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# Organization and Nucleotide Sequences of Two Lactococcal Bacteriocin Operons

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**Two distinct regions of the *Lactococcus lactis* subsp. *cremoris* 9B4 plasmid p9B4-6, each of which specified bacteriocin production as well as immunity, have been sequenced and analyzed by deletion and frameshift mutation analyses. On a 1.8-kb *ScaI-ClaI* fragment specifying low antagonistic activity, three open reading frames (ORFs) were present, which were organized in an operon. The first two ORFs, containing 69 and 77 codons, respectively, were involved in bacteriocin activity, whereas the third ORF, containing 154 codons, was essential for immunity. Primer extension analysis indicated the presence of a promoter upstream of the ORFs. Two ORFs were present on a 1.3-kb *ScaI-HindIII* fragment specifying high antagonistic activity. The first ORF, containing 75 codons, specified bacteriocin activity. The second ORF, containing 98 codons, specified immunity. The nucleotide sequences of both fragments upstream of the first ORFs as well as the first 20 bp of the first ORF of both bacteriocin operons appeared to be identical.**

The ability of various bacteria to inhibit the growth of other bacteria is well documented (9, 19). In many cases it was demonstrated that the antagonistic activity was attributable to molecules of a proteinaceous nature, termed bacteriocins. The first bacteriocins to be discovered were the colicins produced by *Escherichia coli*, and extensive knowledge is now available concerning their genetics (8). In general, three genes are required for colicin production: a gene encoding the functional colicin, an immunity gene, and a gene essential for the release of the colicin. For most of the colicins investigated, the various genes are transcribed from a common promoter, but in some cases the colicin immunity gene is under control of a different promoter.

In contrast to bacteriocins produced by gram-negative bacteria, less is known about bacteriocins and their genetic determinants of gram-positive bacteria. In recent years special attention has been paid to bacteriocin production by lactic acid bacteria used in food fermentation and preservation. Several of these bacteriocins have been investigated with respect to their genetic determinants and biochemical characteristics (10). The gene encoding the precursor of the protein antibiotic nisin of *Lactococcus lactis* subsp. *lactis* ATCC 11454 has been cloned and sequenced (3). For several species of lactic acid bacteria the genetic determinants of bacteriocin activity were shown to be plasmid located (6, 7, 15, 17). One of the plasmids, which has been identified by Neve et al. (15) to encode bacteriocin production, was a 60-kb conjugative plasmid (p9B4-6) from *L. lactis* subsp. *cremoris* 9B4. Recently, we have cloned two fragments of p9B4-6 which were responsible for bacteriocin production and immunity. One was a 1.8-kb *ScaI-ClaI* fragment with low antagonistic activity (pMB225), and the other was a 1.3-kb *ScaI-HindIII* fragment specifying high antagonistic activity (pMB553) (21). Inhibition experiments showed that the two bacteriocins had different specificities.

We report on the sequence analysis of the two bacteriocin regions and show that the open reading frames (ORFs) found on these fragments are involved in bacteriocin production and immunity and are transcribed as an operon.

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used are listed in Table 1. The growth media for *E. coli* and *L. lactis* and the selective conditions for maintaining the various plasmids have been described previously (21).

**Molecular cloning.** Plasmid DNA was isolated from *E. coli* as described by Birnboim and Doly (2). With some modifications (22), the same method was used for the isolation of plasmids from *L. lactis*. Restriction enzymes, the Klenow fragment of the *E. coli* DNA polymerase I, RNasin RNase inhibitor, avian myeoblastosis virus reverse transcriptase, and T4 DNA ligase were purchased from Boehringer, Mannheim, Federal Republic of Germany, and were used as recommended by the supplier. *Asu*II was obtained from Promega Biotec, Madison, Wis., and *Nla*III was from New England BioLabs, Beverly, Mass. DNA cloning and manipulation techniques were performed essentially as described by Maniatis et al. (14). *E. coli* cells were made competent and were transformed by the method of Mandel and Higa (13). Transformation of *L. lactis* was done by electroporation with a Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, Calif.) by the method of Van der Lelie et al. (23).

**Bacteriocin assay.** The method used for screening *L. lactis* transformants for bacteriocin activity has been described previously (21). *L. lactis* IL1403 was used as the indicator strain.

**DNA sequencing.** Nucleotide sequences were determined by sequencing double-stranded DNA in two orientations by the dideoxy-chain method of Sanger et al. (16), using the T7 sequencing kit (Pharmacia, Uppsala, Sweden). Synthetic oligodeoxyribonucleotides were synthesized with an Applied Biosystems 391 PCR-Mate (Applied Biosystems, Foster City, Calif.).

