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 restoration of 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 anthemianibials 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) characterized to date are small (less than 6 kDa), cationic, and amphipathic 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 Ia, the pediocin-like bacteriocins (8, 9), Group Ib, the two-peptide bacteriocins, which require the complementary action of two peptides for full antimicrobial activity (10, 11), and Group Ic, 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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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
TABLE I

Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant phenotype or genotype | Source or reference |
|-------------------|-------------------------------|---------------------|
| *L. lactis* sp. cremoris | Plasmid-free derivative of NCO7012; LmrA+ LmrP+ | Ref. 47 |
| MG1363 | MG1363 derivative with deletion in lmrP | Ref. 39 |
| MG1363/lmrP | MG1363 derivative with deletion in lmrP | Ref. 39 |
| NZ9000 | MG1363 pepN::nisRK | Ref. 35 |
| NZ9070 | Nisin producing transconjugant of MG1363 containing the nisin-sucrose transposon | Ref. 48 |
| NZ9000/lmrA | MG1363 pepN::nisRK with deletion in lmrA | This work |
| NZ9000/aminocillin Ahibited lmrA | MG1363 pepN::nisRK containing deletions in acmA and hraA | Kees J. Leenhouts |
| LL108 | MG1363 derivative carrying the pWV01 repA gene downstream of the promoter P25 in its chromosome | Ref. 25 |
| *L. lactis* sp. lactis | Plasmid-free derivative of BMGN1-5; Bac501+, Bac513+ | Ref. 30 |
| BGMNI-5 | Plasmid-free derivative of BMGN1-5; Bac501+, Bac513+ | Ref. 30 |
| IL1403 | Plasmid-free strain; LmrA+, LmrP+ | Ref. 49 |
| JIM7049 | JIM1403 his::nisRK | Ref. 50 |

pNZ8048 | Cm' inducible expression vector carrying PmnA | Ref. 35 |
| pNHimrA | his6-lmrA of *L. lactis* MG1363 behind Pmna | Ref. 51 |
| pHLp5 | his6-lmrP of *L. lactis* MG1363 behind Pmna | Ref. 52 |
| pORI280 | Em', ori of pJWV01, replicates only in strains carrying repA in trans | Ref. 25 |
| pMN5 | Plasmid carrying lmrB, lsbA, and lsbB | Ref. 30 |
| pNZlsbA | Cm', pNZ8048 carrying lsbA | This work |
| pNZlsbB | Cm', pNZ8048 carrying lsbB | This work |
| pPNHimrB | Cm', pNZ8048 containing his6-lmrB | This work |
| pNZlsbB-lmrB | Cm', pNZ8048 derivative containing lmrB in an operon structure with lsbB | This work |
| pNZlsbB-lmrA | Cm', pNZ8048 derivative containing lmrA in an operon structure with lsbB | This work |
| pNZgfp-mutl | Cm', pGFP-mutl derivative containing lmrB fused C-terminally to gfp | This work |
| pGFP-mutl | Cm', pGFP-mutl containing gfp-mutl | Nathalie Campo |
| pNZ9530 | Em', plL252 derivative carrying nisRK | Ref. 53 |
| pVE6007 | Cm', Ts replication derivative of pWV01 | Ref. 54 |
| pAPL2 | Ap', pUC19 carrying a 6063-bp lacoccol chromosomal DNA fragment with lmrA gene | Koen Venema |
| pAPL3 | Ap', pAPL3 with 1561-bp EcoRV deletion | This work |
| pORI1mr | pOR1280 carrying 3-234-bp ScaI BamHI fragment from pAPL3, containing lmrA | This work |
| pHK10 | Cm', Cm', contains promoterless genes for a-Gal and b-Gal (lacZ) deletion and its flanking regions | Ref. 36 |
| pHK11 | Cm', Cm', contains genes for a-Gal and b-Gal controlled by PmnA and Pshb, respectively | This work |
| pHK22 | Cm', Cm', contains genes for a-Gal and b-Gal controlled by Pshb and PmnA, 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-32P]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 AF065207.

