LsbB^f, sensitivity and resistance to LsbB bacteriocin, respectively.

Previously we have reported that *Lactococcus lactis* subsp. *lactis* BGMN1-5 produces three bacteriocins: lactococcin B, LsbA, and LsbB (9, 10). The genes coding for the biosynthesis of bacteriocins LsbA and LsbB are located on a plasmid (pMN5), while the location of the genes involved in the biosynthesis of lactococcin B are not known (10). Bacteriocin LsbB is a small nonantibiotic bacteriocin of 30 amino acid (aa) residues. It belongs to the same subclass (IId) as lactococcin A and B but is not related to those, based on the amino acid sequence. Further, it is synthesized without a leader peptide, whereas lactococcin A and B and most class II bacteriocins are synthesized with a leader required for export. The

multidrug-resistance protein LmrB is responsible for the transport of LsbB out of the cell, as well as for immunity of the producer (9). In the present work we have developed a novel approach involving cosmid library construction to screen for bacteriocin receptor genes, and we also report the identification of the novel receptor for bacteriocin LsbB, namely, YvjB, a membrane-bound Zn-dependent metallopeptidase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains, their derivatives, and plasmids used in this study are listed in Table 1. *Lactococ-*

TABLE 2 Sequence of specific primers used in this study

Primer name	Sequence of primer ^a	Template(s)
lsbBF	5'-TAACGGATCCAATAGGGAAAATAG-3'	pMN5 plasmid DNA
lsbBR	5'-GCATAATAAAAACTGCAGCTATTG-3'	
ORFR	5'-GGCGTAAAAGATTCAGG-3'	Chromosomal DNA of BGMN1-596, BGMN1-596R2, BGMN1-596R3, BGMN1-596SR, IL1403, and MG7284
ORFR	5'-GAAGGGTTGGTATAAGC-3'	

^a Introduced restriction sites are indicated by underlined sequences.

cus lactis and *Enterococcus faecalis* strains were grown in M17 medium (Merck GmbH, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose (GM17) at 30°C, *Lactobacillus paracasei* was grown in deMan-Rogosa-Sharpe (MRS) medium (Merck), and *Escherichia coli* was grown in Luria broth (LB) at 37°C with aeration. Erythromycin was added to a final concentration of 10 µg ml⁻¹ and 300 µg ml⁻¹ for lactococci and *E. coli*, respectively. Ampicillin was added to a final concentration of 100 µg ml⁻¹ for *E. coli*. When necessary, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Fermentas, Vilnius, Lithuania) was added to LB medium plates at a final concentration of 40 µg ml⁻¹ for blue/white color selection of colonies.

Bacteriocin detection and activity assay. For detection of bacteriocin activity, agar well diffusion assays were performed as described previously by Lozo et al. (11). A spot-on-lawn assay was used for semiquantitative measuring of sensitivity to synthetic LsbB (ChinaPeptides Co., Ltd., Shanghai, China). Precise measurements of resistance were performed using a microtiter plate assay (12).

DNA manipulations. For clonal confirmation, pulse-field gel electrophoresis (PFGE) and DNA-DNA hybridization were performed, as described previously by Kojic et al. (10). Total and plasmid DNA from lactococci was isolated by the modified methods previously described (13, 14). For plasmid isolation from *E. coli*, a QIAprep Spin Miniprep Kit was used according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Digestion with restriction enzymes was conducted according to the supplier's instructions (Fermentas). Plasmid constructs were introduced into lactococci, enterococci, and lactobacilli by electroporation using an Eporator (Eppendorf, Hamburg, Germany) (15). Standard heat shock transformation was used for plasmid transfer into *E. coli* (16). 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. Sets of specific primers used in this study are listed in Table 2. KapaTaq 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, Netherlands). Sequences were compared to the NCBI database using BLAST. The DNA Strider program was used for open reading frame (ORF) prediction. Commercial p-GEM-T-Easy (Promega) vector was used for cloning of PCR products.

