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Novel Mechanism of Bacteriocin Secretion and Immunity Carried Out by Lactococcal Multidrug Resistance Proteins*

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Olivera Gajic‡, Girbe Buist‡, Milan Kojic§, Ljubisa Topisirovic§, Oscar P. Kuipers‡, and Jan Kok‡¶

From the ‡Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands and §Institute for Molecular Genetics and Genetic Engineering, Vojvode Stepe 444a, 11000 Belgrade, Yugoslavia

A natural isolate of *Lactococcus lactis* was shown to produce two narrow spectrum class II bacteriocins, designated LsbA and LsbB. The cognate genes are located on a 5.6-kb plasmid within a gene cluster specifying LmrB, an ATP-binding cassette-type multidrug resistance transporter protein. LsbA is a hydrophobic peptide that is initially synthesized with an N-terminal extension. The housekeeping surface proteinase HtrA was shown to be responsible for the cleavage of precursor peptide to yield the active bacteriocin. LsbB is a relatively hydrophilic protein synthesized without an N-terminal leader sequence or signal peptide. The secretion of both polypeptides was shown to be mediated by LmrB. An *L. lactis* strain lacking plasmid-encoded LmrB and the chromosomally encoded LmrA is unable to secrete either of the two bacteriocins. Complementation of the strain with an active LmrB protein resulted in restored export of the two polypeptides across the cytoplasmic membrane. When expressed in an *L. lactis* strain that is sensitive to LsbA and LsbB, LmrB was shown to confer resistance toward both bacteriocins. It does so, most likely, by removing the two polypeptides from the cytoplasmic membrane. This is the first report in which a multidrug transporter protein is shown to be involved in both secretion and immunity of antimicrobial peptides.

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a large variety of bacteria (1, 2). This group of antibacterial compounds is also found in plants (*e.g.* thionins (3)) and animals (*e.g.* defensins, magainins, and cecropins (4)). The eukaryotic antimicrobials share a number of structural similarities with bacterial bacteriocins such as a very small size and considerable amphiphilicity.

Most bacteriocins produced by the Gram-positive lactic acid bacteria (LAB)[1] characterized to date are small (less than 6 kDa), cationic, and amphiphatic membrane permeabilizing peptides (2). They can be classified into three main groups (5). Group I comprises the lantibiotics that contain post-translationally modified amino acids, such as lanthionine and β-methyllanthionine and the dehydrated residues dehydroalanine and dehydrobutyrine (6, 7). Group II, consisting of the unmodified heat-stable peptide bacteriocins (the non-lantibiotics), can be further divided into Group IIa, the pediocin-like bacteriocins (8, 9), Group IIb, the two-peptide bacteriocins, which require the complementary action of two peptides for full antimicrobial activity (10, 11), and Group IIc, other unmodified bacteriocins. Group III contains larger and heat-labile bacteriocins. Bacteriocins are mainly synthesized as precursor peptides with an N-terminal leader sequence (12). The primary translation product of most non-lantibiotics and some lantibiotics contains a leader peptide of the double glycine-type (Gly^{-2}-Gly^{-1}), which is cleaved off during export across the cytoplasmic membrane by dedicated ATP-binding cassette (ABC) transporters and their accessory proteins (13, 14). Some bacteriocins, *e.g.* divergicin A, enterocin P, and listeriocin 743A (9, 15), are exported across the cytoplasmic membrane by the general secretory pathway (16). They contain canonical *sec* signal peptides consisting of a positively charged N terminus, a hydrophobic core, and a defined cleavage site that is removed by a specific signal peptidase during translocation. Some bacteriocins produced by *Enterococcus faecium*, *e.g.* enterocin L50A and L50B, and enterocin Q have been shown recently (17, 18) to be synthesized without an N-terminal leader sequence or signal peptide.

All bacteriocin producers are insensitive to the bacteriocin(s) they produce. The genetic determinants proposed or confirmed to confer immunity are frequently found downstream of the bacteriocin structural gene(s) in the bacteriocin operon (2, 12). These immunity proteins usually have a high pI, and those that are associated with one-peptide bacteriocins are generally small in size (51 to 113 amino acids) and contain no or only a few (one to two) potential transmembrane helices (10, 12, 19).

Here, we report the characterization of two novel and highly different bacteriocins, LsbA and LsbB, produced by *Lactococcus lactis*. The cognate genes were cloned and sequenced, and their transcription was analyzed. Also, the processing mechanism of LsbA, the secretion of both bacteriocins, and the resistance mechanism against both peptides were studied. Both secretion and immunity were found to rely on the activity of a single multidrug resistance (MDR) transporter protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media

Bacterial strains and plasmids used in this study are listed in Table I. *L. lactis* was grown at 30 °C in chemically defined medium CDM (20), M17 (Difco; West Molesey, United Kingdom), or ½ M17 broth (containing 0.95% β-glycerophosphate; Sigma) as standing cultures and on M17 agar plates containing 1.5 or 0.75% (w/v) agar. All media contained 0.5% (w/v) glucose, whereas 5 μg/ml chloramphenicol (Sigma) or 5 μg/ml erythromycin (Roche Applied Science) were added when needed.

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¶ To whom correspondence should be addressed. Tel.: 31-50-3632111; Fax: 31-50-3632348; E-mail: j.kok@biol.rug.nl.

[1] The abbreviations used are: LAB, lactic acid bacteria; ABC, ATP-binding cassette; MDR, multidrug resistance; GFP, green fluorescent protein; PAA, polyacrylamide; RT, reverse-transcribed; ORF, open reading frame.

TABLE I
Bacterial strains and plasmids used in this study

Em^r, Cm^r, Ap^r, resistance to erythromycin, chloramphenicol, and ampicillin, respectively. P_{lsbB}; *lsbB* promoter; P_{lmrB}; *lmrB* promoter; P_{nisA}, nisin inducible *nisA* promoter; Gal, galactosidase.

