Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance proteins

Gajic, O; Buist, G; Kojic, M; Topisirovic, L; Kuipers, OP; Kok, J

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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.

**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 1/2 M17 broth (containing 0.95% (w/v) glucose) whereas 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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Olivera Gajic‡, Girbe Buist‡, Milan Kojić‡, Ljubisa Topisirovic§, Oscar P. Kuipers‡, and Jan Kok‡¶

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

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Table I

Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant phenotype or genotype</th>
<th>Source or reference</th>
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<tr>
<td><strong>L. lactis sp. cremoris</strong></td>
<td></td>
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<tr>
<td>MG1363</td>
<td>Plasmid-free derivative of NCO7012; LmrA’ LmrP’</td>
<td>Ref. 47</td>
</tr>
<tr>
<td>MG1363/lmrP</td>
<td>MG1363 derivative with deletion in lmrP</td>
<td>Ref. 39</td>
</tr>
<tr>
<td>NZ9000</td>
<td>MG1363 pepN::nisRK</td>
<td>Ref. 35</td>
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<td>NZ9700</td>
<td>Nisin producing transconjugant of MG1363 containing the nisin-sucrose transposon</td>
<td>Ref. 48</td>
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<td>This work</td>
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<tr>
<td>NZ9000/aczA SchrA</td>
<td>MG1363 pepN::nisRK containing deletions in acmA and htrA</td>
<td>Kees J. Leenhouts Ref. 25</td>
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<td>LL108</td>
<td>MG1363 derivative carrying the pWVO1 repA gene downstream of the promoter P_{ac} in its chromosome</td>
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<td><strong>L. lactis sp. lactis</strong></td>
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<tr>
<td>BGMN1–5</td>
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<td>BGMN1–596</td>
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<td>Ref. 30</td>
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<tr>
<td>IL1403</td>
<td>Plasmid-free strain; LmrA’, LmrP’</td>
<td>Ref. 49</td>
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<td>JIM7049</td>
<td>IL1403 his6::nisRK</td>
<td>Ref. 50</td>
</tr>
<tr>
<td>pNZ8048</td>
<td>Cm’ inducible expression vector carrying P_{nisA}</td>
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<td>pNHImrA</td>
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<td>pHLp5</td>
<td>his6-lmrP of L. lactis MG1363 behind P_{nisA}</td>
<td>Ref. 52</td>
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<td>pORI280</td>
<td>Em’, ori’ of JWV01, replicates only in strains carrying repA in trans</td>
<td>Ref. 25</td>
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<td>pMN5</td>
<td>Plasmid carrying lmrB, lsbA, and lsbB</td>
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<td>pNZlbkBa</td>
<td>Cm’, pNZ8048 carrying lsbA</td>
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<td>pNHlmrB</td>
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<td>pVE6007</td>
<td>Cm’, Ts replication derivative of pWVO1</td>
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<td>Koen Venema</td>
</tr>
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<td>pAPL3</td>
<td>Ap’, pAPL2 with 1561-bp EcoRV deletion</td>
<td>This work</td>
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<td>pORI280 carrying 3-234-kb ScaI/BamHI fragment from pAPL3, containing nisA</td>
<td>This work</td>
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<td>pGKH10</td>
<td>deletion and its flanking regions</td>
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<td>pGKH1</td>
<td>Em’, Cm’, contains promoterless genes for a-Gal and b-Gal (lacZ)</td>
<td>Ref. 36</td>
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<tr>
<td>pGKH2</td>
<td>Em’, Cm’, contains genes for a-Gal and b-Gal controlled by P_{nisA} and P_{nisB}, respectively</td>
<td>This work</td>
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<td>5-Bromo-4-choro-3-indolyl-b-D-galactopyranoside (Sigma)</td>
<td>was used at a concentration of 1 mM.</td>
<td></td>
</tr>
</tbody>
</table>

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 dyeoxy chain termination method (22) was used with [a-35S]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'-CATGACCACCCATGGGCTCATCACCATCAGTGAGCTGACGAACAGCGGAAAAAGG) and LMRB2 (5'-ATACTAGAGTTGCTGACCA), introducing the underlined NcoI restriction enzyme site upstream of the His6 tag (italic), and LMRB2 (5'-ATACTAGAGTTGCTGACCA), introducing the underline XbaI restriction enzyme site downstream of the stop codon (italic) and lmrB. The purified 1764-bp PCR product was digested with NcoI and XbaI and ligated into the corresponding sites in pNZ8048, resulting in pNHImrB. The lsbB gene was amplified with oligonucleotides LSBB1 (5'-CCCGGTCTAGAACACATCTCCTG) and LSB2 (5'-CTGCAGTCTAGGAGCTTTCCAGC) by using pMN5 as a template. An XcoI and XbaI (underlined) digested PCR product was ligated into the NcoI and XbaI sites of pNZ8048, resulting in pNZlbkB. Plasmid 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'-CCCGGTCTAGCTAAAGAGAAGAATTATGTAG) and LMRB2 (5'-CCGGATCTCATGAAATTTTGAGACAAATC) and LMRB2 as oligonucleotides. The PCR product was digested with EcoRI and XhoI (underlined) and ligated into the corresponding sites in pNZ8048 resulting in pNZlgfp/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).

