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The Genes for Secretion and Maturation of Lactococcins Are Located on the Chromosome of *Lactococcus lactis* IL1403

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Southern hybridization and PCR analysis were used to show that *Lactococcus lactis* IL1403, a plasmid-free strain that does not produce bacteriocin, contains genes on its chromosome that are highly homologous to *lcnC* and *lcnD* and encode the lactococcin secretion and maturation system. The *lcnC* and *lcnD* homologs on the chromosome of IL1403 were interrupted independently by Campbell-type integrations. Both insertion mutants were unable to secrete active lactococcin. Part of the chromosomal *lcnC* gene was cloned and sequenced. Only a few nucleotide substitutions occurred, compared with the plasmid-encoded *lcnC* gene, and these did not lead to changes in the deduced amino acid sequence. No genes homologous to those for lactococcin A, B, or M could be detected in IL1403, and the strain does not produce bacteriocin activity.

Lactococcins are bacteriocins produced by *Lactococcus lactis*. van Belkum et al. (29, 30) determined the nucleotide sequences of three bacteriocin determinants from the lactococcal plasmid p9B4-6. In detailed mutational analyses, the lactococcin structural and immunity genes were identified (29, 30). Conclusive evidence that *lcnA* is indeed the structural gene of lactococcin A came from Holo et al. (10). By comparing the amino acid sequence determined from the purified bacteriocin with that deduced from the structural gene, it was evident that lactococcin A was synthesized as a 75-amino-acid precursor with an N-terminal extension of 21 amino acids. Processing of this precursor takes place behind a glycine doublet. Recently, LcnB has also been purified and its N-terminal amino acid sequence has been determined (31). This bacteriocin is also processed after a double glycine. The glycine processing site is present in many class II peptide bacteriocins of lactic acid bacteria (13), even in some lantibiotics, and in colicin V, an *Escherichia coli* peptide bacteriocin (5, 7, 8, 11, 17, 19, 27).

lcnA is also present on pNP2 of *L. lactis* subsp. *lactis* bv. diacetylactis WM4 (25), and by Tn*5* transposon mutagenesis, it was shown that two additional genes are required for bacteriocin activity. These genes, designated *lcnC* and *lcnD*, are located in an operon immediately upstream of the lactococcin A structural and immunity genes (25). Comparisons of LcnC and LcnD with protein database sequences revealed that these proteins have significant similarities to gram-negative proteins implicated in signal sequence-independent secretion pathways (2, 9, 24). It is now generally accepted that LcnC and LcnD are required for the secretion of lactococcins via a system dedicated to bacteriocin export (14, 25). Similarly dedicated secretion systems have now been proposed for other lactic acid bacterium bacteriocins (15, 17).

In retrospect, it is surprising that lactococcin production and secretion were found with plasmids which carry only the lactococcin structural and immunity genes but do not encode the postulated secretion function. Holo et al. (10) were able to detect only reduced production of lactococcin A in *L. lactis* IL1403. This strain was also used throughout the works of van Belkum et al. (28–30). In all other strains, no lactococcin production was detectable. In this study, we investigated why strain IL1403 is capable of producing and externalizing active lactococcins in the absence of the plasmid-encoded *lcnC* and *lcnD* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was cultured in M17 broth (26) supplemented with 0.5% glucose. Agar (1.5%) was added to plates. *E. coli* was cultured on TY medium (21). Erythromycin and chloramphenicol were used at final concentrations of 5 μg/ml each for *L. lactis* and of 100 and 10 μg/ml, respectively, for *E. coli.*

Molecular cloning and DNA sequencing. Transformations of *L. lactis* and *E. coli* were done as described earlier (32). General DNA cloning and manipulation techniques and Southern hybridizations were carried out essentially as described by Sambrook et al. (22). Probes for Southern hybridizations were labelled with an ECL labelling kit (Amersham, Little Chalfont, Buckinghamshire, United Kingdom). DNA sequencing was done on double-stranded DNA by the dideoxy chain termination method (23), using a T7 sequencing kit and the manufacturer's (Pharmacia, Uppsala, Sweden) protocol.

Bacteriocin assay. The bacteriocin spot test was used to determine bacteriocin activity, as described before (31). One arbitrary unit was defined as the reciprocal of the highest dilution forming a visible halo. Overlayer assays on colonies were done as described before (31). *L. lactis* IL1403 was used as the lactococcin indicator strain throughout this study.