Strain or plasmid	Relevant phenotype or genotype	Source or reference
L. lactis sp. *cremoris*		
MG1363	Plasmid-free derivative of NCDO712; $LmrA^+$ $LmrP^+$	Ref. 47
MG1363Δ*lmrP*	MG1363 derivative with deletion in *lmrP*	Ref. 39
NZ9000	MG1363 *pepN*::*nisRK*	Ref. 35
NZ9700	Nisin producing transconjugant of MG1363 containing the nisin-sucrose transposon Tn*5276*	Ref. 48
NZ9000Δ*lmrA*	MG1363 *pepN*::*nisRK* with deletion in *lmrA*	This work
NZ9000Δ*acmA* Δ*htrA*	MG1363 *pepN*::*nisRK* containing deletions in *acmA* and *htrA*	Kees J. Leenhouts
LL108	MG1363 derivative carrying the pWV01 *repA* gene downstream of the promoter P_{23} in its chromosome	Ref. 25
L. lactis sp. *lactis*		
BGMN1–5	Wild type strain, $Bac501^+$, $Bac513^+$	Ref. 30
BGMN1–596	Plasmid-free derivative of BGMN1–5; $Bac501^+$, $Bac513^+$	Ref. 30
IL1403	Plasmid-free strain; $LmrA^-$, $LmrP^-$	Ref. 49
JIM7049	IL1403 *his*::*nisRK*	Ref. 50
pNZ8048	Cm^r inducible expression vector carrying P_{nisA}	Ref. 35
pNH*lmrA*	*his6-lmrA* of *L. lactis* MG1363 behind P_{nisA}	Ref. 51
pHLP5	*his6-lmrP* of *L. lactis* MG1363 behind P_{nisA}	Ref. 52
pORI280	Em^r, ori^+ of pWV01, replicates only in strains carrying *repA in trans*	Ref. 25
pMN5	Plasmid carrying *lmrB, lsbA,* and *lsbB*	Ref. 30
pNZ*lsbA*	Cm^r, pNZ8048 carrying *lsbA*	This work
pNZ*lsbB*	Cm^r, pNZ8048 carrying *lsbB*	This work
pNH*lmrB*	Cm^r, pNZ8048 containing *his6-lmrB*	This work
pNZ*lsbB-lmrB*	Cm^r, pNZ*lsbB* derivative containing *lmrB* in an operon structure with *lsbB*	This work
pNZ*lsbB-lmrA*	Cm^r, pNZ*lsbB* derivative containing *lmrA* in an operon structure with *lsbB*	This work
pNZ*gfp-lmrB*	Cm^r, pGFP-*mutI* derivative containing *lmrB* fused C-terminally to *gfp*	This work
pGFP-*mutl*	Cm^r, pNZ8048 containing *gfp mutI*	Nathalie Campo
pNZ9530	Em^r, pIL252 derivative carrying *nisRK*	Ref. 53
pVE6007	Cm^r, Ts replication derivative of pWV01	Ref. 54
pAPL2	Ap^r, pUC19 carrying a 6063-bp lactococcal chromosomal DNA fragment with *lmrA* gene	Koen Venema
pAPL3	Ap^r, pAPL2 with 1561-bp *Eco*RV deletion	This work
pORI*lmr*	pORI280 carrying 3.234-kb *Sca*I/*Bam*HI fragment from pAPL3, containing *lmrA* deletion and its flanking regions	This work
pGKH10	Em^r, Cm^r, contains promoterless genes for α-Gal and β-Gal (*lacZ*)	Ref. 36
pGKH1	Em^r, Cm^r, contains genes for α-Gal and β-Gal controlled by P_{lmrB} and P_{lsbB}, respectively	This work
pGKH2	Em^r, Cm^r, contains genes for α-Gal and β-Gal controlled by P_{lsbB} and P_{lmrB}, respectively	This work

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma) was used at a concentration of 1 mM.

DNA Techniques and Transformation

Molecular cloning techniques were performed essentially as described by Sambrook *et al.* (21). Restriction enzymes, T4 DNA ligase, and Expand™ High Fidelity DNA polymerase (Roche Applied Science) were used according to the instructions of the supplier. Synthetic oligonucleotides were obtained from Invitrogen. The High Pure PCR product purification kit (Roche Applied Science) was used to purify PCR products. For nucleotide sequence analysis the dideoxy chain termination method (22) was used with [α-^{35}S]dATP and the T7 sequencing kit (Amersham Biosciences). *L. lactis* was transformed by electroporation using a gene pulser (Bio-Rad) as described by Leenhouts and Venema (23). The DNA sequence of the pMN5 was deposited in GenBank™ under the accession number AF056207.

Plasmid Construction

The *lmrB* gene was amplified from pMN5 by PCR with oligonucleotides LMRB1 (5′-TCTAGACCACCATGGGG*CATCACCATCACCATCA-C*GATGACGATGACAAAGCCGAAAGAGG), introducing the underlined *Nco*I restriction enzyme site upstream of the His_6 tag (italic), and LMRB2 (5′-ATATCTAGAG*TTA*ATTGATTCTGAAC), introducing the underlined *Xba*I restriction enzyme site downstream of the stop codon (italic) of *lmrB*. The purified 1764-bp PCR product was digested with *Nco*I and *Xba*I and ligated into the corresponding sites in pNZ8048, resulting in pNH*lmrB*. The *lsbB* gene was amplified with oligonucleotides LSBB-1 (5′-CCGGTCATGAAAACAATCCTACG) and LSBB-2 (5′-CTGGTCTAGATTAAGCTTTTCCACG) by using pMN5 as a template. An *Rca*I and *Xba*I (underlined) digested PCR product was ligated into the *Nco*I and *Xba*I sites of pNZ8048, resulting in pNZ*lsbB*. Oligonucleotides LSBA-1 (5′-CCGGTCATGATAATTGTAGGAATTATATTTTTG) and LSBA-2 (5′-CTGGTCTAGATTATGCATATCTTGGTAC) were used to amplify *lsbA* gene from plasmid pMN5. The PCR product was digested with *Rca*I and *Xba*I (underlined) and ligated into the *Nco*I and *Xba*I sites of pNZ8048, resulting in pNZ*lsbA*. To make an *lmrB-lsbB* operon, *lmrB* was amplified by PCR using oligonucleotides LMRB3 (5′-CCGGAGCTCTAAAAAGGAAGTGATAAATTTATG) and LMRB2. *Sac*I and *Xba*I (underlined) digested PCR product was ligated into the corresponding sites in pNZ*lsbB*, resulting in pNZ*lsbB-lmrB*. To construct an in-frame N-terminal fusion with the green fluorescent protein (GFP), *lmrB* was amplified by PCR using LMRB4 (5′-CCGGAATTCATATGAAATTTTTGAAAGACAAATC) and LMRB2 as oligonucleotides. The PCR product was digested with *Eco*RI and *Xba*I (underlined) and ligated into the corresponding restriction enzymes sites of pGFP-mut1, containing *gfp-mut1* downstream of the *nisA* promoter (P_{nisA}), resulting in pNZ*gfp-lmrB*. All plasmids were introduced in *L. lactis* NZ9000 to enable nisin induction. Nisin induction of P_{nisA} in the pNZ8048 derivatives was performed as described by de Ruyter *et al.* (24).