**Primer extension.** RNA was isolated from *L. lactis* by the method described by Van der Vossen et al. (24). Synthetic primer (1 μg) was hybridized to 10 μg of RNA in a total volume of 40 μl containing 50 mM Tris hydrochloride (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 30 U of RNasin. This mixture was heated for 3 min at 65°C and was subsequently allowed to cool to room temperature. The volume was increased to 50 μl by the addition of dCTP,

TABLE 1. Bacterial strains and plasmids

| Bacterial strain or plasmid | Description[a] | Reference |
|---|---|---|
| *E. coli* MH1 | MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA* | 4 |
| *L. lactis* subsp. *lactis* IL1403 | Plasmid-free | 5 |
| Plasmid | | |
| pMB225 | Em$^r$ Bac$^+$, 6.2 kb; containing 1.8-kb *ScaI-ClaI* fragment with low antagonistic activity | 21 |
| pMB553 | Em$^r$ Bac$^+$, 5.1 kb; containing 1.3-kb *ScaI-Hin*dIII fragment with high antagonistic activity | 21 |
| pGKV210 | Em$^r$, 4.4 kb | 24 |
| pGKV410 | Em$^r$, 4.4 kb | 24 |
| pGKV259 | Em$^r$ Cm$^r$, 5 kb; pGKV210 derivative containing promoter P59 in multiple cloning site | 24 |

[a] Bac$^+$, Bacteriocin producer; Em$^r$ and Cm$^r$, resistance to erythromycin and chloramphenicol, respectively.

dGTP, and dTTP to final concentrations of 100 μM each. Then 1 μl of dATP from a 0.5 mM stock, 1 μl of [α-$^{35}$S]dATP (1 μCi; 1,000 Ci/mmol), and 1 μl of avian myeloblastosis reverse transcriptase (30 U) were added. After 1 h of incubation at 37°C, the reaction mixture was precipitated with ethanol. The pellet was washed with 80% ethanol and dissolved in 6 μl of distilled water and 4 μl of sequencing dye, and the sample was heated for 3 min in a boiling water bath. To determine the start site of transcription, the extension reaction mixtures were run on an acrylamide sequencing gel. Sequence ladders primed with the same oligonucleotides were used as molecular weight markers.

**Assay for bacteriocin activity in SDS-polyacrylamide gels.** To estimate the size of the bacteriocin molecule, 12.5 μl of the supernatant of a bacteriocin-producing late-exponential-phase culture of *L. lactis* was applied to a sodium dodecyl sulfate (SDS)-polyacrylamide (15%) gel (12) next to a molecular weight standard (Rainbow protein molecular weight marker; Amersham International, Amersham, England). The sample was mixed in a 1:1 ratio with 2× loading buffer for polyacrylamide gel electrophoresis (PAGE) (18) and was heated to 100°C for 10 min. After electrophoresis, the gel was washed as described by Bhunia et al. (1) and put on a glucose-M17 (20) agar plate. An indicator lawn of 4 ml of soft glucose-M17 agar (0.7%), containing 100 μl of an overnight culture of *L. lactis* IL1403, was poured on the surface. After 15 h of incubation at 30°C, the plate was examined for zones of inhibition of growth.

## RESULTS

**Nucleotide sequence of the bacteriocin genes on pMB225 and pMB553.** The nucleotide sequence of the 1.8-kb DNA fragment specifying low antagonistic activity is shown in Fig. 1. Three ORFs, each preceded by a putative ribosome binding site, were identified within this sequence. The first ORF (ORF-A1) could encode a polypeptide of 69 amino acids and is followed by an ORF (ORF-A2) able to encode a polypeptide of 77 amino acids. ORF-A2 is followed by a third ORF (ORF-A3) with 154 codons. The nucleotide sequence of the region downstream of ORF-A3 revealed neither additional ORFs nor palindromic sequences which might function as rho-independent transcriptional termination sites. Directly upstream of ORF-A1 two putative promoters were found, designated promoter P1 and promoter P2. The −35 sequence of promoter P1 corresponded more closely to the consensus −35 sequence of lactococcal promoters than promoter P2 (24). Also, the spacing of 16 bp between the −35 region and the −10 region of promoter P1 agreed more closely to the usual spacing in *L. lactis* promotors than to that in the promoter sequence P2.

