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Cloning of Two Bacteriocin Genes from a Lactococcal Bacteriocin Plasmid

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***Lactococcus lactis* subsp. *cremoris* 9B4 plasmid p9B4-6 (60 kilobases [kb]), which specifies bacteriocin production and immunity, was analyzed with restriction endonucleases, and fragments of this plasmid were cloned into shuttle vectors based on the broad-host-range plasmid pWVO1. Two regions on p9B4-6 were identified which specify inhibitory activity on *L. lactis* indicator strains: one that could be confined to a 1.8-kb *Sca*I-*Cla*I fragment with low antagonistic activity and a 15-kb *Xba*I-*Sal*I fragment specifying high antagonistic activity. The inhibitory substances produced by these two clones were sensitive to proteolysis. A 4-kb *Hin*dIII fragment derived from the 15-kb fragment strongly hybridized with the 1.8-kb fragment. The antagonistic activity specified by the 4-kb fragment was somewhat reduced as compared with that of the 15-kb fragment. A 1.3-kb *Sca*I-*Hin*dIII subfragment of the 4-kb fragment contained both the immunity and bacteriocin genes. Inhibition studies showed that the two bacteriocins had different specificities.**

Many strains of lactic acid bacteria produce antagonistic substances that have been identified as bacteriocins (7). Bacteriocins are bactericidal proteins that are secreted by the cells. The inhibition spectrum of bacteriocins produced by lactic acid bacteria on gram-positive bacteria varies but is mostly confined to closely related species. They have long been known and are important in food fermentation and food preservation. Several bacteriocins from lactic streptococci have been purified and characterized. They appear to be heterogeneous in molecular weight, biochemical characteristics, optimal conditions for activity, and mode of action.

In several cases it has been demonstrated that bacteriocin production is associated with plasmid DNA. Production of the bacteriocin of *Lactococcus lactis* subsp. *cremoris* 346, termed diplococcin, was demonstrated by Davey (5) to be specified by a conjugative plasmid of 54 megadaltons. By using curing experiments, bacteriocin production in *Pediococcus pentosaceus* FBB61, designated pediocin A, was demonstrated by Daeschel and Klaenhammer (4) to be encoded by a 13.6-megadalton plasmid. Scherwitz et al. (13) showed by conjugation experiments that bacteriocin production in *L. lactis* subsp. *lactis* WM4 was linked to an 88-megadalton plasmid, pNP2. By using cloning techniques, Scherwitz-Harmon and McKay (14) isolated *Bcl*I fragments from pNP2 containing the bacteriocin determinants. Geis et al. (6) investigated 280 strains of lactococci for the production of antagonistic compounds. On the basis of secretion into liquid medium, precipitation by ammonium sulfate, inactivation by proteolytic enzymes, and their narrow inhibitory spectrum, the substances produced by 16 of these strains were classified as bacteriocins. Neve et al. (11) have shown, by means of conjugation and curing experiments, that a 60-kilobase (kb) plasmid (p9B4-6) from one of these strains, *L. lactis* subsp. *cremoris* 9B4, is responsible for bacteriocin production. In addition to the inhibitory activity against other lactococci, 9B4 was also capable of inhibiting several *Clostridium* strains (6). By conjugation, the bacteriocin production could be transferred to a plasmid-free strain of *L. lactis* (11).

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In this paper we report the cloning of two different fragments of plasmid p9B4-6; both fragments encoded bacteriocin production as well as immunity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* was grown in TY broth (12) at 37°C. For plates, TY broth was solidified with 1.5% agar. Glucose-M17 broth and glucose-M17 agar (15) were used to grow *L. lactis* at 30°C. Selective concentrations of erythromycin, kanamycin, and chloramphenicol for growing *L. lactis* cells containing the various plasmids were 5, 50, and 5 μg/ml, respectively. For growing *E. coli* cells containing the various plasmids, the selective concentrations of erythromycin and kanamycin were 100 and 50 μg/ml, respectively.

Molecular cloning. For large-scale preparation of plasmid p9B4-6 from *L. lactis* subsp. *lactis* Bu2-61, the method of Neve et al. (11) was used. Plasmids from *E. coli* were isolated as described by Birnboim and Doly (1). With some modifications (16), the same method was used for minipreparations of plasmid DNA from *L. lactis*. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer (Mannheim, Federal Republic of Germany) and used as recommended by the supplier. General procedures for DNA manipulations and cloning were employed essentially as described by Maniatis et al. (10). Competent cells of *E. coli* were transformed by the method of Mandel and Higa (9). 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. (16) or by the liposome-enhanced transformation procedure of Van der Vossen et al. (17).