To investigate the transcription of *lsbB* and *lmrB* the DNA fragment containing the expression signals of the divergently transcribed *lsbB* and *lmrB* genes was amplified from pMN5 by PCR using oligonucleotides P1 (5′-CTCGTGATCATAGGTTTACTTCCTTTC) and P2 (5′-CAGGTGATCATAAATTTATCACTTCC) containing *Bcl*I restriction sites (underlined) that overlap the *lsbB* and *lmrB* initiation codons. *Bcl*I digested PCR product was ligated into the *Bam*HI site of pGKH10, resulting in pGKH1 and pGKH2. In plasmid pGKH1, the *lacZ* gene is under the control of the *LsbB* promoter, whereas the *lmrB* promoter directs the transcription of the α-galactosidase gene. The promoter fragment is present in the reverse orientation in pGKH2.

Construction of an lmrA Deletion Strain

The non-autonomously replicating vector pORI280 (25) was used to construct an *lmrA* replacement plasmid. The 1561-bp *Eco*RV fragment encoding the N-terminal portion of LmrA was deleted from pAPL2.[2] The resulting plasmid pAPL3 was digested with *Sca*I and *Bam*HI yielding a

[2] K. Venema, unpublished data.

3.234-kb fragment with the *lmrA* deletion and its flanking regions. This fragment was ligated into corresponding sites in pORI280. The resulting plasmid, pORILmr, was used to transform *L. lactis* LL108, which carries the *repA* gene on the chromosome, thereby allowing the pORI280 derivative to replicate. pORILmr isolated from this strain was introduced, together with pVE6007, into *L. lactis* NZ9000. As this strain does not contain the *repA* gene, selection for growth in the presence of erythromycin and increased temperature (37 °C) forces pORILmr to integrate into the chromosome by homologous recombination. A number of integrants were subsequently grown for about 30 generations under non-selective conditions allowing a second recombination event to occur, which results in either the deletion or the wild-type gene *lmrA*. The Δ*lmrA* mutation was confirmed by PCR, as well as Southern hybridization experiments.

Assay of β-Galactosidase Activity

The activity of β-galactosidase was measured during growth of *L. lactis* in a 96-well microtiter plate (Greiner Bio-One B.V., Alphen, The Netherlands) using the GENios microtiterplate reader and Magelan software (Tecan, Grödig, Austria). β-Galactosidase activity was measured by conversion of T657 substrate (trifluoromethylumbelliferyl-β-D-galactopyranoside; Molecular Probes) into T659 fluorescent product. Fluorescence was followed using excitation and emission wavelengths of 360 and 535 nm, respectively. Culture optical densities were measured at 595 nm. Specific β-galactosidase activity was calculated as arbitrary fluorescence units divided per time and optical absorbance ($AFU \times min^{-1} \times A_{595}{}^{-1}$).

Bacteriocin Activity Assays

Bacteriocin activity was detected using an agar-well diffusion assay (1). To this end, wells made in the lawn of soft agar with an indicator strain (10^6 cells/ml), which was poured onto agar plates, were filled with 50-μl aliquots of supernatant. To detect bacteriocin activity on SDS-polyacrylamide (PAA) gels, an overlay assay was used (26). The supernatant of a nisin-induced culture of a bacteriocin producer (2 ml) was concentrated 20-fold by phenol/ether extraction (27), after which 15 μl was loaded onto an SDS-20% (w/v) PAA gel. After electrophoresis the gel was treated twice for 30 min with a mixture of isopropanol (20%) and acetic acid (10%), washed with several changes of demineralized water, and overlaid with soft agar containing 10^6 cells/ml of an indicator strain, followed by overnight incubation at 30 °C.

Bacteriocin Purification and N-terminal Amino Acid Sequence Determination

LsbA and LsbB were purified from 50 ml of nisin-induced cultures of *L. lactis* NZ9000 carrying pNZ*lsbA* or pNZ*lsbB*, respectively. Cells were removed by centrifugation, after which the supernatant was concentrated 20-fold by phenol/ether extraction (27). The supernatant was dialyzed against several changes of demineralized water at 4 °C, using cellulose ester membranes with a molecular mass cut-off of 1 kDa (SpectraPor® CE; Spectrum Laboratories). Quantification of protein was done by the Bradford method, using bovine serum albumin as a standard. The purified sample was subjected to SDS 20% (w/v)-PAA gel electrophoresis (28) using the Rainbow pre-stained low range molecular weight protein marker (Amersham Biosciences) as a size reference. The protein band corresponding to active bacteriocin was excised from a Coomassie Brilliant Blue-stained SDS-PAA gel and destained for 1 h at room temperature in a solution of 45% methanol, 10% acetic acid. The purified protein was subjected to N-terminal amino acid sequencing (Eurosequence, Groningen, The Netherlands) by means of Edman degradation on an automated sequenator (model 477A; Applied Biosystems) using protocols, chemicals, and materials from Applied Biosystems (Foster City, CA).

RNA Analysis

Primer Extension Analysis—RNA was isolated from exponentially growing *L. lactis* cells as described by van Asseldonk *et al.* (29). Synthesis of cDNA was performed using SUPERSCRIPT transcriptase (Invitrogen). mRNA (3.5 μg) was reverse-transcribed using 25 ng of synthetic oligonucleotide REP1 (5′-AATTAAGATAGCCTAACTCC), which anneals at the 5′ of the *repA*, or LSBA (5′-GTACAAAATAATGCTATAGC), which anneals at the 5′ of *lsbA*, and dATP, dGTP, dTTP, and [α-P^{32}]dCTP (Amersham Biosciences). Reaction mixtures were incubated for 10 min at 42 °C, after which an excess in cold dCTP was added, and incubation was continued for another 10 min at 42 °C. The reaction products were separated by electrophoresis on a 6% polyacrylamide urea gel and analyzed by autoradiography.