N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) mutagenesis of LsbB-sensitive strain BGMN1-596. *L. lactis* BGMN1-596 was grown overnight at 30°C in 10 ml of GM17 broth. The overnight culture was diluted 100-fold in 50 ml of GM17 broth and incubated at 30°C until the end of the exponential growth phase. Pelleted cells were washed with 10 ml of sodium phosphate buffer (100 mmol, pH 7), harvested by centrifugation, and finally resuspended in 1 ml of sodium phosphate buffer (100 mmol, pH 7). The cell suspension was divided into smaller volumes and

mixed with 1 ml of MNNG (Sigma) dissolved in sodium phosphate buffer (pH 7) to obtain the final concentrations of 12.5, 25, 50, and 100 µg ml⁻¹. The resulting mixtures were incubated for 1 h at 30°C. The cells were recovered by centrifugation, washed with 1 ml of sodium phosphate buffer (pH 7), resuspended in 1 ml of GM17 broth, and incubated for 1 h at 30°C. The mixtures were serially diluted (from 10⁻¹ to 10⁻⁷), and cells were then grown on GM17 plates for determination of bacterial survival after mutagenesis (17). In addition, spontaneously LsbB-resistant mutants of BGMN1-596 were isolated as colonies growing in inhibition zones.

Construction of cosmid library of LsbB-sensitive strain BGMN1-596. Total genomic DNA isolated from BGMN1-596 was partially digested with EcoRI at room temperature (ca. 23°C), and samples were collected at different time points. EDTA was immediately added to a final concentration of 10 mM (pH 8) to stop digestion. pAZILcos vector (18) was digested with EcoRI, dephosphorylated, and ligated with partially digested total DNA. Ligation mixtures were checked for the presence of high-molecular-weight concatemers by agarose gel electrophoresis before encapsulation into phage particles using a packaging kit (Agilent Technologies) and transduction of *E. coli* HB101 cells. Clones were selected on Luria-Bertani agar (LA) plates containing 300 µg ml⁻¹ erythromycin. For long-term storage, the constructed cosmid library in *E. coli* was stored in LB medium containing 15% (vol/vol) glycerol at -80°C.

Construction of the *yvjB* gene knockout mutants. The gene *yvjB* was insertionally inactivated in the bacterial genome by homologous integration using a previously described protocol (19) but with some minor modifications. A fragment containing part of the *yvjB* gene (EcoRI-ScaI; 946 bp) was cloned into pGhost9 vector, resulting in the construct pGhost9/ES. The LsbB-sensitive strains BGMN1-596 and IL1403 were transformed with pGhost9/ES and grown on GM17 plates at 28°C for 48 h. Obtained transformants were transferred to 28°C and 37°C and incubated for 48 h. In the next step, transformants grown at both temperatures were used as indicator strains in agar well diffusion assays. Strain MG7284/pAZIL-lsbB was used as a bacteriocin producer.

Global phenotypic testing of carbon source utilization. Cellular respiration of *L. lactis* was measured in triplicate by the Phenotype MicroArrays system (Biolog, USA) according to the manufacturer's instructions. *L. lactis* strains (BGMN1-596WT and mutants resistant to LsbB) were streaked on GM17 agar plates. Colonies were scraped from the plates and resuspended in Biolog inoculating fluid IF-0a with growth supplements and Biolog redox dye mixture according to standard protocols recommended for *Streptococcus* species. Aliquots (100 µl) were added to each well of carbon source plates (PM1 and PM2). The plates were incubated at 30°C in an aerobic OmniLog incubator plate reader, and cellular respiration was measured kinetically by determining the colorimetric reduction of tetrazolium dye. Data were collected approximately every 10 min over a 72-h period and analyzed with Biolog kinetic and parametric software. The PM1 and PM2 Biolog assays assess the ability of a bacterium to utilize any of 190 carbon compounds as the sole carbon source.

Whole-genome sequencing. DNA samples from wild-type and two mutant strains were sequenced at the Norwegian Sequencing Centre (sequencing.uio.no) using an Illumina MiSeq instrument, according to the manufacturer's recommendations. For each strain about 1.5 million paired-end reads of 2 by 250 bp were obtained. A preliminary assembly of the wild-type genome was obtained using ABySS (20), resulting in 157 contigs covering 2,465,230 bp. Genetic differences between the reference genome and the mutants were then detected using the polymorphism discovery algorithm VAAL from the Broad Institute (21), installed on the Abel Computing Cluster at the University of Oslo.

Nucleotide sequence accession number. The nucleotide sequence of the cloned DNA fragment from cosmid pAZILcos/MN2 that carries the *yvjB* gene was submitted to EMBL and GenBank under accession number HG008906.