Bacteriocin assay. By using sterile toothpicks, *L. lactis* or *E. coli* transformants were transferred to glucose-M17 plates. After growth overnight, an indicator lawn of 3 ml of soft glucose-M17 agar (0.7%), containing 100 μl of an overnight culture of *L. lactis* Bu2-60, was poured on the surface. After 15 h of incubation the colonies were examined for zones of inhibition.

To confirm that a bacteriocinlike substance was produced,

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Reference
E. coli		
MH1	MC1061 derivative; *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	2
JM83	*ara (lac-proAB) rpsL* φ80 *lacZ*ΔM15	19
L. lactis subsp. *lactis*		
Bu2-60	Plasmid free	11
Bu2-61	Bac$^+$, Bu2-60 transconjugant containing plasmid p9B4-6	11
IL1403	Plasmid free	3
Plasmids		
p9B4-6	Bac$^+$, 60 kb	11
pGV1	Km^r, 4 kb	20
pGK11	Cm^r, 3.2 kb	8
pGKV210	Em^r, 4.4 kb	18
pGKV410	Em^r, 4.4 kb; *Bam*HI site of pGKV210 changed into a *Cla*I site	
pMG24	Km^r, 4.4 kb; pWVO1 (8) derivative containing kanamycin resistance gene from pJH1 and multiple cloning site of pUC18 (M. van de Guchte, personal communication)	

a hole in the agar was punched next to a colony transferred with a toothpick. The hole was filled with 10 μl of a solution containing either bovine serum albumin, proteinase K, or pronase (each at 20 mg/ml), after which the plate was incubated for 2 h at 30°C. An overlayer of indicator cells was applied, and after incubation for 15 h the plates were examined to judge whether the inhibitory substance was sensitive to proteolysis.

To see whether *E. coli* cells contained active bacteriocin, 12.5 ml of a late-exponential-phase culture was centrifuged, and the cells were suspended in 0.5 ml of glucose-M17 broth. The cell suspension was sonicated at 0°C (amplitude, 4 μm; three cycles of 20 s with intervals of 20 s), and 10-μl samples of the lysates were spotted onto glucose-M17 plates to check for inhibitory activity on a lawn of indicator cells.

Southern hybridization. DNA was transferred to Gene Screen Plus membranes as described by Maniatis et al. (10). Nonradioactive DNA probes were made with a random-primed labeling and detection kit (Boehringer). Hybridization and immunological detection were done as recommended by the supplier.

RESULTS

Subcloning of the bacteriocin plasmid p9B4-6. The 60-kb bacteriocin plasmid p9B4-6, was transferred by conjugation from *L. lactis* subsp. *cremoris* 9B4 to the plasmid-free strain *L. lactis* subsp. *lactis* Bu2-60 (11). The resulting transconjugant, strain Bu2-61, was used in this study as a source of the bacteriocin plasmid.

A restriction enzyme map of p9B4-6 was constructed by single and double digestions with several restriction endonucleases (Fig. 1). Various digests of p9B4-6 were ligated into the multiple cloning sites of the pWVO1 (8)-derived cloning vector pGKV210, pGV1, or pMG24. Plasmids carrying the pWVO1 origin of replication replicate in both *Lactococcus* species and *E. coli* (8). The ligation mixtures

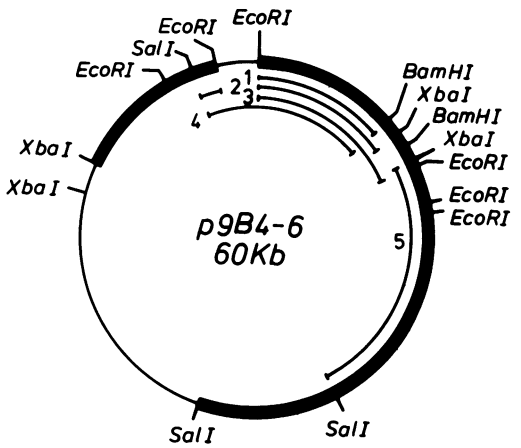

FIG. 1. Restriction enzyme map of the bacteriocin plasmid p9B4-6. The thick bars represent the parts of p9B4-6 cloned in *E. coli*. Only those parts associated with bacteriocin activity are further specified as follows: 1, 7.9-kb *Eco*RI-*Bam*HI fragment in pMB200; 2, 8.6-kb *Xba*I-*Eco*RI fragment and 1.5-kb *Eco*RI-*Sal*I fragment in pMB201; 3, 11-kb *Eco*RI fragment in pMB202; 4, 11.6-kb *Bam*HI-*Sal*I fragment in pMB203; 5, 15-kb *Sal*I-*Xba*I fragment in pMB500.