RT-PCR—First strand cDNA synthesis with reverse transcriptase was carried out with the first strand cDNA synthesis kit for RT-PCR from Roche Applied Science. mRNA (2 μg) was reverse-transcribed with 50 ng of synthetic oligonucleotide LMRB (5′-CTATATTGATACCTTGAC). The cDNA thus obtained was subsequently amplified by PCR using REP2 (5′-GAAATTGGCAACAACG) in combination with REP3 (5′-CCCAATTCCAAATCGC) or LSBA-3 (5′-GTACAAAATAATGCTATAGC). The size of the obtained PCR products was checked on a 1% (w/v) agarose gel.

Northern Hybridization—RNA for Northern blot analysis was fractionated on a 1% formaldehyde-agarose gel (21). The RNA size marker (0.5–9 kb) was from Ambion (Austin, TX). Purified PCR products obtained with the oligonucleotides LMRA1 (5′-TGATTGGGATTGTGGCTGG) and LMRA2 (5′-CAAAACCAATTTGACTCCGCC), containing the 5′ end of *lmrA*, or LMRP1 (5′-CAATGATTTTTGGAAGTGGGC) and LMRP2 (5′-CTCATCAACTTTGACTGTGG), containing the 5′ end of *lmrP*, were used as probes. Labeling of probes and transcript detection were performed with the ECL detection system (Amersham Biosciences) according to the manufacturer's instructions.

RESULTS

Sequence Analysis of the Locus Responsible for Bacteriocin Production in L. lactis BGMN1–5—L. lactis sp. *lactis* BGMN1–5 has been shown previously (30) to produce two class II bacteriocins, Bac513 and Bac501. By means of plasmid curing, derivatives of *L. lactis* BGMN1–5 have been obtained that lack one or more of the five resident plasmids. Bac513 has been shown to be only produced when a 5.65-kb plasmid (pMN5) was present. The genetic information for the production of Bac501 is located on a 80-kb plasmid (30) and will not be discussed further. Subcloning of the two *Eco*RI fragments of pMN5 revealed that the genetic information for the production of and immunity toward Bac513 is located on the 3.28-kb *Eco*RI fragment of pMN5 (Fig. 1*A*). Three open reading frames (ORFs) could be discerned in the nucleotide sequence of this region (Fig. 1*C*). The first ORF could encode a peptide of 67 amino acid residues with a calculated molecular mass of 7.8 kDa. The ATG start codon is preceded by a potential ribosome binding site (GAGGA), but no obvious −35 and −10 consensus promoter regions were present. Two 12-bp inverted repeats separated by four nucleotides are present immediately downstream of this ORF, which could form a stable stem-loop structure with an estimated ΔG° of −15.2 kcal/mol (−63.6 kJ/mol) and could serve as a bidirectional *rho*-independent transcription terminator (31). The divergently oriented ORF could specify a 30-amino acid peptide with a calculated molecular mass of 3.4 kDa. The gene is preceded by a strong potential ribosome binding site (AGAAAGGAAG). The third ORF could encode a protein of 567 amino acid residues with a calculated molecular mass of 63.8 kDa. This ORF is preceded by a potential ribosome binding site (AAAGGAAG) and is located immediately downstream of the oppositely oriented second ORF. A 96-bp intergenic region separates both genes.

The deduced amino acid sequences of the two small ORFs do not share mutual similarity nor do they show homology with any entry in the protein databases. The product of the large ORF shares 34% sequence identity with the multidrug transporter protein LmrA of *L. lactis* MG1363 (32) and, like LmrA, is a half-size version of the human multidrug resistance P-glycoprotein (33). Based on this homology the gene was labeled *lmrB*. LmrB is homologous to many pro- and eukaryotic ABC transporters and to the hop resistance protein HorA of the beer-spoilage bacterium *Lactobacillus brevis* (34). Strain *L. lactis* BGMN1–5 contains neither *lmrA* nor *lmrB* on its chromosome.

Bac513 Activity Is a Mixture of Two Bacteriocins—The *L. lactis* BGMN1–5 plasmid pMN5 has been shown to specify bacteriocin activity, which has been named Bac513 (30). To investigate which of the two small ORFs carried by the 3.28-kb *Eco*RI fragment of pMN5 encodes Bac513 activity, the ORFs were