Construction of an lmrA Deletion Strain

The non-autonomously replicating vector pORI280 (25) was used to construct an lmrA replacement plasmid. The 1561-bp EcoRV fragment encoding the N-terminal portion of lmrA was deleted from pAPL2. The resulting plasmid pAPL3 was digested with ScaI and BamHI yielding a

* 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* NZ2000. 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 lmrA mutation was confirmed by PCR, as well as by 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 microtiter plate reader and Magellan software (Tecan, Grodig, Austria). β-Galactosidase activity was measured by conversion of *p*-nitrophenyl-β-D-galactopyranoside (Molecular Probes) into *p*-nitrophenol at 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 × min⁻¹ × A₅₉₅⁻¹).

**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 (10⁶ 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/ethanol 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 10⁵ 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* NZ2000 carrying pNZlsbA or pNZlsbB, respectively. Cells were removed by centrifugation, after which the supernatant was concentrated 20-fold by phenol/ethanol 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 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 sequence determination (Eurosequence, Groningen, The Netherlands) by means of Edman degradation on an automated sequencer (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 with 25 ng of synthetic oligonucleotide REP1 (5′-AATTAAGATAGCTGCATTCC), which anneals at the 5′ of the repA, or LSBA (5′-GTCAAAATATGGTATAC), which anneals at the 5′ of lsbA, and dATP, dGTP, dTTP, and [α-²⁵⁴P]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′-CTATAATGGTACCTT-GAC). The cDNA thus obtained was subsequently amplified by PCR using REP2 (5′-GAAATTGGCAACAAAGC) in combination with REP3 (5′-CCAAATTCAATGATCG) or LSRB-5 (5′-GTACAATGCTATAGC). 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 LMR1A (5′-GTAGGGAATTGTCG) and LMR2A (5′-GAAATTGGCAACAAAGC) containing the 5′ end of lmrA, or LMR1P (5′-CATGATTTTTGGAAGTG) and LMR2P (5′-CTCAATATTTGGCTG), 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 EcoRI fragments of pMN5 revealed that the genetic information for the production of and immunity toward Bac513 is located on the 3.28-kb EcoRI fragment of pMN5 (Fig. 1A). Three open reading frames (ORFs) could be discerned in the nucleotide sequence of this region (Fig. 1C). 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 (GGAGA), 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 (GGAGA). 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 (AAAGAGAAG) 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 named 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 EcoRI fragment of pMN5 encodes Bac513 activity, the ORFs were
cloned separately in a lactococcal expression vector, down-
stream of the nisin-inducible promoter P\textsubscript{nisA} (35). The two
plasmids pNZ\textsubscript{lsbA} and pNZ\textsubscript{lsbB}, carrying the 67- and the 30-
codon ORFs, respectively, were introduced in the naturally
resistant \textit{L. lactis} strain NZ9000. This \textit{L. lactis} MG1363 deriv-
ative contains the nisRK genes needed for inducible expression
of both ORFs from P\textsubscript{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 \textit{L. lactis} sp. \textit{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

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**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.
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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 × A₅₉₅⁻¹).

Gram-positive species Lactobacillus, Leuconostoc, Bacillus, Enterococcus, and Staphylococcus or that of several Gram-negative bacteria tested (Escherichia coli C600, Salmonella typhimurium 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 (P1) is present upstream of the preceding gene, that of the plasmid replication protein RepA (Fig. 1A). 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. 1B). 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, P₂ and P₃ (Fig. 1A). 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 (TTGAGA and AATAAT, respectively, with a spacing of 16 nucleotides), could constitute promoter P₃ (Fig. 1C). 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.

Fig. 3. Detection of purified LsbA and LsbB and their activities in an SDS 20% polyacrylamide gel. A, Comassie 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 (pNZlmbA (lanes 1)) or L. lactis NZ9000 (pNZlmbB (lanes 2)) were applied. Molecular masses (in kDa) of reference proteins are shown on the left.

LsbA Is Processed by HtrA—LsbA and LsbB were purified from the supernatant of L. lactis NZ9000 containing either pNZlmbA or pNZlmbB, 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. 1C). 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. 4A, no active LsbA was present in the supernatant. Moreover, in a total cell extract of L. lactis NZ9000ΔacmAΔhtrA, LsbA was detected as an inactive pre-bacteriocin by SDS-20% PAGE (Fig. 4B). 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. 4B). 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. 1C).

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 PnisA promoter. After nisin induction the GFP::LmrB fusion protein was shown to be distributed all along the cytoplasmic

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

To examine the possible involvement of LmrB in immunity against LsbA and LsbB, a histidine (His6)-tagged variant of the protein was overexpressed in L. lactis by using the nisin controlled gene expression system (35). His6-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, shown). L. lactis clonal antibodies directed against the histidine tag (data not shown). These findings demonstrate that resistance to LsbA and LsbB, whereas LmrP is not.

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, His6-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 NZ9000ΔlmrA. Upon disruption of the cells, active LsbB was shown to be present intracellularly by an SDS-PAGE 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 pNZsB-B-lmrB or pNZsB-lmrA in NZ9000ΔlmrA; 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. 4. LsbA production in L. lactis strains NZ9000 and NZ9000 ΔhsrA. 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 (pNZsB-A); lanes 2 and 3, total cell extract and 10-fold concentrated supernatant of L. lactis NZ9000 ΔhsrA (pNZsB-A), 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).

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).
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**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, dide-hydrobutyrine, lanthionine, and β-methylthionationine (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 1c.

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-LabA (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 LabA 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). LabA 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 *Hae-mophilus 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. LeiA, are envisaged to interact with and block the receptor for the bacteriocin. By binding to the receptor, LeiA 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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