**Plasmid Construction**

The *lmrB* gene was amplified from pMN5 by PCR with oligonucleotides LMRB1 (5'-TCTAGACCACTGGGGCACAATCACACAGTACGAGTAGTACACAAGCGGAAGAGGAGG, introducing the underlined NcoI restriction enzyme site upstream of the His6 tag (italic), and LMRB2 (5'-ATATCTAGATTTGAATCTTCGAGA, introducing the underlined XbaI restriction enzyme site downstream of the stop codon (italic) for lmrB). The purified 1764-bp PCR product was ligated into the NcoI and XbaI sites of pNZ8048, resulting in pNHimrB. The *lsbB* gene was amplified with oligonucleotides LSBB-1 (5'-CCGGCTGTACTAAACAATCTTCGAGA) and LSBB-2 (5'-CTGGTCTAGATTTAGCTTTCCACCAGC) by using pMN5 as a template. An XbaI and XbaI (underlined) digested PCR product was ligated into the NcoI and XbaI sites of pNZ8048, resulting in pNZlsbB. Oligonucleotides LSHA-1 (5'-CCGGCTGTATTTAATGAGAATTTTTTTTGCAG) and LSB2-1 (5'-CTGGTCTAGATTTAGCTTTCCACCAGC) were used to amplify *lsbA* gene from plasmid pMN5. The PCR product was digested with *SalI* and XbaI (underlined) and ligated into the NcoI and *XbaI* sites of pNZ8048, resulting in pNZlsbA. To make an *lmrB-32P* operon, *lmrB* was amplified by PCR using oligonucleotides LMRB3 (5'-CCGGAGCTGTTTAAAGGGAAGAATGTTATTATGAGATGATGGATAGAATC) and LMRB2. *lacZ* was ligated into the corresponding site in pNZlsbB, resulting in pNZlsbB-lmrB. To construct an in-frame N-terminal fusion with the green fluorescent protein (GFP), *lmrB* was amplified by PCR using LMRB4 (5'-CCGGAGCTGTTTAAAGGGAAGAATGTTATTATGAGATGATGGATAGAATC) and LMRB2 as oligonucleotides. The PCR product was digested with EcoRI and XbaI (underlined) and ligated into the corresponding restriction enzyme sites of gfp-mut1, resulting in pGFP-mut1 downstream of the *nisA* promoter (PnisA) in pNZgfp-lmrB. All plasmids were introduced in *L. lactis* NZ9000 to enable nisin induction. Nisin induction of PnisA 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'-CTCGTGATCTAATTGCTTCTTCTTCC) and P2 (5'-CAGTGATCATAAATGCTTCTTCC) containing BclI restriction sites (underlined) that overlap the *lsbB* and *lmrB* initiation codons. BclI digested PCR product was ligated into the BamHI site of pGKH10, resulting in pGKH10 and pGKH2. In plasmid pGKH1, the lacZ gene is under the control of the *lmbB* promoter, whereas the *lmbB* 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 EcoRV fragment encoding the N-terminal portion of *lmrA* was deleted from pAPL2.2 The resulting plasmid pAPL3 was digested with *ScaI* and BamHI yielding a

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*2 K. Venema, unpublished data.*
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3.234-kb fragment with the lmrA deletion and its flanking regions. This fragment was ligated into corresponding sites in pORI290. The resulting plasmid, pORILmr, was used to transform L. lactis LL108, which carries the repA gene on the chromosome, thereby allowing the pORI290 derivative to replicate. pORILmr isolated from this strain was introduced, together with pVE6007, into L. lactis NZ2900. 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 ΔlmrA 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 microtitre plate (Greiner Bio-One B.V., Alphen, The Netherlands) using the GENios microtitre plate reader and Magelan software (Tecan, Groeningen, Aardria). 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 380 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 × OD₅₉₅⁻¹).

Bacteriocin Activity Assays

Bacteriocin activity was detected using a agar-well diffusion assay (1). To this end, wells made in the lawn of soft agar with an indicator strain ( toned 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 10⁶ cells/ml of an indicator strain, followed by overnight incubation at 30 °C.