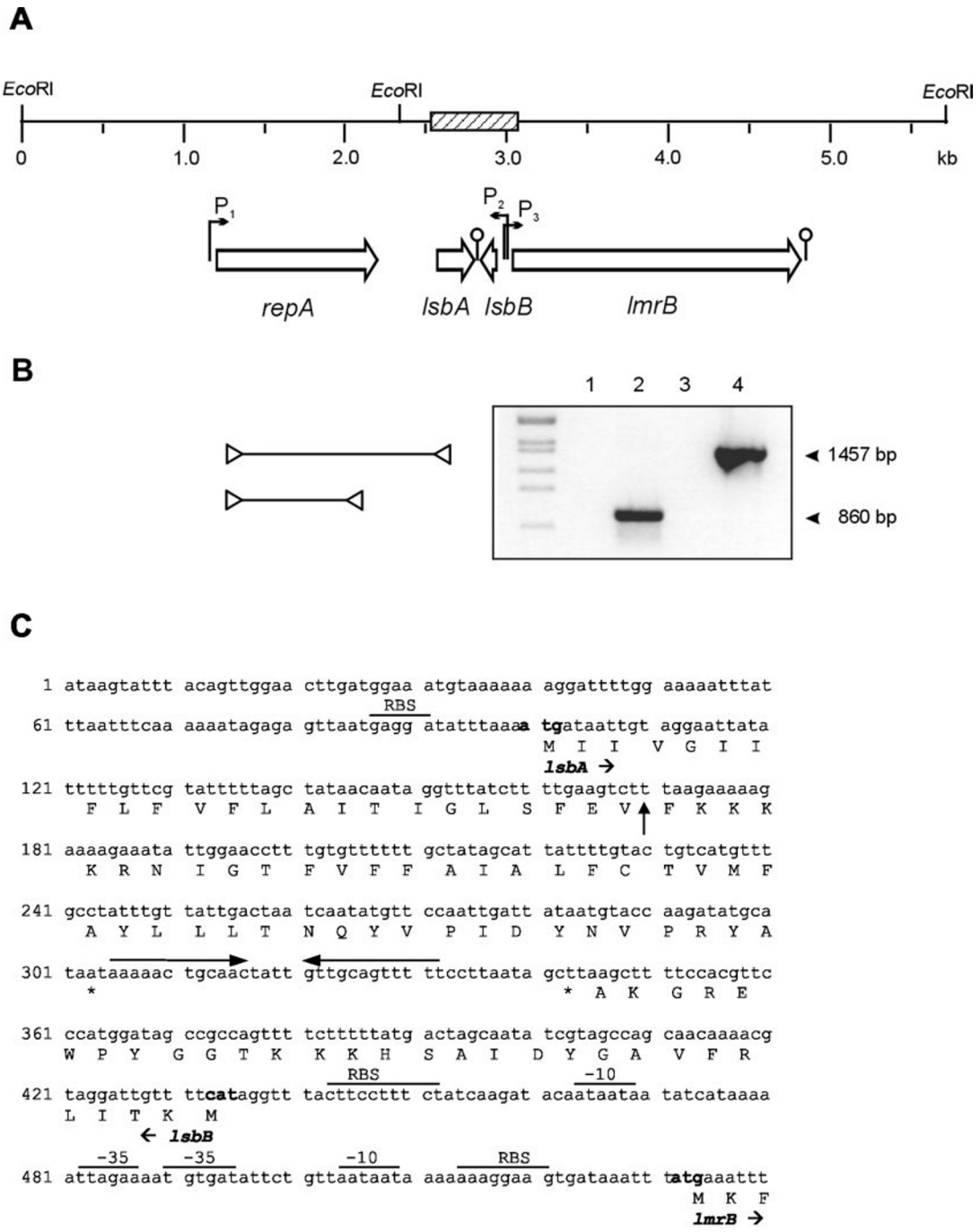

FIG. 1. *A*, linear map of pMN5. Positions and orientation of genes are indicated by the *block arrows*, promoters are indicated by *vertical arrows*, and putative terminator structures are indicated by *ball-and-stick symbols*. The DNA fragment of which the sequence is presented in *C* is *boxed*. *B*, analysis of transcription of *lsbA*. The products obtained by PCR amplification of RT-mRNA using the oligonucleotides located within *repA* (*lane 2*) and at the 5′-end of *repA* and within *lsbA* (*lane 4*). PCR amplification of the control samples without RT reaction is shown in *lanes 1* and *3*. The sizes of the products are indicated in the *right margin*. In the *left panel* the location of the PCR products on the map in *panel A* is shown. *C*, nucleotide sequence of a 530-bp DNA fragment from pMN5 containing the structural genes of LsbA and LsbB and the start of *lmrB*. The deduced amino acid sequences are shown *below* the DNA sequence. Putative ribosomal binding sites (*RBS*) and −35 and −10 promoter sequences are *overlined*. Start codons are indicated in *bold*. The *horizontal arrows* indicate a potential *rho*-independent transcription terminator sequence. A *vertical arrow* indicates the cleavage site in pre-LsbA.

cloned separately in a lactococcal expression vector, downstream of the nisin-inducible promoter P_{nisA} (35). The two plasmids pNZ*lsbA* and pNZ*lsbB*, carrying the 67- and the 30-codon ORFs, respectively, were introduced in the naturally resistant *L. lactis* strain NZ9000. This *L. lactis* MG1363 derivative contains the *nisRK* genes needed for inducible expression of both ORFs from P_{nisA} (35). Moreover, this strain specifies LmrA, which, as we will show below, is needed for LsbA and LsbB secretion. The supernatants of both strains were shown to inhibit the growth of *L. lactis* sp. *lactis* IL1403, a strain that does not produce LmrA or LmrP, indicating that the gene products of both ORFs are secreted and have antimicrobial activity. The two peptides do not act synergistically, because the titer of a mixture of both was the same as the sum of the individual titers. Hereafter, the gene products are designated LsbA and LsbB for the 67- and the 30-residue peptides, respectively.

Neither LsbA nor LsbB inhibits the growth of strains of the

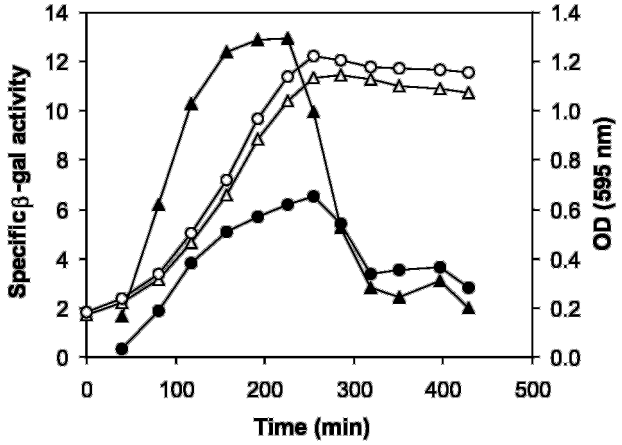

FIG. 2. **Analysis of transcription of *lsbB:lacZ* (in pGKH1) and *lmrB:lacZ* (in pGKH2) transcriptional fusions.** Specific β-galactosidase activity of *L. lactis* MG1363 carrying pGKH1 (▲) or pGKH2 (●) during growth in GM17 containing the fluorescent β-galactosidase substrate trifluoromethylumbelliferyl-β-D-galactopyranoside is shown. Optical density of MG1363 (pGKH1) (△) and MG1363 (pGKH2) (○) was measured at 595 nm. The excitation and emission wavelengths of trifluoromethylumbelliferyl-β-D-galactopyranoside were 355 and 457 nm, respectively. Specific β-galactosidase activity was calculated as arbitrary fluorescence units divided per time and optical absorbance (AFU × min^{-1} × $A_{595}{}^{-1}$).

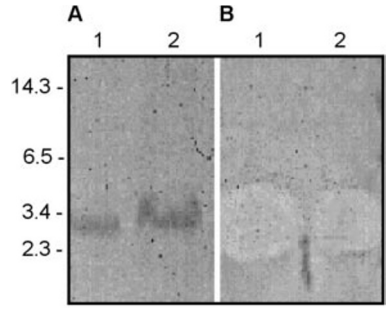

FIG. 3. **Detection of purified LsbA and LsbB and their activities in an SDS 20% polyacrylamide gel.** *A*, Coomassie Brilliant Blue staining. *B*, bacteriocin activity as visualized by an activity overlay assay using *L. lactis* IL1403 as the indicator strain. 15 μl of a 10-fold concentrated supernatant of a nisin-induced culture of *L. lactis* NZ9000 (pNZ*lsbA*) (*lanes 1*) or *L. lactis* NZ9000 (pNZ*lsbB*) (*lanes 2*) were applied. Molecular masses (in kDa) of reference proteins are shown on the *left*.