Construction of plasmids. Internal fragments from the plasmid-derived *lcnC* and *lcnD* genes (*Hin*dIII-*Nsi*I and *Hin*cII-*Hae*III fragments for *lcnC* and *lcnD*, respectively) were cloned in pORI28 (16) to give pINT4C and pINT1D, respectively. These plasmids were maintained in the *E. coli* helper strain EC1000, which contains on its chromosome a copy of *repA*, encoding the pWV01 replication protein RepA. The plasmids from this strain were isolated and used to transform *L. lactis* IL1403. Campbell-type integrants were selected on plates containing erythromycin, as described by Leenhouts and Venema (16).

pHVB was constructed to test bacteriocin production in integrants. This plasmid is a theta-replicating plasmid derived from pAMβ1 and does not interfere with the integrated state of the pINT plasmids. To obtain pHVB, pMB580 was cut with *Eco*RI and *Xba*I. The fragment carrying *lcnB* was isolated and cloned into pHV1432 cut with the same restriction endonucleases.

PCR. PCR was performed according to standard protocols (22) with *Taq* polymerase (Boehringer Mannheim GmbH, Mannheim, Germany). The primers used for PCR were synthesized with model 381A DNA synthesizer (Applied Biosystems, Foster City, Calif.).

Plasmid rescue. Chromosomal DNA of *L. lactis* IL1403::pINT4C was isolated and cut with various restriction endonucleases. After self-ligation, the DNA was used to transform *E. coli* EC1000, with selection for erythromycin resistance.

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant properties[a]	Source or reference
Bacteria		
L. lactis		
IL1403	Plasmid-free strain; indicator strain for lactococcin B	1
MG1363	Plasmid-free strain	6
E. coli EC1000	Helper strain; MC1000 containing pWV01 *repA* on chromosome	Laboratory collection
Plasmids		
pMB580	Em^r; containing the lactococcin B operon	30
pMB551	Em^r; containing most of *lcnC* and *lcnD*; used as probe in Southern hybridization	30
pHV1432	Cm^r; cloning vector; pAMβ1 derivative	21
pORI28	Em^r; cloning vector lacking *repA*	16
pINT4C	Em^r; integration vector; pORI28 with an internal fragment of *lcnC*	This work
pINT1D	Em^r; integration vector; pORI28 with an internal fragment of *lcnD*	This work
pHVB	Cm^r; pHV1432 derivative containing lactococcin B operon	This work
pKV4	Em^r; pIL253 derivative containing *lcnCDA* and *lciA*	30a

[a] Em^r, erythromycin resistance; Cm^r, chloramphenicol resistance.

RESULTS AND DISCUSSION

The fact that *lcnC* and *lcnD* are essential for extracellular lactococcin activity (25), combined with the observation that in *L. lactis* IL1403 these genes need not be present on a plasmid together with the *lcnA* structural gene (10, 28, 30), prompted us to investigate whether IL1403, a plasmid-free strain, carries genes homologous to *lcnC* and *lcnD* on its chromosome. A DNA fragment encompassing most of plasmid-derived *lcnC* and *lcnD* was used as the probe in Southern hybridizations. A signal was indeed obtained with the chromosome of IL1403 (Fig. 1B, lanes 6 to 9). Subfragments of *lcnC* and *lcnD* also reacted in Southern hybridizations (data not shown). In PCRs on IL1403 chromosomal DNA with primers derived from and spanning almost the entire plasmid-encoded *lcnC* and *lcnD* region (3, 4), fragments with sizes similar to those expected from plasmid-borne *lcnC* and *lcnD* were obtained (Fig. 2A). No PCR products were obtained with primers specific for the 3′ end of *lcnD*. By using DNA probes encompassing this part of the gene in a Southern hybridization, a signal was found, however, suggesting that a homologous region is preserved on the chromosome of IL1403. Apparently, the nucleotide sequence of the part of *lcnD* against which the primers were directed differs significantly from that of the plasmid-encoded gene, preventing primer annealing. Taken together, these results indicate that the chromosome of strain IL1403 contains