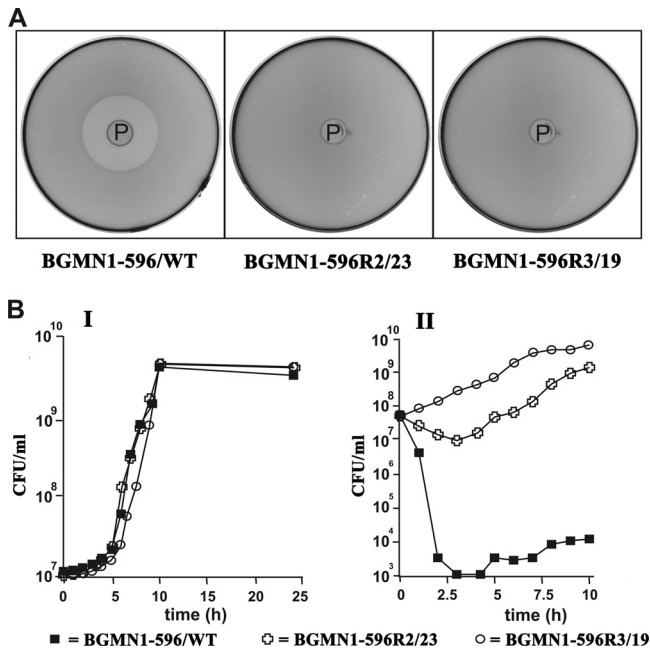


FIG 1 (A) Inhibition plate assay comparing the wild-type strain BGMN1-596/WT with resistant mutants (BGMN1-596R2/23 and BGMN1-596R3/19) used as indicators. LsbB-producing (P) cells of *L. lactis* MG7284 pAZIL-lsbB were applied into the wells made in the indicator lawns. Plates were incubated overnight at 30°C for development of inhibition zones. (B) Growth rate of WT cells compared with that of resistant cells in the medium without (I) and with (II) 25 $\mu\text{g ml}^{-1}$ LsbB.

RESULTS

Heterologous expression of the *lsbB* gene in *L. lactis* MG7284.

As the original LsbB bacteriocin producer *L. lactis* BGMN1-5 is a multibacteriocin producer, the *lsbB* gene was cloned and expressed in the nonproducer *L. lactis* MG7284 to produce LsbB alone. The *lsbB* gene was amplified from plasmid pMN5 (isolated from BGMN1-596T) using primers lsbBF and lsbBR with incorporated restriction sites (BamHI and PstI, respectively) (Table 2). An amplicon of the expected size was purified, digested, and ligated into pAZIL vector predigested with the same restriction enzymes. A clone carrying the *lsbB* gene was first selected in *E. coli* DH5 α and then transferred into *L. lactis* MG7284. The resulting LsbB-producing clone, *L. lactis* MG7284/pAZIL-lsbB, was used to screen for LsbB-resistant mutants.

Isolation of LsbB-resistant mutants from a random mutant bank of *L. lactis* BGMN1-596. MNNG was used to generate random mutations in the wild-type *L. lactis* BGMN1-596. An appropriate survival rate of about 1% was achieved when MNNG was used at a concentration of 100 $\mu\text{g ml}^{-1}$. LsbB-resistant mutants were selected on GM17 agar plates containing cell-free spent culture supernatant of the LsbB-producing clone *L. lactis* MG7284/pAZIL-lsbB. Thirty-nine colonies appeared on the selective plates after incubation for 48 h at 30°C. Cells from 5 of these 39 colonies were found to be sensitive to LsbB, while the remaining colonies still displayed a resistance phenotype, as shown for two such resistant mutants in Fig. 1A.

Analysis of LsbB-resistant mutants. Using synthetic LsbB in the bacteriocin test, we found that the resistant mutants could be divided into two groups, designated BGMN1-596R2 and BGMN1-

596R3 (Table 3), based on their tolerance levels toward LsbB. Resistant mutants of the BGMN1-596R3 group had the ability to grow at LsbB concentrations above 1 mg ml^{-1} , whereas mutants of the BGMN1-596R2 group could grow at LsbB concentrations of up to 62.5 $\mu\text{g ml}^{-1}$. In contrast, the wild-type strain BGMN1-596 was capable of growth only in medium with less than 125 ng ml^{-1} of LsbB. Five mutants from each group were stored and used for further analysis.

The growth patterns of the strain BGMN1-596 and mutants BGMN1-596R2/23 and BGMN1-596R3/19 were very similar in LsbB-free medium but differed greatly from each other when the strains were grown in a medium containing LsbB (25 mg ml^{-1}), with BGMN1-596R3/19 having the best growth, followed by BGMN1-596R2/23, while the growth of the wild-type control strain BGMN1-596 as expected was severely affected (Fig. 1B).