Gram-positive species *Lactobacillus*, *Leuconostoc*, *Bacillus*, *Enterococcus*, and *Staphylococcus* or that of several Gram-negative bacteria tested (*Escherichia coli* C600, *Salmonella thyphimurium* LT2, *Pseudomonas* sp.). In fact, only closely related *L. lactis* strains are inhibited, showing that both bacteriocins exhibit a very narrow antibacterial spectrum.

Analysis of Transcription of the Bacteriocin Encoding Genes—No consensus promoter sequence is present immediately upstream of *lsbA*. In accordance with this observation, no transcription initiation start site could be determined by primer extension mapping. A promoter sequence (P_1) is present upstream of the preceding gene, that of the plasmid replication protein RepA (Fig. 1*A*). Primer extension analysis confirmed the position of this postulated promoter, 80 nt upstream of the *repA* start codon (data not shown). RT-PCR on total RNA isolated from *L. lactis* (pMN5) with a primer located within *lsbA* and one immediately upstream of *repA* gave a product of the expected size (1457 bp; see Fig. 1*B*). An equal amount of total RNA sample was amplified with the same primers without a prior RT-PCR to confirm that no contaminating DNA material was present. These data indicate that *lsbA* and *repA* are located in one operon and form a transcriptional unit.

The region between *lsbB* and *lmrB* contains two putative promoters, P_2 and P_3 (Fig. 1*A*). Gene *lsbB* is preceded by possible −35 (ATCACA) and −10 (TATTAT) sequences that are 17 nucleotides apart. The −35 and −10 promoter sequences upstream of *lmrB* (TTAGAA and AATAAT, respectively, with a spacing of 16 nucleotides), could constitute promoter P_3 (Fig. 1*C*). A DNA fragment carrying the expression signals of the divergently transcribed *lsbB* and *lmrB* genes was inserted, in two orientations, between the two promoterless reporter genes in pGKH10 in such a way that translational fusions were created. The reporter genes in pGKH10 encode *E. coli* β-galactosidase and *Cyamopsis tetragonoloba* α-galactosidase (36). The *lmrB* and *lsbB* genes were mainly expressed during the exponential growth phase (Fig. 2). By comparing β-galactosidase activity levels it was shown that the expression signals of *lsbB* are about 2.5-fold stronger than those of *lmrB*. Taken together these results show that *lsbB* and *lmrB* are both actively transcribed.

LsbA Is Processed by HtrA—LsbA and LsbB were purified from the supernatant of *L. lactis* NZ9000 containing either pNZ*lsbA* or pNZ*lsbB*, respectively, and the purified peptides were subjected to N-terminal amino acid sequencing. The molecular mass of the secreted form of LsbA, estimated after SDS-20% PAGE, was ~3 kDa (Fig. 3), indicating that LsbA is synthesized as a pre-protein. The first five amino acids obtained by Edman degradation were Phe-Lys-Lys-Lys-Lys, indicating that the bacteriocin is processed between the two putative membrane spanning domains, leaving a highly positive charge on the N terminus of the mature protein (Fig. 1*C*). No consensus signal peptidase I or II cleavage site is present in the deduced amino acid sequence of LsbA. Instead, the region of cleavage shows similarity with the cleavage site of the housekeeping protease HtrA (37). The production of LsbA was examined in a lactococcal strain in which the chromosomally located *htrA* gene was inactivated by single cross-over homologous recombination. As can be seen in Fig. 4*A*, no active LsbA was present in the supernatant. Moreover, in a total cell extract of *L. lactis* NZ9000Δ*acmA*Δ*htrA*, LsbA was detected as an inactive pre-bacteriocin by SDS-20% PAGE (Fig. 4*B*). The supernatant of this strain contained neither pre-LsbA nor the mature bacteriocin. By contrast, processed LsbA was detected in the supernatant of a wild-type strain (Fig. 4*B*). These data clearly show the involvement of HtrA in LsbA processing during or immediately after translocation of pre-LsbA across the cytoplasmic membrane.

The amino acid sequence Met-Lys-Thr-Ile-Leu-Arg-Phe-Val-Ala-Gly was obtained after Edman degradation of purified LsbB. Apparently, the product of *lsbB* is secreted without N-terminal processing (Fig. 1*C*).

LmrB Renders Cells Resistant to LsbA and LsbB—Hydropathy analysis of LmrB suggests the presence in the N terminus of six putative α-helical transmembrane segments and a C-terminal, highly conserved hydrophilic nucleotide binding domain. This latter domain contains features diagnostic for ABC-type ATPases, such as the ABC signature sequence and the Walker A and B motifs (38). LmrB was shown to be an active MDR transporter protein involved in the extrusion from the cytoplasmic membrane of the typical MDR protein substrates ethidium bromide and the amphiphilic compound Hoechst 33342.[3] To visualize the protein *in situ*, LmrB was N-terminally fused to GFP and overexpressed from the nisin inducible P_{nisA} promoter. After nisin induction the GFP:LmrB fusion protein was shown to be distributed all along the cytoplasmic

[3] O. Gajic, G. Buist, A. Margolles, W. N. Konings, L. Topisirovic, J. Kok, and O. P. Kuipers, unpublished data.

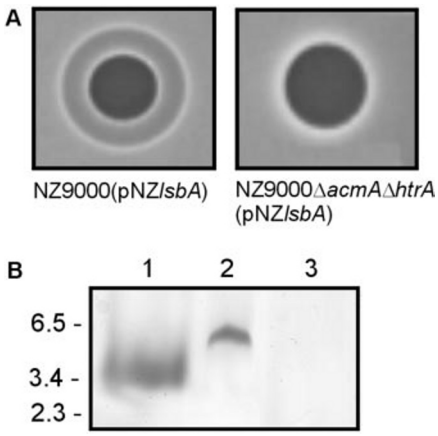

FIG. 4. **LsbA production in *L. lactis* strains NZ9000 and NZ9000Δ*acmA*Δ*htrA*.** *A*, detection of LsbA activity using an agar-well diffusion assay. The indicator strain used was *L. lactis* IL1403. *B*, detection of LsbA in an SDS 20% polyacrylamide gel stained with Coomassie Brilliant Blue. *Lane 1*, 10-fold concentrated supernatant of a nisin-induced culture of *L. lactis* NZ9000 (pNZ*lsbA*); *lanes 2* and *3*, total cell extract and 10-fold-concentrated supernatant of *L. lactis* NZ9000Δ*acmA*Δ*htrA* (pNZ*lsbA*), respectively. Molecular masses (in kDa) of reference proteins are shown on the *left*.