Figure 2 shows the first 1,048 nucleotides of the 1.3-kb *ScaI-Hin*dIII fragment specifying high bacteriocin activity. Two ORFs (ORF-B1 and ORF-B2) were present which could encode polypeptides of 75 and 98 amino acids, respectively. Both ORFs were preceded by a putative ribosome binding site. In the nucleotide sequence two inverted repeats were identified which could act as rho-independent terminators, one 11 bp and one 104 bp downstream of the TAA stop codon of ORF-B2. The putative stem-and-loop structures had ΔG's of −130 and −96 kJ/mol, respectively. The first 378 nucleotides of the 1.8- and 1.3-kb fragments (starting at *ScaI* site present at the 5′ end of the fragment) were identical. This implies that the regulatory region preceding ORF-A1 and ORF-B1 as well as the first 20 bp of ORF-A1 and ORF-B1 were identical. Downstream of these identical regions the sequences on the two fragments presented in Fig. 1 and 2 did not reveal further nucleotide sequence similarity. The deduced amino acid sequences of the different ORFs were compared for sequence similarities. Only between ORF-A1 and ORF-B1 was significant similarity found (Fig. 3).

**Determination of the transcription start sites.** The start sites of transcription were determined by primer extension. RNAs from *L. lactis* IL1403(pMB225) and IL1403(pMB553) were hybridized with oligonucleotides specific for ORF-A1 (5′-CCACCAACAGCACCGCC-3′) and ORF-B1 (5′-CATC CATTAACAATGGT-3′), respectively. Comparison of the DNA bands synthesized from these primers with DNA sequencing ladders as standards showed that transcription started 12 bp downstream of the proposed (−10) TATAAT box of promoter P1 on the 1.8-kb fragment and 14 bp downstream of this box on the 1.3-kb fragment (Fig. 4).

**Mutation analyses.** The sequence data of the cloned inserts of pMB225 and pMB553 were analyzed for restriction sites, using MicroGenie software (Beckman, Palo Alto, Calif.). Several restriction sites were found which could be used for in vitro deletion, for the introduction of frameshift mutations, and for subcloning fragments in the pWV01-derived (11) shuttle vector pGKV210, pGKV410, or pGKV259 in *E. coli*. The mutated derivatives were transferred to *L. lactis* IL1403 and screened for bacteriocin activity and immunity.

The results of these experiments with pMB225 carrying the 1.8-kb *ScaI-ClaI* fragment specifying low bacteriocin activity are presented in Fig. 5. Plasmid pMB229, containing a frameshift mutation in ORF-A1, was constructed by filling in the *Asu*II site with Klenow DNA polymerase, resulting in an *Nru*I site. This mutation caused loss of antagonistic activity, but not of immunity, implying that ORF-A1 is involved in bacteriocin production. Deleting the *AluI-ClaI* fragment downstream of ORF-A1 resulted in plasmid pMB242. *L. lactis* cells containing this plasmid showed a Bac$^-$ Imm$^-$ phenotype, indicating that sequences in addition to ORF-A1 were involved in bacteriocin production. To