In addition to the MNNG-generated resistant mutants, two spontaneously resistant mutants, named BGMN1-596SR1 and BGMN1-596SR2 (group BGMN1-596SR), were isolated from cultures exposed to high concentrations of LsbB (50 to 250 mg ml^{-1}). In agar well diffusion assays these two mutants showed a resistance level similar to that of the BGMN1-596R3 group of mutants (Table 3).

LsbB bacteriocin does not use sugar transporters as a receptor. It is known that pediocin-like bacteriocins (class IIa) and lactococcin A (a class IIb bacteriocin from *L. Lactis*) employ man-PTS as a receptor. Resistant mutants to these bacteriocins normally harbor mutations that lead to downregulation of man-PTS genes (22) or to a nonfunctional man-PTS (mutations within the man-PTS genes themselves) (7). To determine whether LsbB employs man-PTS as a receptor, agar well diffusion assays with the wild-type *L. lactis* IL1403 and a man-PTS deletion mutant (strain B464) were performed. It was observed that LsbB killed bacterial cells of both strains with equal efficiency, confirming that man-PTS is not a receptor for LsbB. In addition, a metabolic assay

TABLE 3 Summary of mutations within the *yyjB* gene in LsbB-resistant mutants

Designation of mutant	Location of mutations in the <i>yyjB</i> gene	Size (aa)
MNNG-induced mutants		
Group BGMN1-596R2		
BGMN1-596R2/12	Gly→Ser (188)	428
BGMN1-596R2/16	Gly→Ser (188); Phe→Leu (414); Val→Gly (415); Asn→Lys (428)	428
BGMN1-596R2/22	Gly→Ser (188); Phe→Leu (414); Val→Gly (415); Asn→Met (428)	428
BGMN1-596R2/23	Gly→Ser (188)	428
BGMN1-596R2/27	Gly→Ser (188); Phe(TTT)→STOP codon TAA(414)	413
Group BGMN1-596R3		
BGMN1-596R3/11	Gln(CAA)→STOP codon TAA(172)	171
BGMN1-596R3/17	Gln(CAA)→STOP codon TAA(172)	171
BGMN1-596R3/19	Trp(TGG)→STOP codon TAG(248)	247
BGMN1-596R3/21	Base C at position 558 deleted; STOP codon TGA(570)	189
BGMN1-596R3/25	Gln(CAA)→STOP codon TAA(172)	171
Spontaneous mutants		
Group BGMN1-596SR		
BGMN1-596SR1	Trp(TGG)→STOP codon TAG(26)	25
BGMN1-596SR2	Trp(TGG)→STOP codon TGA(248)	247