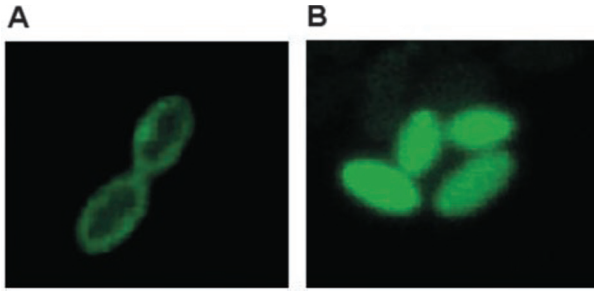

FIG. 5. **Fluorescence microscopy analysis of *L. lactis* NZ9000 cells expressing GFP:LmrB fusion protein (*A*) or GFP (*B*).** Fluorescence was visualized using a Zeiss Axiophot (Zeiss) microscope and an Axion Vision camera (Axion Technologies, Houston, TX).

membrane by fluorescence microscopy (Fig. 5*A*). In contrast, when expressed alone, GFP was present in the cytoplasm (Fig. 5*B*).

To examine the possible involvement of LmrB in immunity against LsbA and LsbB, a histidine (His_6)-tagged variant of the protein was overexpressed in *L. lactis* by using the nisin controlled gene expression system (35). His_6-LmrB was of the expected molecular size (65.4 kDa) in Western blotting using monoclonal antibodies directed against the histidine tag (data not shown). *L. lactis* strain IL1403 is very sensitive for LsbA and LsbB. A derivative of this strain, *L. lactis* JIM7049, containing the *nisRK* genes needed for nisin induction of P_{nisA} was used as the host for His_6-LmrB expression. *L. lactis* JIM7049 (pNH*lmrB*) became resistant to both LsbA and LsbB when His_6-LmrB expression was induced with nisin. Moreover, expression in *L. lactis* JIM7049 of two other lactococcal multidrug transporter proteins, LmrA and LmrP (32, 39), also resulted in resistance against both bacteriocins to the same level as *L. lactis* MG1363 (data not shown). These findings demonstrate that resistance to LsbA and LsbB can be accomplished via all three MDR proteins.

LmrA and LmrP Are Not Expressed in L. lactis IL1403—In contrast to *L. lactis* IL1403, the growth of *L. lactis* sp. *cremoris* NCDO712 or its plasmid-free derivative MG1363 is not inhibited by LsbA and LsbB. Strain MG1363 produces both MDR proteins, LmrA and LmrP (32, 39). The *lmrA* gene is also present in the chromosome of *L. lactis* IL1403 (40), but it is preceded by a nucleotide sequence that is different from that of

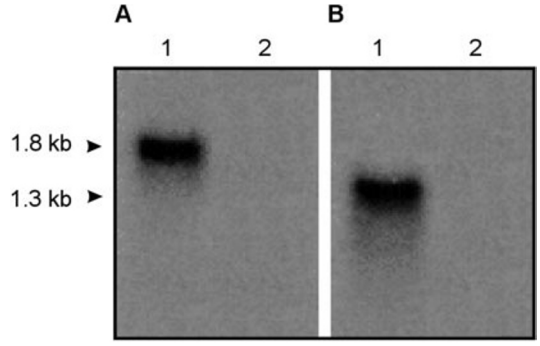

FIG. 6. **Northern analysis of *lmrA* and *lmrP* transcription.** Total RNA (5 μg) isolated from *L. lactis* MG1363 (*lane 1*) or *L. lactis* IL1403 (*lane 2*) was hybridized with an *lmrA*-specific probe (*A*) or an *lmrP*-specific probe (*B*).

the region preceding *lmrA* in MG1363. The gene in strain IL1403 is preceded by a potential ribosome binding site (AAAGGAAG), but no obvious −35 and −10 consensus promoter regions could be discerned. Possibly, *lmrA* is in an operon with the upstream-located *yhbF*, encoding a hypothetical protein. However, two 14-bp inverted repeats separated by 15 nucleotides are present immediately upstream of the RBS and could form a stable stem-loop structure with an estimated ΔG° of −14.9 kcal/mol (−62.3 kJ/mol). This structure could serve as a *rho*-independent transcription terminator blocking transcription of *lmrA*. Also, the −35 and −10 consensus promoter regions of the *lmrP* genes of *L. lactis* strains MG1363 and IL1403 are different. In MG1363, *lmrP* is preceded by possible −35 (TTGACT) and −10 (TATAAA) sequences with a spacing of 16 nucleotides (39). The putative −35 and −10 promoter sequences upstream of *lmrP* in strain IL1403 (TTGCAG and TTTAAA, respectively) most likely do not constitute an active promoter. Immediately downstream of both *lmrP*s, the oppositely oriented *sipX* is located.

Northern analysis of the *lmr* genes in both strains after hybridization with an *lmrA*-specific probe revealed a transcript of 1.8 kb only in RNA isolated from MG1363. A transcript of 1.3 kb was observed with the same RNA when an *lmrP*-specific probe was used. No transcripts were detected in RNA isolated from strain IL1403 with either of the two probes (Fig. 6). These findings demonstrate that, although *lmrA* and *lmrP* are present on the chromosome of IL1403, they are not transcribed.

LmrB Is Involved in Bacteriocin Secretion—The secretion of both bacteriocins by *L. lactis* was not affected by the addition of 2 mM azide, a known inhibitor of the Sec translocation pathway in *B. subtilis* (41), to the growth medium (data not shown). Next, we examined whether LmrB mediates the secretion of both bacteriocins. To this end, His_6-LmrB was overexpressed in either *lmrA*- or *lmrP*-deficient isogenic *L. lactis* MG1363 derivatives. The experiments could not be done in an *lmrA*, *lmrP* double mutant as such a strain is, apparently, not viable. Lack of active LmrP did not have any effect on the secretion of LsbB (Fig. 7). In contrast, no secretion of LsbB was observed in *L. lactis* NZ9000Δ*lmrA*. Upon disruption of the cells, active LsbB was shown to be present intracellularly by an SDS-PAA gel overlay assay (data not shown). The function of LmrA could be complemented either by LmrB or by LmrA, as was shown by the introduction of pNZ*lsbB-lmrB* or pNZ*lsbB-lmrA* in NZ9000Δ*lmrA*; nisin-induced co-expression of LsbB with LmrA or LmrB from these plasmids resulted in the secretion of active LsbB (Fig. 7). The same observations were made with respect to the secretion of LsbA (data not shown). Taken together these findings show that the multidrug transporter proteins LmrA and LmrB are directly involved in the secretion of LsbA and LsbB, whereas LmrP is not.