were used to transform competent *E. coli* MH1 or JM83 cells. *E. coli* transformants were screened for their plasmid content, and recombinant plasmids were analyzed with respect to their sizes and restriction enzyme patterns. In this way about 70% of plasmid p9B4-6 was subcloned in *E. coli* (Fig. 1). *E. coli* transformants were overlaid with indicator cells to screen for bacteriocin activity as described in Materials and Methods. However, no inhibition zones were detected. Also, in lysates prepared by sonication from the various *E. coli* clones no inhibitory activity was observed.

At least two bacteriocin determinants are present on p9B4-6. Plasmid DNA isolated from the various *E. coli* clones was transferred to *L. lactis* IL1403, and the transformants were screened for antagonistic activity. Transformants containing plasmid pMB200 (7.9-kb *Bam*HI-*Eco*RI fragment in pGKV210) or pMB201 (8.6-kb *Xba*I-*Eco*RI and 1.5-kb *Eco*RI-*Sal*I fragments in pMG24) (Fig. 1) inhibited the growth of the bacteriocin-sensitive strain Bu2-60 but did not inhibit the bacteriocin-producing strain Bu2-61. The zones of inhibition caused by these transformants were clearly smaller than those formed by Bu2-61, which contains the complete bacteriocin plasmid (Fig. 2; compare stabs 2 and 4). To examine whether this size difference was due to host-specific differences, pMB200 and pMB201 were transferred to *L. lactis* Bu2-60. However, the inhibitory activity in Bu2-60 harboring the recombinant plasmids was also significantly less than that of p9B4-6 in this strain (results not shown).

Pronase and proteinase K, but not bovine serum albumin, prevented inhibition of growth of the indicator strain Bu2-60 by the pMB200-specified product (Fig. 2, stabs 3 and 4), which confirms that the inhibitory substance is proteinaceous.

L. lactis IL1403 used as indicator strain showed the same low degree of sensitivity to the Bu2-60 transformants, unless the IL1403 indicator strain also contained pMB200 or pMB201. This indicates that an immunity gene(s) is also located on the cloned inserts. However, this immunity determinant did not give resistance against *L. lactis* Bu2-61: Bu2-61 formed large halos when *L. lactis* cells were used with pMB200 or pMB201 as the indicator (data not shown).

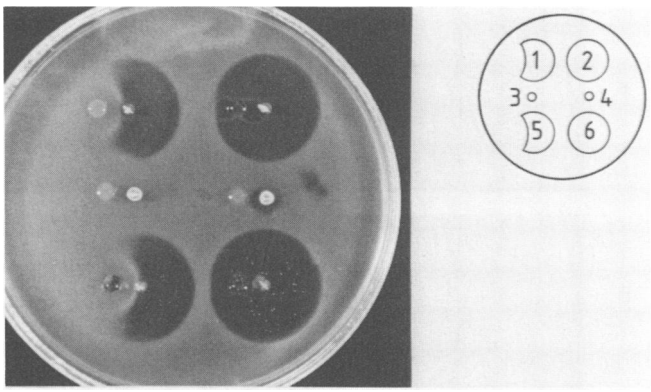

FIG. 2. Antagonistic activity by the various *L. lactis* strains and its sensitivity to proteolysis. Stabs: 1 and 2, *L. lactis* Bu2-61; 3 and 4, *L. lactis* IL1403(pMB200); 5 and 6, *L. lactis* IL1403(pMB500). In the wells next to 1, 3, and 5 pronase was applied; next to 2, 4, and 6 bovine serum albumin was added. *L. lactis* Bu2-60 was used as indicator strain. For details, see the text.