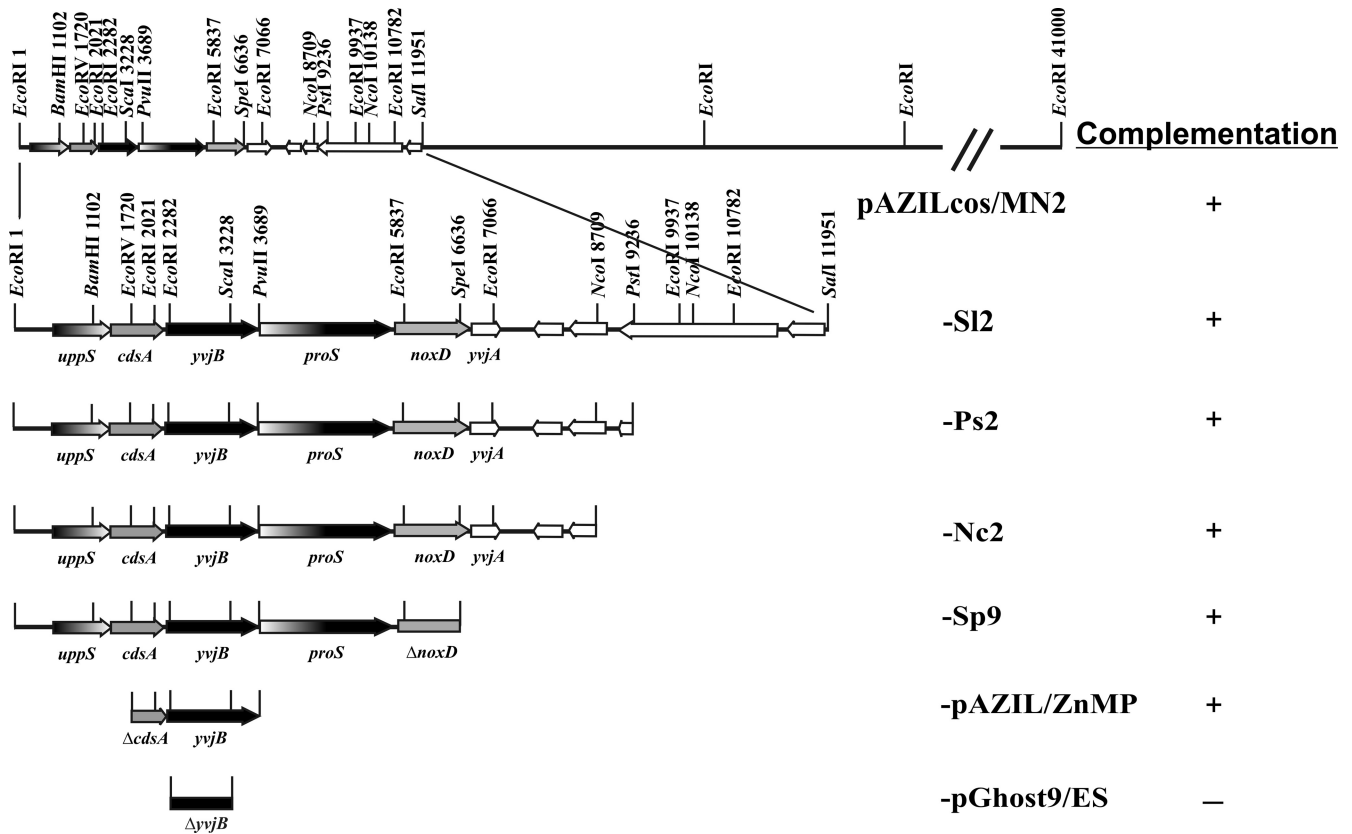


FIG 2 Linear gene map of cosmid pAZILcos/MN2 and the scheme of constructed clones used for homologous and heterologous expression of the *yvjB* gene. Relevant restriction sites are indicated. The size and orientation of predicted ORFs are indicated by arrows.

(Biolog) with a panel of 190 different carbon sources was performed for two different LsbB-resistant mutants and the wild-type strain in order to examine whether another sugar transporter could be involved as a receptor. In this assay, we also included lactococcin A-resistant mutants and garvicin ML-resistant mutants as controls since the receptors for these bacteriocins (manPTS for lactococcin A and maltose-ABC transporter for garvicin ML) are involved in sugar metabolism. As expected, the control experiment clearly demonstrated that lactococcin A-resistant mutants and garvicin ML-resistant mutants could not ferment glucose and maltose, respectively, substrates of the corresponding transporters. For the two LsbB-resistant mutants, some changes were found in the metabolic patterns compared with wild-type cells, but none of these changes were shared by both mutants.

Reversion of the resistant mutant BGMN1-596R3/19 to wild-type sensitivity by transformation with cosmids. A cosmid library of the sensitive strain BGMN1-596 was constructed, and DNA samples from 148 cosmid clones with average insert sizes of 40 kb were individually isolated and transferred by electroporation into the resistant mutant BGMN1-596R3/19 for complementation. One cosmid clone, termed pAZILcos/MN2, was found to restore sensitivity to LsbB. This cosmid was also found to complement all other resistant mutants (BGMN1-596R2, BGMN1-596R3, and BGMN1-596SR, representing all three groups), indicating that they all contain mutations within the region that is contained in the cosmid pAZILcos/MN2. Using PFGE SmaI macrorestriction pattern analysis, it was confirmed that all isolated and complemented mutants were derivatives of the corresponding parental strain.

A gene encoding a membrane-bound metallopeptidase is required for LsbB sensitivity. To identify the minimal genetic unit on the cosmid pAZILcos/MN2 that could transfer sensitivity to LsbB, pAZILcos/MN2 was digested with different restriction enzymes and subcloned into pAZIL before being transferred to the resistant mutant BGMN1-596R3/19 for functional analysis. Several constructs were able to restore sensitivity in BGMN1-596R3/19 (Fig. 2). These clones were used for mapping and sequencing the region of the pAZILcos/MN2 cosmid responsible for bacteriocin sensitivity (Fig. 2). The smallest DNA fragment that led to regained LsbB sensitivity, construct Sp9, was completely sequenced by primer walking. The fragment was found to encode five open reading frames (ORFs), one of which is truncated (*uppS*, *cdsA*, *yvjB*, *proS*, and truncated *noxD*) (Fig. 2). To dissect further, the individual ORFs were subcloned and again transferred into BGMN1-596R/19. Only the clone (pAZIL/ZnMP) that contained the *yvjB* gene was able to restore sensitivity. This clone also rendered all other LsbB-resistant mutants sensitive to LsbB. Thus, the gene *yvjB* appears to be the minimal genetic unit required to restore bacteriocin sensitivity.