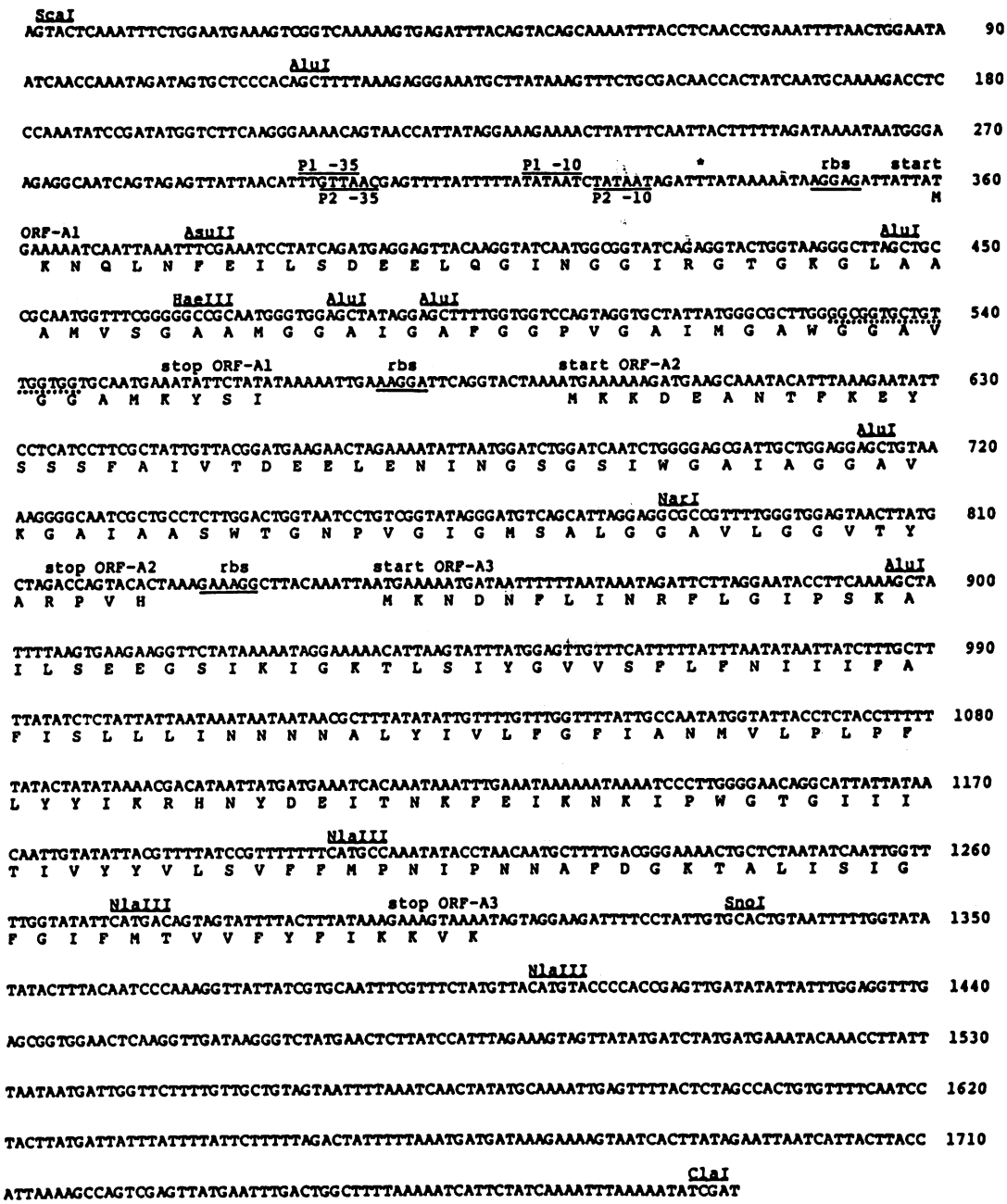


FIG. 1. Nucleotide and deduced amino acid sequences of the 1.8-kb *Sca*I-*Cla*I fragment. The deduced amino acid sequences of the three ORFs are shown below the nucleotide sequence. The −35 and −10 regions of the possible promoters P1 and P2 are indicated. Putative ribosome binding sites (rbs) are underlined. The asterisk above the sequence indicates the start site of transcription determined by primer extension. Restriction sites for mutagenesis are indicated. The dotted line represents the sequence used for the primer extension assay.

identify these sequences also, a frameshift mutation was made in ORF-A2 by filling in the unique *Nar*I site, changing it into a *Bss*HII site. Cells carrying the resulting plasmid pMB246 were Bac$^-$ Imm$^+$. Cells that carried plasmid pMB248, containing an *Asu*II-*Nar*I deletion encompassing both ORF-A1 and ORF-A2, were also Bac$^-$ Imm$^+$. From these data we conclude that both ORF-A1 and ORF-A2 are required for bacteriocin activity but not for immunity against bacteriocin and that ORF-A3 was probably responsible for immunity. Two lines of evidence support the supposition that the three ORFs on pMB225 constitute a single operon, as would be expected from the nucleotide sequence of the 1.8-kb *Sca*I-*Cla*I fragment. First, removal of the region upstream of the *Asu*II site, which includes the promoter and the 5′ part of ORF-A1, resulted in a Bac$^-$ Imm$^-$ phenotype (pMB226). Apparently, transcription of ORF-A3 is dependent on the promoter upstream of ORF-A1. Second, when the 1.4-kb *Hae*III-*Cla*I fragment of pMB226 was cloned behind the lactococcal promoter P59 of pGKV259 (24), yielding pMB241, the immunity-positive phenotype was