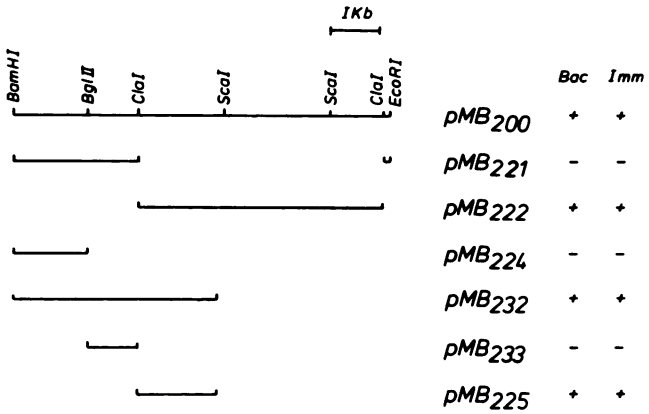

FIG. 3. Bacteriocin production and immunity associated with various fragments of pMB200. The restriction enzyme map of the 7.9-kb *Eco*RI-*Bam*HI fragment in pMB200 is shown. The lines represent the parts of the *Bam*HI-*Eco*RI fragment present in the various subclones. Bacteriocin production (Bac) and immunity (Imm) expressed by the different plasmids are indicated.

To see whether the reduction in halo size was caused by information lacking on the cloned fragments, e.g., a promotor and/or a ribosome-binding site, the adjacent regions of the 7.9-kb fragment were cloned (Fig. 1). The 11-kb *Eco*RI fragment of p9B4-6 was extracted from a gel and cloned into the *Eco*RI site of pGKV210 in *E. coli*. The recombinant plasmid, pMB202, was then transferred to *L. lactis* IL1403. Cloning of the 11.6-kb *Bam*HI-*Sal*I fragment in pGKV210 proved to be unsuccessful in *E. coli*. After transformation of *L. lactis* IL1403 with the ligation mixture, a transformant was obtained containing a plasmid, pMB203, with the expected size and restriction pattern. However, both pMB202 and pMB203 did not change the bacteriocin activity in *L. lactis*.

A second locus on the bacteriocin plasmid encoding bacteriocin activity was identified upon cloning of the 15-kb *Sal*I-*Xba*I fragment of p9B4-6. The fragment was inserted in the *Sal*I-*Xba*I site of pMG24, resulting in plasmid pMB500. *L. lactis* IL1403 containing this plasmid gave rise to a large halo, which was comparable to that formed by Bu2-61, when Bu2-60 was used as the indicator strain (Fig. 2, stabs 2 and 6). With *L. lactis* Bu2-61 as the indicator no halo was observed. The inhibitory substance produced by this clone was sensitive to proteolysis (Fig. 2, stab 5). Apparently, p9B4-6 carries at least two bacteriocin determinants, one with a low activity and one with a high activity when Bu2-60 is used as the indicator.

Deletion analysis of pMB200 and pMB500. Restriction enzyme analysis of pMB200 showed that the 7.9-kb *Bam*HI-*Eco*RI fragment contained two *Cla*I sites, one *Bgl*II site, and two *Sca*I sites. These sites were used for in vitro deletion experiments and for subcloning of the fragment in pWVO1 derivatives in *E. coli*. Bacteriocin activity was tested by transfer of the mutated derivatives to *L. lactis* (Fig. 3).

Plasmids pMB221, pMB224, and pMB233 were Bac^- and Imm^-, whereas plasmids pMB222 and pMB232 were Bac^+ and Imm^+. These results indicated that the bacteriocin gene(s) of pMB200 was located on a 1.8-kb *Sca*I-*Cla*I fragment. This fragment was cloned into the *Cla*I-*Sma*I site of pGKV410, giving plasmid pMB225. Transformants of *L. lactis* IL1403 containing pMB225 were Bac^+ and Imm^+.

In the course of this work we observed that *L. lactis* spontaneously acquired, with rather high frequency, a slight degree of resistance to Bu2-61 bacteriocin (unpublished data). To confirm that immunity was actually specified by pMB225 and to rule out the possibility that pMB225 transformants had been picked up containing a mutation that would render them sufficiently resistant to their own low bacteriocin activity, the following experiment was done. pGK11 (Cm^r) was introduced in *L. lactis* cells containing pMB225 (Em^r). By selective growth it was possible to eliminate pMB225 from the cells on the basis of plasmid incompatability, since both plasmids are pWVO1 derivatives (16). When the double transformant IL1403(pMB225) (pGK11) was grown in the presence of chloramphenicol and in the absence of erythromycin, the loss of pMB225 was accompanied by the loss of the Bac^+ Imm^+ phenotype. This implies that the 1.8-kb *Cla*I-*Sca*I fragment indeed determined both traits.