***yvjB* knockout mutants confer resistance to LsbB.** To confirm that the gene *yvjB* is required for LsbB sensitivity in other *L. lactis* strains as well, *yvjB* knockout mutants of BGMN1-596 and IL1403 were constructed by insertional inactivation using the clone pGhost9/ES. pGhost9/ES contains a heat-labile replicon and a fragment of the *yvjB* gene and allows inactivation of *yvjB* by homologous recombination at elevated temperature (37°C). The results showed that transformants grown at 28°C (controls) were

sensitive to LsbB bacteriocin, whereas transformants grown at 37°C became resistant to LsbB, indicating that inactivation of the *yyjB* gene confers resistance to LsbB.

Comparative DNA sequence analyses of the *yyjB* gene in LsbB-resistant mutants. In parallel to the cloning approach described above, the genomes of two resistant mutants (BGMN1-596R2/23 and BGMN1-596R3/19) and the wild-type strain BGMN1-596 were sequenced to identify genes that may be involved in the resistance phenotype. It was found that both mutants indeed harbored mutations in the *yyjB* gene (Table 3), in addition to a number of other mutations dispersed in the genome. The *yyjB* mutation in strain BGMN1-596R2/19 generated a stop codon at position 248 of the encoded protein, leading to a truncated protein of 247 aa compared to the wild-type protein of 428 aa. The *yyjB* mutation in BGMN1-596R3/23 generated a frame-shift mutation in codon 188. Sequencing of PCR-amplified *yyjB* from the 10 remaining resistant mutants (groups BGMN1-596R2, BGMN1-596R3, and BGMN1-596SR) gave similar results as all were shown to contain different mutations within this gene (Table 3). In all five mutants of the BGMN1-596R2 group, Gly-188 was replaced with Ser in the YvjB protein, giving a mutated protein of the same size (428 aa) as the wild type. Mutant BGMN1-596R2/27 in addition had a stop codon at position 414 producing a truncated form of the YvjB protein (413 aa). All mutants of group BGMN1-596R3 and the spontaneously resistant mutants (group BGMN1-596SR) carried stop codons at different positions, leading to various truncated forms of the YvjB protein (Table 3). Taken together, the sequencing results are in line with the results from the cloning approach, all of which indicates that gene *yyjB* is required for sensitivity to LsbB and likely serves as a receptor for the bacteriocin.

Heterologous expression of the *yyjB* gene confers sensitivity to LsbB. To examine whether the *yyjB*-dependent sensitivity to LsbB is strain or species specific, this gene was transferred into cells of various naturally resistant strains: *L. lactis* MG7284, *E. faecalis* BGZLS10-27, and *L. paracasei* BGHN14. For this purpose, the complete *yyjB* gene was cloned into pAZIL and pAZIL-SJ, giving rise to the constructs pAZIL/ZnMP and pAZILSJ/ZnMP, respectively. The construct pAZIL/ZnMP replicates in *L. lactis* and *E. faecalis*, whereas pAZILSJ/ZnMP replicates only in *L. paracasei*. Transformation of these bacteria with the cloned *yyjB* indeed rendered all of the strains sensitive to LsbB (Fig. 3), indicating that the observed *yyjB*-dependent sensitivity is not strain or species specific.

DISCUSSION

Most bacteriocins have relatively narrow inhibition spectra, often displaying activity toward species closely related to the bacteriocin producer. Due to this strong specificity, it is generally believed that most, if not all, bacteriocins recognize specific receptors on target cells. Although numerous bacteriocins from Gram-positive bacteria have been characterized over the years, only a few receptors so far have been reported; these are lipid II recognized by nisin and several other lantibiotics, as well as the class II d bacteriocin lactococcin 972 (3, 4, 23), man-PTS, targeted by class II a pediocin-like bacteriocins and the class II d lactococcins A and B (5, 24), and maltose-ABC transporter, recognized by the circular bacteriocin garvicin ML (8). The possibility that LsbB employs sugar transporters as receptors was also explored. The results of a bacteriocin test (with strain B464 as an indicator) and Biolog assay strongly indicate that the resistance phenotype observed for the LsbB mu-

tants did not result from a defective sugar metabolic pathway. In the present work, we report the discovery of a new receptor, namely, the membrane-bound Zn-dependent metallopeptidase YvjB, targeted by LsbB.