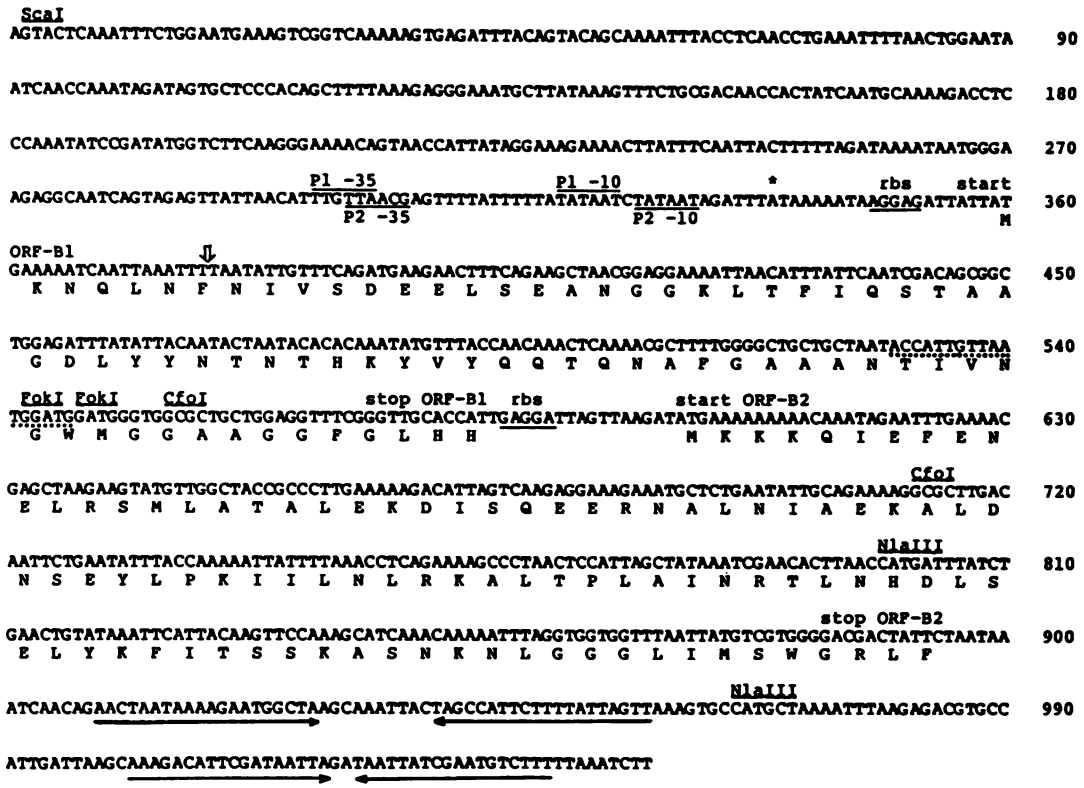


FIG. 2. Nucleotide and deduced amino acid sequences of the first 1,048 nucleotides of the 1.3-kb *Sca*I-*Hin*dIII fragment. The deduced amino acid sequences of the two ORFs are shown below the nucleotide sequence. The putative −35 and −10 regions of promoters P1 and P2 as well as the putative ribosome binding sites of the two ORFs are indicated. The start site of transcription as determined by primer extension is marked with an asterisk. Restriction sites for mutagenesis are indicated. The inverted repeats downstream of the second ORF are underlined by solid arrows. The region upstream of the vertical open arrow represents the region of identity between the 1.3-kb fragment and the first 378 nucleotides of the 1.8-kb *Sca*I-*Cla*I fragment. The dotted line represents the sequence used for the primer extension assay.

restored. As expected from the sequence data, deletion of the *Sno*I-*Cla*I fragment of pMB225 (pMB249) did not interfere with immunity.

To investigate the effect on transformation of *L. lactis* with a plasmid carrying undisturbed ORFs A1 and A2, but carrying a 3′ deletion in ORF-A3, pMB250 was constructed in *E. coli*. In pMB250 ORF-A3, the presumed immunity gene had been partly deleted by removal of a 0.14-kb *Nla*III fragment. Upon introduction of pMB250 in *L. lactis*, transformants were detected and appeared to produce bacteriocin, but the colonies were small, indicating impeded growth of cells producing a nonfunctional ORF-A3 product. No transformants developed on plates in which the growth medium had been supplemented with a 50% (vol/vol) supernatant culture of the bacteriocin-producing strain IL1403 (pMB225). Apparently, the initial concentration of bacteriocin produced by the transformants in the plates was insufficient to completely suppress the growth of the transformed cells. These results strongly suggest that ORF-A3 specified immunity.

```
ORF-A1   M K N Q L N F E I L S D E E L Q G I N G G         I
         : : : : : : :   : . : : : : :       : : :       :
ORF-B1   M K N Q L N F N I V S D E E L S E A N G G K L T F I

ORF-A1   R G T G K G   L   A A A M V S G A A M G G A I G A F
         :   :   :   :   .           .           .   :   :
ORF-B1   Q S T A A G D L Y Y N T N T H K Y V Y Q Q T Q N A F

ORF-A1   G G P V G A I   M G A W G G A V G G A M K Y S I
         :   .   :   :   :   :   :   :   :
ORF-B1   G A A A N T I V N G W M G G A A G G F G L H H
```