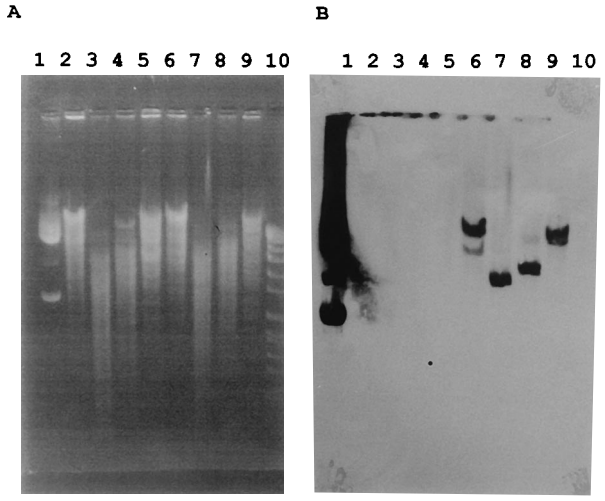

FIG. 1. (A) Agarose (0.8%) gel; (B) corresponding Southern hybridization film. Lanes 1, plasmid pMB551 (positive control); lanes 2 to 5, chromosomal DNA of *L. lactis* MG1363 cut with *Eco*RV, *Hin*dIII, *Hpa*II, and *Pvu*II, respectively; lanes 6 to 9, chromosomal DNA of *L. lactis* IL1403 cut with *Eco*RV, *Hin*dIII, *Hpa*II, and *Pvu*II, respectively; lanes 10, SPP1 marker DNA cut with *Eco*RI. Plasmid pMB551 was used as the probe.

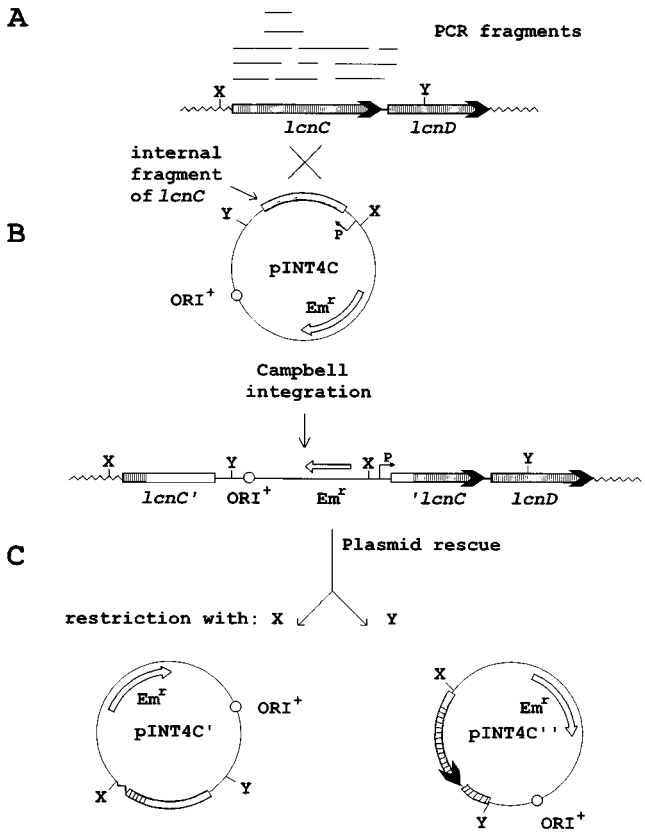

FIG. 2. (A) Schematic representation of the PCR products obtained from the chromosome of IL1403. Schematic outlines for the Campbell-type integration of pINT4C into the chromosome of IL1403 to produce an insertion mutant of *lcnC* (B) and plasmid rescue (C). ORI^+, origin of replication; Em^r, erythromycin resistance marker; X and Y, restriction endonuclease sites; P, promoter; open box, internal fragment of *lcnC*; hatched box, chromosomally located gene.

genes that are highly homologous to plasmid-encoded *lcnC* and *lcnD*.

By using probes encompassing the structural genes for lactococcins A, B, and M and their corresponding immunity genes, no signal was obtained by Southern hybridization (data not shown). In addition, no bacteriocin activity was detectable when overlayer assays were performed on IL1403 with several other lactococcal and nonlactococcal strains as indicators (data not shown), indicating that IL1403 itself does not produce bacteriocin activity.

Two small fragments containing internal parts of *lcnC* (a *Hin*dIII-*Nsi*I fragment) and *lcnD* (a *Hin*cII-*Hae*III fragment) were cloned in pORI28, and the resulting integration vectors were used to disrupt both genes in the IL1403 chromosome in a Campbell-type integration (Fig. 2B; exemplified for pINT4C only). Disruption of *lcnC* had no polar effects on the expression of *lcnD* because of the presence of a promoter on the integration plasmid (Fig. 2B). Strain IL1403 and both insertion mutants were transformed with pHVB, a plasmid containing the lactococcin B structural and immunity genes. IL1403(pHVB) produced bacteriocin, as judged by the appearance of halos around the colonies in an overlayer assay. However, extracellular production of lactococcin B was abolished in both insertion mutants, indicating that both chromosomally encoded genes are functional and necessary for the secretion of active lactococcin B.