The identification of the membrane-bound Zn-dependent metallopeptidase YvjB as a receptor for LsbB is based on several lines of evidence: (i) by cosmid construction and gene subcloning, we were able to find the minimum genetic unit capable of recovering sensitivity to LsbB in LsbB-resistant mutants, and this minimum genetic unit contains only the gene *yyjB*; (ii) all 12 independently isolated LsbB-resistant mutants contain mutations within *yyjB*; (iii) inactivation of *yyjB* by directed gene disruption in LsbB-sensitive *L. lactis* strains BGMN1-596 and IL1403 rendered them resistant to the bacteriocin; and (iv) perhaps most convincingly, *yyjB* also conferred sensitivity to LsbB when expressed in heterologous hosts outside the *Lactococcus* genus, such as *Lactobacillus paracasei* and *Enterococcus faecalis*, which are normally resistant to LsbB. The last observation also suggests that the protein YvjB most likely serves as the sole receptor unit (i.e., without the requirement of additional factors) and acts in a species-independent manner.

Comparative DNA sequence analysis of *yyjB* genes from all three groups of mutants (BGMN1-596R2, BGMN1-596R3, and BGMN1-596SR) revealed two types of mutations within *yyjB* that gave rise to the LsbB resistance phenotype. The resistance arose either when Gly-188 was replaced with Ser, as shown for the BGMN1-596R2 group of mutants, or when truncated forms of YvjB lacking the C-terminal part were produced, as observed in the BGMN1-596R3 and BGMN1-596SR groups of mutants. Different levels of resistance to LsbB were observed for the different groups of mutants, with BGMN1-596R3 and BGMN1-596SR mutants (encoding truncated versions of YvjB) more resistant than the BGMN1-596R2 mutants (YvjB containing the Gly-to-Ser substitution at position 188). It is reasonable to believe that the truncated forms of YvjB are structurally deficient and incapable of serving as receptors. The molecular basis of the resistance resulting from the replacement of Gly-188 with Ser is less obvious, as there are several possibilities for how such a substitution might cause a resistance phenotype. The substitution might cause a structural change, somehow becoming a nonfunctional or less functional receptor for LsbB. (ii) The amino acid Gly-188 might play an important role in receptor binding; the nonconserved substitution to Ser which is both larger and more hydrophilic than Gly-188, could interfere with the interaction with LsbB. (iii) Gly-188 might directly be involved in pore formation; a nonconserved substitution at this residue might somehow affect the properties of the pore (i.e., blocking pore formation). Arguably, other possibilities might likely exist. However, shedding light on the role of this interesting residue in terms of bacteriocin sensitivity and mode of resistance development requires further and more detailed investigation. It is worth noting that Gly-188 is almost completely conserved (present in 7,947 out of 8,451 sequences) in the corresponding position in the Pfam family PF02163 (peptidase_M50), pointing to an essential role for Gly in this position.

Interestingly, the strain *L. lactis* MG1363 also contains a *yyjB* gene in its genome; the encoded YvjB protein has the same length (428 aa) and shows a relatively high sequence identity (93%; 397 of 428 aa), to the YvjB ortholog in the LsbB-sensitive strains BGMN1-596 and IL1403. Nevertheless, MG1363 is resistant to LsbB. The sequence around Gly-188 is identical in all three strains (KMLT^NFGG^LPLNNFILG; Gly-188 is underlined), suggesting