FIG. 3. Homology comparison of the deduced amino acid sequences of ORF-A1 and ORF-B1. Colons indicate amino acid identity; periods indicate conservative substitution.

Figure 6 shows the different mutation derivatives of pMB553. Deleting the 0.1-kb *Cfo*I fragment, encompassing the 3′ part of ORF-B1 and the 5′ part of ORF-B2 (pMB560), resulted in loss of both bacteriocin production and immunity. *L. lactis* transformants containing a plasmid in which the promoter region and the 5′ part of ORF-B1 were removed by deleting the 0.54-kb *Sca*I-*Fok*I fragment of pMB553 (pMB562) were Bac$^-$ Imm$^-$. Immunity but not bacteriocin production could be restored when promoter 59 was cloned upstream of the remaining part of the bacteriocin operon of pMB562 (yielding plasmid pMB563). These results indicate that ORF-B1 is involved in bacteriocin production and ORF-B2 specifies immunity and that these ORFs are transcribed as an operon. Further support for ORF-B2 being the immunity gene was provided by the observation that transformantion of *L. lactis* with plasmid pMB561, carrying a deletion in ORF-B2, gave rise to only very small colonies of transformants producing bacteriocin. When the plates were supplemented with a 30% (vol/vol) supernatant culture of the bacteriocin-producing strain IL1403(pMB553), no transformants of *L. lactis* were obtained with pMB561. Apparently also in this case, the initial concentration of the

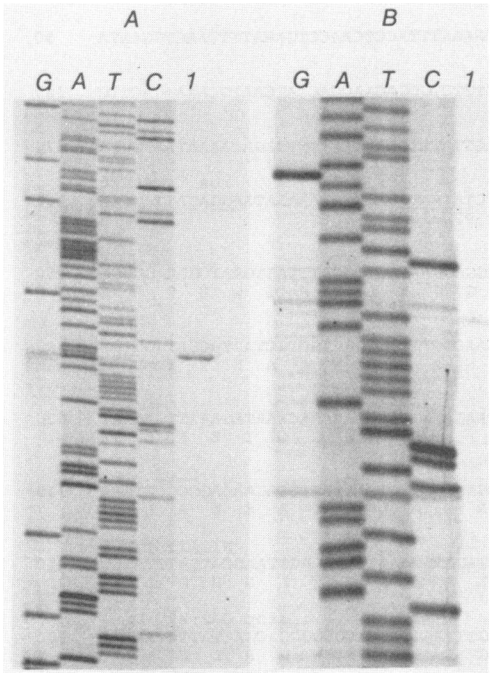


FIG. 4. Determination by primer extension of start site of transcription of the two bacteriocin operons. (A) Lane 1 shows the primer extension product of RNA from IL1403(pMB225). (B) Lane 1 shows the primer extension product of RNA from IL1403(pMB553). The sequence ladders in panels A and B were produced by using the same primers and are shown for each reaction (GATC).