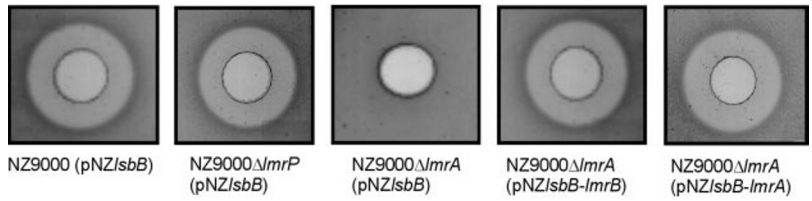

FIG. 7. **Detection of LsbB activity in *L. lactis* strains NZ9000, NZ9000-Δ*lmrP*, and NZ9000Δ*lmrA*, all carrying pNZ*lsbB*, and of *L. lactis* NZ9000Δ*lmrA*, carrying pNZ*lsbB-lmrB* or pNZ*lsbB-lmrA*.** The indicator strain used in this agar-well diffusion assay was *L. lactis* IL1403.

DISCUSSION

The data presented here show that *L. lactis* BGMN1–5 produces three bacteriocins. The antimicrobial activity that had previously been labeled Bac513 (30) is, in fact, the result of the concerted action of two distinct bacteriocins, LsbA and LsbB. Although LsbB is smaller than LsbA (30 instead of 44 amino acid residues) and possesses less pronounced cationic and hydrophobic properties, both bacteriocins share the characteristic physico-chemical properties of LAB bacteriocins (size, molecular weight, isoelectric point, and hydrophobicity) (2). They are not post-translationally modified, because problems typically encountered when determining the amino acid sequence of proteins containing residues such as didehydroalanine, didehydrobutyrine, lanthionine, and β-methyllanthionine (6, 7) were not observed. Both peptides contain a stretch of four to five positively charged amino acid residues. These are located at the N terminus of LsbA and in the middle of LsbB. LsbA and LsbB are apparently one-peptide bacteriocins, because each is active on its own, and no additional bacteriocin encoding genes could be discerned on pMN5. Based on the features described above LsbA and LsbB can be regarded as members of LAB bacteriocin group IIc.

Most bacteriocins are synthesized as precursor peptides containing an N-terminal leader peptide with two conserved glycine residues at positions −1 and −2 relative to the cleavage site (14). Translocation across the cytoplasmic membrane and the subsequent removal of the leader peptide during maturation is carried out by a dedicated ABC transporter (14, 42, 43). Only a few LAB bacteriocins described to date contain a typical signal peptidase cleavage site (44) and are secreted by the general secretory pathway (9, 15, 16, 45). Comparing the amino acid sequence of purified LsbA with the deduced amino acid sequence revealed that the bacteriocin is first produced as a precursor peptide. Instead of a leader peptide of the double glycine-type (14) or a consensus signal peptidase cleavage site (44) a possible HtrA cleavage site could be discerned in pre-LsbA (37). HtrA is a surface housekeeping protease in *L. lactis* that was shown to have a dual function; it acts as a chaperone and as a protease. It is responsible for both the degradation and maturation of exported proteins (46). The activation of LsbA by HtrA during or immediately after translocation of the bacteriocin across the cellular membrane is a mechanism of processing that is distinctly different from that of all other known bacteriocins (14, 44).

For most non-lantibiotics, the gene encoding bacteriocin immunity is usually located immediately downstream of and in the same operon as the bacteriocin structural gene(s) (2, 12). LsbA and LsbB are exceptions to this rule. The gene conferring immunity, *lmrB*, is located immediately downstream of and in the opposite orientation to *lsbB*. LmrB is a member of the ABC protein superfamily. It is homologous to LmrA of *L. lactis* MG1363 (32), to prokaryotic ABC transporters of *B. subtilis*, *Staphylococcus aureus*, *E. coli*, *Campylobacter jejuni*, and *Haemophilus influenzae* and to the hop resistance protein HorA of the beer-spoilage bacterium *Lactobacillus brevis* (34). LmrB is also homologous to eukaryotic ABC transporters, *e.g.* human multidrug resistance P-glycoprotein (33). LmrB and the other two MDR proteins of *L. lactis*, LmrA and LmrP, were shown here to confer immunity to LsbA and LsbB. In contrast to LmrB and LmrA, LmrP is a proton motive force-driven transporter (39). These three MDR proteins do not render cells resistant to other lactococcal bacteriocins *e.g.* lactococcins A, B, M/N, or nisin; they are rather specific for LsbA and LsbB.

It is not clear what the exact mechanism(s) are by which immunity proteins function. Those predicted to have transmembrane helices, *e.g.* LciA, are envisaged to interact with and block the receptor for the bacteriocin. By binding to the receptor, LciA prevents lactococcin A from inserting into the membrane, although binding of lactococcin A to the receptor still occurs (19). Bacterial strains that produce multiple bacteriocins also produce different bacteriocin-specific immunity proteins (11, 12). Here, we report that immunity against two distinct bacteriocins relies on the activity of only one protein; in the case of LsbA and LsbB, the protein that is responsible for immunity is a multidrug transporter protein. The common feature of most MDR proteins is their ability to extrude a wide range of hydrophobic and amphiphilic compounds from the cytoplasmic membrane (33). As both bacteriocins are hydrophobic molecules, it seems likely that all three lactococcal multidrug transporters mediate bacteriocin resistance by removing bacteriocin that enters the cytoplasmic membrane from the outside. Besides conferring immunity, we show that LmrB and LmrA also function as exit pumps for the two bacteriocins, extruding the molecules from their site of production, the cytoplasm, to the extracellular medium. Although LsbA is cleaved during this process by HtrA, precursor cleavage *per se* is not necessary for transport via LmrA or LmrB. LmrP clearly is not involved in bacteriocin secretion and seems to be only capable of removing the bacteriocins from (the outer leaflet of) the cytoplasmic membrane, resulting in bacteriocin resistance. Bacteriocin secretion via MDR proteins is a route of secretion that is different from that of all other known bacteriocins and also suggests a novel function of MDR proteins, namely the secretion (extrusion) of natural biologically active peptides.

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