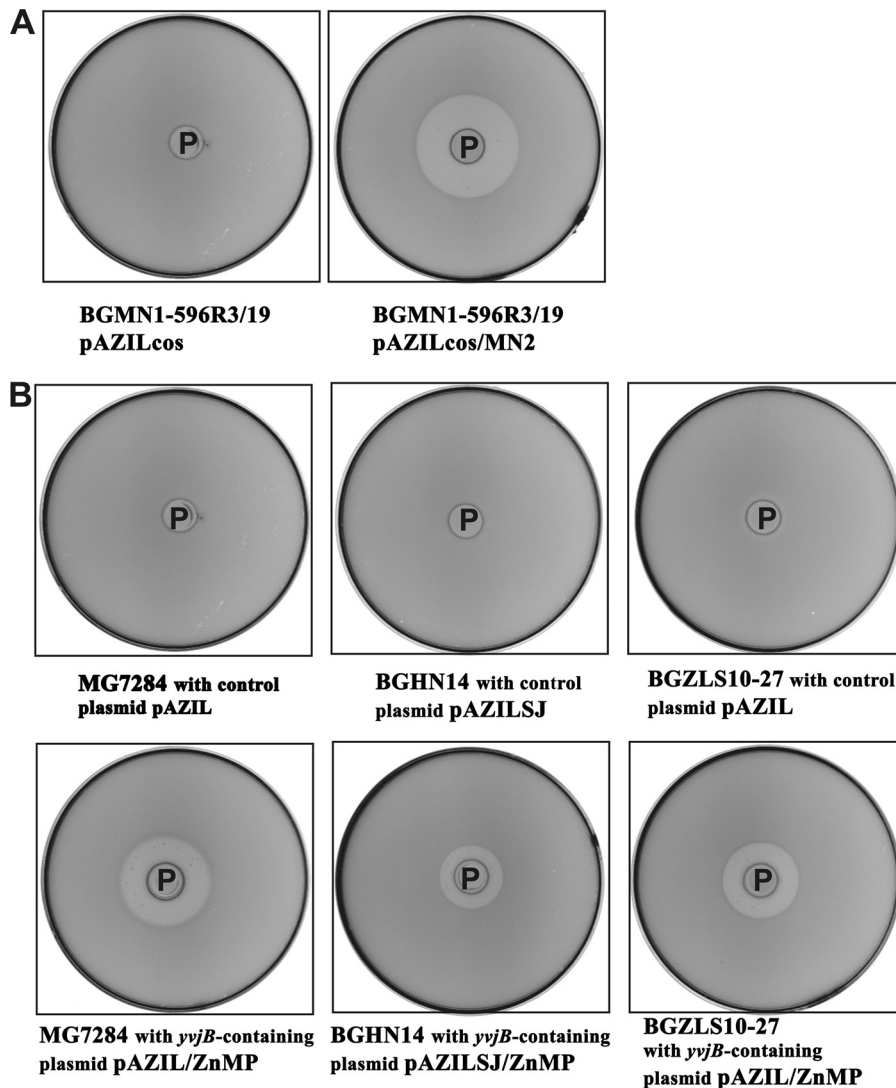


FIG 3 Complementation assay. (A) Resistant mutant BGMN1-596R3/19 containing the empty plasmid (pAZILcos) or a plasmid carrying the *yvjB* gene (pAZIL-MN2). *L. lactis* MG7284/pAZIL-lsbB was used as an LsbB producer. (B) Heterologous expression of *yvjB* renders resistant cells of *L. lactis* subsp. *lactis* MG7284, *Lactobacillus paracasei* BGHN14, and *Enterococcus faecalis* BGZLS10-27 sensitive to the bacteriocin. Strains and plasmids are as indicated. See the legend of Fig. 1 for the experimental setup.

that some of the other substitutions (31 in total) in MG1363 YvjB may be responsible for the observed resistance in this particular strain.

Class II bacteriocins are numerous and very diverse in terms of sequence, structure, and physicochemical properties and are thus believed to recognize different receptors on target cells. Similarly, bacterial cells contain a plethora of membrane proteins that are involved in diverse activities across the membrane, including transport of diverse salts and sugars, energy harvesting, cell-to-cell communication, structural functions, etc. It is reasonable to believe that any of these proteins have the potential to serve as receptors for bacteriocins. However, so far only a few membrane-located proteins are known as receptors for class II bacteriocins; these comprise a man-PTS, a maltose-ABC transporter, and, from this work, a Zn-dependent metallopeptidase. The limited progress in receptor identification is probably due to a lack of efficient strategies for this purpose. In the present work, we demonstrate an

efficient and novel strategy involving generation of resistant mutants, complementation by cosmid cloning, genome sequencing, and metabolic analysis to identify novel bacteriocin receptors. Indeed, we are currently exploiting this strategy to identify receptors also for other bacteriocins, and some promising preliminary results have been obtained. Importantly, the combination of the generation of resistant mutants and genome sequencing proves to be a very powerful approach to identify potential receptor loci, which can be further assessed by other molecular tools (e.g., gene cloning). As genome sequencing has become a very cost-effective and hence more available genetic tool, more receptors are likely to be discovered in the near future.

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