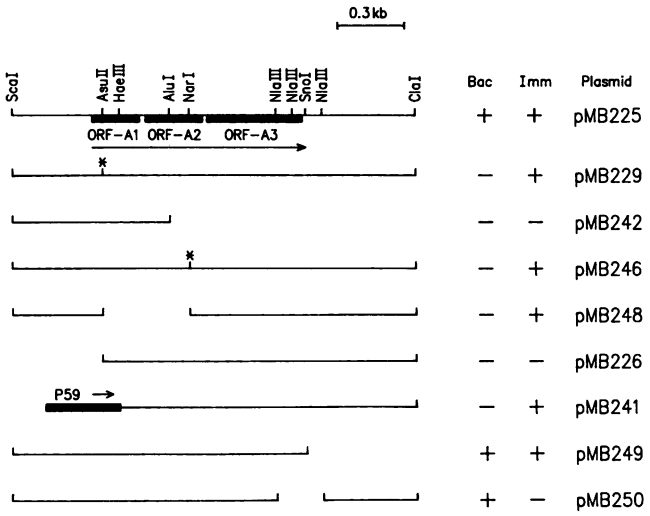


FIG. 5. Phenotype of various mutation derivatives of pMB225. The restriction enzyme map and the location of ORF-A1, -A2, and -A3 on the 1.8-kb *Sca*I-*Cla*I fragment of pMB225 are shown. Of the six *Alu*I sites, only one is presented. The lines represent the parts of the 1.8-kb fragment present in the various subclones. Asterisks indicate frameshift mutations of the ORFs (for details, see text). The thick bar represents promoter P59. The arrow indicates direction of transcription. Bacteriocin production (Bac) and immunity (Imm) expressed by the various plasmids are indicated.

bacteriocin produced by the *L. lactis* transformants was too low to completely inhibit the growth of the transformants.

**SDS-PAGE.** To examine whether the size of the bacteriocin molecule specified by pMB553 is in agreement with the expected molecular weight of the polypeptide that could be encoded by ORF-B1 (approximately 8,100), a sample of the supernatant of a culture of IL1403(pMB553) was subjected to SDS-PAGE and subsequently examined for bacteriocin activity. A zone of inhibition of growth could be detected at a position around 3.4 kDa (Fig. 7). This indicates that the bacteriocin molecule specified by pMB553 has an aberrant mobility on SDS-PAGE and/or is subject to processing. A sample of the supernatant of IL1403 did not show antagonistic activity in an SDS-polyacrylamide gel (Fig. 7). When *L. lactis* indicator cells were used which contained pMB553, no zones of inhibition of growth were detected (results not shown). The same procedure was used to test the pMB225-specified bacteriocin. Probably because of its lower antagonistic activity, no zone of inhibition of growth was observed with IL1403 as the indicator strain (results not shown).

## DISCUSSION

We present the results of the nucleotide sequence analysis of two DNA regions which specify bacteriocin production as well as immunity from the lactococcal plasmid p9B4-6. Several ORFs on these fragments could be identified. The sequence data of the 1.8-kb *Sca*I-*Cla*I fragment present on pMB225, specifying low antagonistic activity, indicate the presence of three ORFs (Fig. 1). Analysis of different mutation derivatives of pMB225 indicated that the first two ORFs (ORF-A1 and ORF-A2) were involved in bacteriocin production whereas a third (ORF-A3) appeared to be responsible for immunity. Sequence and mutation analyses of the 1.3-kb *Sca*I-*Hin*dIII fragment specifying the higher activity present on pMB553 showed that one ORF was responsible for bacteriocin activity (ORF-B1) and another was responsible for immunity (ORF-B2). Transformation of an *L. lactis* strain which originally did not show antagonistic activity, using plasmid pMB553, resulted in the production by the transformant of a bacteriocin with a specificity identical to that of the strain from which the plasmid-borne DNA fragment was obtained (21). This observation, together with the

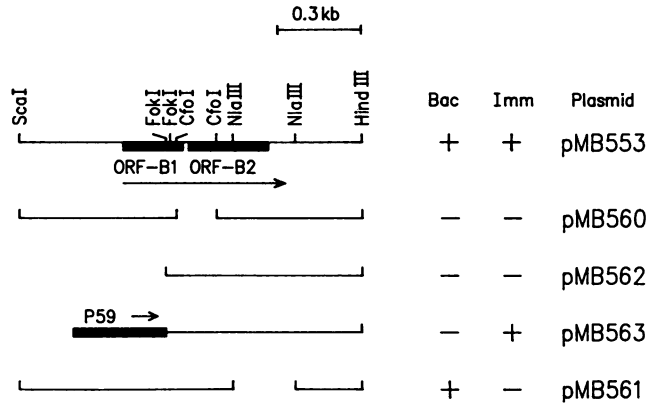


FIG. 6. Phenotype of various mutation derivatives of pMB553. The restriction sites, as well as the locations of ORF-B1 and ORF-B2 on the 1.3-kb *Sca*I-*Hin*dIII fragment of pMB553, are shown. The lines indicate the parts of the 1.3-kb fragment remaining in the various subclones; the thick bar represents promoter P59. The arrow indicates direction of transcription. Bac and Imm indicate the phenotypes for bacteriocin production and immunity of the different constructs, respectively.