The reduced production of lactococcins in IL1403 carrying a plasmid with only the bacteriocin structural and immunity genes, compared with that of the wild-type strain carrying all of the essential genes on a plasmid (10, 28), can be explained by assuming that the lower copy number of the chromosomally located genes (most probably one) results in lower secretion efficiency. This conclusion was corroborated by introducing *lcnC* and *lcnD*, originating from p9B4-6, on plasmid pKV4 into IL1403. This led to lactococcin production that was increased at least 10-fold (data not shown).

The integrated plasmid pINT4C was rescued from the chromosome by cutting chromosomal DNA of *L. lactis* IL1403::pINT4C with various restriction endonucleases (Fig. 2C). After self-ligation, the DNA was used to transform *E. coli* EC1000 ($RepA^+$). Ligated fragments carrying a functional origin of replication of pWV01 can replicate in this *E. coli* strain because of the fact that it produces the plasmid replication protein RepA. Only from the *Hin*dIII ligation mixture was obtained a plasmid that was larger than pINT4C. The constructs rescued from the other ligation mixtures were either smaller than (because of deletion formation) or of the same size (because of homologous recombination) as pINT4C. All constructs rescued from IL1403::pINT1D carried deletions, since all were smaller than pINT1D. The insert in the plasmid rescued from the *Hin*dIII ligation mixture of IL1403::pINT4C was sequenced. It contained the nucleotide sequence corresponding to nucleotides 1260 to 1786 of *lcnC*, as published by Stoddard et al. (25). Several nucleotide substitutions were found compared with the sequence of plasmid-encoded *lcnC*, but none of these led to amino acid changes in the translation product (Fig. 3). It was impossible to clone the complete secretion mechanism from the chromosome of *L. lactis* IL1403 in *E. coli* or *L. lactis*. Apparently, the products encoded by these genes or by the DNA flanking these genes are deleterious when provided to the cell in more than one copy, as is the case on the plasmids used here. In this respect, it is noteworthy that we have also been unable to subclone plasmid-encoded *lcnC* (30a), indicating that it is this gene, encoding an integral membrane protein, that causes the problems.

Apart from the lactococcins, the secretion mechanism on the chromosome of IL1403 is also capable of secreting lacticin 481, a lantibiotic produced by *L. lactis* (18, 20). This lantibiotic contains a leader sequence of the double-glycine type. We have previously established that the N-terminal domain of the ABC transporter involved in the secretion of bacteriocins with this type of leader is responsible for cleavage of the leader (33). Apparently, chromosomally encoded *lcnC* is able to recognize the leader of lacticin 481 and the apparatus is able to secrete this lantibiotic.

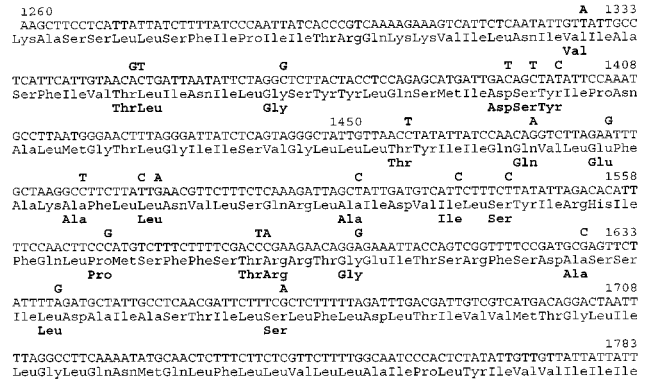

FIG. 3. Nucleotide sequence and deduced amino acid sequence of plasmid-located *lcnC* from nucleotides 1260 to 1786, as published by Stoddard et al. (25). Above the nucleotide sequence the changes in chromosomally located *lcnC* are in bold. The amino acids specified by the changed codons are in bold below the amino acid sequence.

It appears that strains of *L. lactis* can carry a bacteriocin secretion and maturation mechanism without producing bacteriocin. The reason for this phenomenon is unknown, but one explanation might be that a bacteriocin plasmid has integrated into the chromosome of IL1403 with subsequent or consequent loss of the bacteriocin structural and immunity genes. Alternatively, we may speculate that bacteriocins are not the only substrates for the secretion apparatus and that other proteins, peptides, or even nonproteinaceous substances are also secreted via this mechanism.

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