LmrB is homologous to many pro- and eukaryotic ABC transporters and to the hop resistance protein HorA of L. lactis MG1363 (32) and, like LmrA, carries the multidentate β-lactamase binding site (AGAAAGGAAG). The third ORF could encode a 34-amino acid peptide with a calculated molecular mass of 3.4 kDa. The gene is preceded by a strong potential ribosome binding site (GAGGA), but no obvious ATG start codon is preceded by a potential ribosome binding site (GAGGA), but no obvious ATG start codons are present. The genetic information for the production of Bac501 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 32.8-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 (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 (GAGGA). 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 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 32.8-kb EcoRI fragment of pMN5 encodes Bac513 activity, the ORFs were

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 LMBR (5'-CTATATTGACATTCTCTGGG). The cDNA thus obtained was subsequently amplified by PCR using REP2 (5'-GAAATTGCACAAGC) in combination with REP3 (5'-CCAAATCTCAATGGC) or LSRB-3 (5'-GTCAAAAAGCTATGGAC). 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 mRNA size marker (0.5−9 kb) was from Amblon (Austin, TX). Purified PCR products obtained with the oligonucleotides LMR1 (5'-TGATGGAAGAACTTGCGG) and LMR2 (5'-GCAAGAGATTTGGCTC), containing the 5’ end of lmrA, or LMR1 (5'-CATGAATTTTGGAATTTGGG) and LMR2 (5'-CTCATCACTTGGCTTG), 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 32.8-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 (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 (GAGGA). 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.

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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 derivative contains the \textit{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
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 (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* (TTGAAAA 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.

**Lmr-mediated Bacteriocin Secretion and Immunity**

The amino acid sequence Met-Lys-Thr-Ile-Leu-Arg-Phe-Val-Ala-Gly-Gly was obtained after Edman degradation of purified LsbA indicating that the in vitro sequence Met-Lys-Thr-Ile-Leu-Arg-Phe-Val-Ala-Gly is cleaved at this position in *L. lactis* NZ9000. The removal of this signal sequence results in a mature LsbA of 457 amino acids which is consistent with the mass of 53 kDa determined by SDS-PAGE analysis (Fig. 3A). The detection of this protein was used to confirm the purification of LsbA. The LsbA immunoreactivity was used to detect strains that were purified from the supernatant of *L. lactis* NZ9000 containing either pNZlsmB or pNZlsBA, respectively, and the purified peptides were subjected to N-terminal amino acid sequencing. The molecular mass of the secreted forms of LsbA was estimated after SDS-20% PAGE, was ~3 kDa (Fig. 3B). The N-terminal of this strain contained neither pre-LsbB nor the mature bacteriocin. By contrast, processed LsbB was detected in the supernatant of a wild-type strain (Fig. 3B). 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. To visualize the protein in *L. lactis* IL1403 as the indicator strain. 15 μl of a 10-fold concentrated supernatant of a nisin-induced culture of *L. lactis* NZ9000 (pNZlsmB) (lanes 1) or *L. lactis* NZ9000 (pNZlsBA) (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 pNZlsmB or pNZlsBA, 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* NZ9000 ΔacmAΔhtrA, LsbB was detected as an inactive pre-bacteriocin by SDS-20% PAGE (Fig. 4B). The supernatant of this strain contained neither pre-LsbB nor the mature bacteriocin. By contrast, processed LsbB 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-LsbB across the cytoplasmic membrane.
Fig. 4. LsbA production in L. lactis strains NZ9000 and NZ9000::acmAΔhtrA. 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 (pNZlsbA); lanes 2 and 3, total cell extract and 10-fold-concentrated supernatant of L. lactis NZ9000::acmAΔhtrA (pNZlsbA), 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 Axiohot (Zeiss) microscope and an Axion Vision camera (Axion Technologies, Houston, TX).

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, L. lactis JIM7049, containing the nisRK genes needed for nisin induction of PnisA, was used as the host for His6-LmrB expression. L. lactis JIM7049 (pNH/lmrB) became resistant to both LsbA and LsbB when His6-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 the region preceding lmrA in MG1363. The gene in strain IL1403 is preceded by a potential ribosome binding site (AAAG-GAAG), but no obvious −35 and −10 consensus promoter regions could be discerned. Possibly, lmrA is in an operon with the upstream-located ykbF, 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 lmrPs, 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, 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 LmrB did not have any effect on the secretion of LsbB (Fig. 7). In contrast, no secretion of LsbA was observed in L. lactis NZ9000ΔlmrA. 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 pNZlsbB-lmrB or pNZlsbB-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.
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 dihydroxyalanine, dihydrobutyryl, 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-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 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 S. 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. lactococcin 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 amphipathic 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 LabA 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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