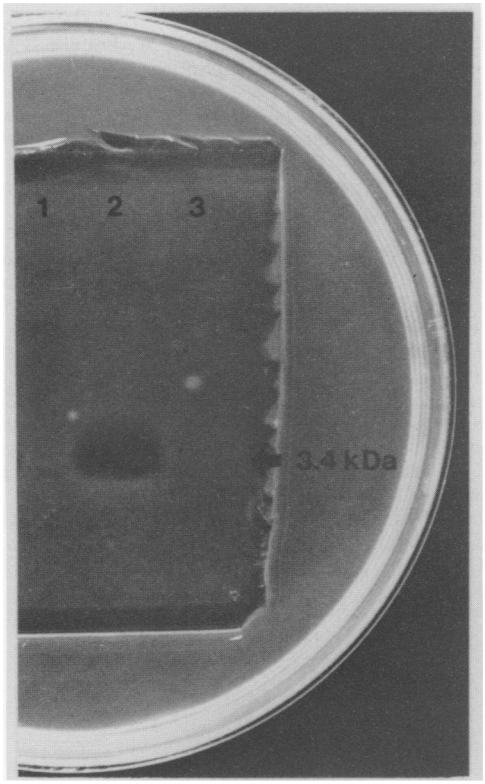


FIG. 7. Detection of bacteriocin activity in an SDS-polyacrylamide (15%) gel by overlayering the gel with glucose-M17 soft-agar medium containing *L. lactis* IL1403. Lane 1, Molecular weight standard; lane 2, supernatant of IL1403(pMB553); lane 3, supernatant of IL1403.

mutation analysis of the bacteriocin operon present on pMB553 presented here, strongly suggests that ORF-B1 is the structural bacteriocin gene of pMB553. If this idea is correct, then we have to assume that the bacteriocin molecule has an aberrant mobility in SDS-PAGE and/or is subject to processing, as the size of the product that could be specified by ORF-B1 is 8.1 kDa while the active bacteriocin has a size of around 3.4 kDa. Considering the degree of amino acid similarity of the products of ORF-A1 and ORF-B1 (Fig. 3), it is conceivable that ORF-A1 encodes the bacteriocin of pMB225. Why a second gene (ORF-A2) is involved in pMB225-specified bacteriocin activity is unclear. Conceivably, the polypeptide encoded by ORF-A2 is necessary for the maturation and/or the secretion of the bacteriocin, or the bacteriocin encoded by pMB225, might be a heterodimer of the ORF-A1 and ORF-A2 products.

The deduced amino acid sequence of ORF-B2 did not show significant similarity to that of ORF-A3, which would be expected on the basis of our previous observation that ORF-A3-specified immunity does not give protection against ORF-B1-specified bacteriocin, and vice versa.

The results of analysis of the nucleotide sequence and the bacteriocin phenotype of the different mutation derivatives suggest that on both fragments on pMB225 and pMB553 the different ORFs are transcribed as an operon. The noncoding region upstream of the ORFs, which contains two possible promoters according to the sequence data, appeared to be identical on both fragments. Thus, the difference in activity we observed between IL1403(pMB225) and IL1403(pMB553) (21) cannot be caused by a difference in promoter strength. That the autoradiograms of the primer extension experiments showed only one major band for both fragments strongly suggests that only one of the two possible promoters is used. The nucleotide sequence of promoter P1 conforms more to the *L. lactis* consensus promoter sequence than that of promoter P2; thus, we think that promoter P1 is the actual promoter used for transcription of the bacteriocin operons. The sequence TAT is fairly common as the +1 region in which A is the initiating nucleotide. The primer extension experiments indicate that the initiation of transcription of the bacteriocin operon of pMB553 conforms to this pattern, whereas transcription on the bacteriocin operon of pMB225 started at a T 2 bp upstream of the central A. Since the bacteriocin promoters are embedded in completely identical regions on both fragments, we speculate that the choice of different start sites of transcription is dictated by the downstream region, which may cause a different nucleotide conformation at the transcription start site.

The complete identity of the regions upstream of the ORFs of the two bacteriocin operons, including the first part of their first ORF, is intriguing. Preliminary results indicate that downstream of the bacteriocin operon on the 1.3-kb fragment a sequence is present which is very similar to the identical nucleotide sequences upstream of the two bacteriocin operons. Whether this sequence is followed by a third bacteriocin operon is presently under investigation. The finding that the bacteriocin operon on the 1.3-kb fragment is flanked by similar nucleotide sequences, which are similar to the 5′ flanking region of the bacteriocin operon on the 1.8-kb fragment, suggests that these operons may be moved in the lactococcus genome by transposition-like events.

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