To examine whether the 15-kb fragment in pMB500, specifying strong bacteriocin production, showed homology with the 1.8-kb *Cla*I-*Sca*I fragment in pMB225, encoding the weaker activity, pMB500 was digested with *Hin*dIII. After agarose gel electrophoresis, the DNA was transferred to nitrocellulose filters. The blots were then used for hybridization with the labeled 1.8-kb *Cla*I-*Sca*I fragment. The 1.8-kb fragment strongly hybridized, even under stringent conditions, with a 4-kb *Hin*dIII fragment (Fig. 4, lane 2). This 4-kb *Hin*dIII fragment consisted entirely of p9B4-6 DNA. The 4-kb fragment was isolated from a gel and cloned into the unique *Hin*dIII site of pGKV210. *L. lactis* IL1403 carrying the resulting plasmid, pMB551 (Fig. 5), produced bacteriocin. The size of the halo on the indicator lawn was slightly reduced as compared with that of IL1403 containing pMB500. pMB551 was digested with either *Eco*RV or *Sca*I and with *Sma*I and self-ligated. The resulting plasmids, pMB552 and pMB553, respectively (Fig. 5), gave rise to a halo on Bu2-60, indicating that both the immunity and bacteriocin genes were located on a 1.3-kb *Sca*I-*Hin*dIII fragment. pMB552 and pMB553 were digested with *Hin*dIII and *Eco*RI (the *Eco*RV site in pMB552 and the *Sca*I site in pMB553 were lost as a consequence of the ligation, and therefore the *Eco*RI site of the multiple cloning site was used) and hybridized to the 1.8-kb *Cla*I-*Sca*I fragment. There was still hybridization with the cloned inserts (Fig. 4). Especially the observation that the 1.3-kb *Eco*RI-*Hin*dIII fragment of pMB553 hybridized with the 1.8-kb *Cla*I-*Sca*I

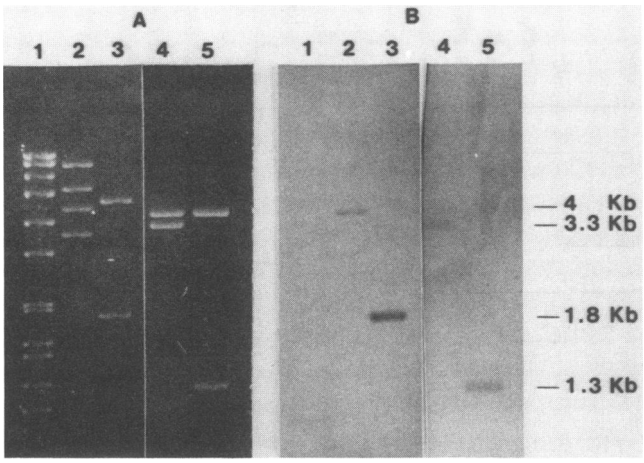

FIG. 4. Restriction enzyme and Southern hybridization analyses of the plasmids pMB225, pMB500, pMB552, and pMB553. (A) Agarose (0.8%) gel electrophoresis. Lanes: 1, molecular weight marker, phage SPP1 DNA digested with *Eco*RI; 2, pMB500 digested with *Hin*dIII; 3, pMB225 digested with *Eco*RI and *Cla*I (*Sca*I site was lost as a consequence of the cloning, and therefore the *Eco*RI site of the multiple cloning site was used); 4, pMB552 digested with *Eco*RI and *Hin*dIII; 5, pMB553 digested with *Eco*RI and *Hin*dIII. (B) Southern hybridization of the gel in panel A with the 1.8-kb *Sca*I-*Cla*I fragment derived from pMB200 as a probe.

fragment of pMB200 strongly suggests that the hybridization signal resulted from homology between the two different sets of bacteriocin and immunity genes.

Inhibition experiments showed that IL1403(pMB200) and IL1403(pMB551) were capable of mutual inhibition (Fig. 6). This result suggests that the specificities of the two bacteriocin activities were not identical.

DISCUSSION

From the results presented here it is clear that there are at least two regions on the bacteriocin plasmid p9B4-6 which contain information for antagonistic activity as well as immunity. The inhibitory substances produced by the clones are sensitive to proteolysis (Fig. 2). Apparently, we have succeeded in cloning the DNA fragments specifying the bacteriocin production in strain 9B4, originally described by Geis et al. (6). The results clearly indicate that plasmid p9B4-6 contains at least two bacteriocin genes. In contrast to the situation in *L. lactis*, inhibitory activity against sensitive *L. lactis* cells could not be detected in *E. coli* cells or lysates

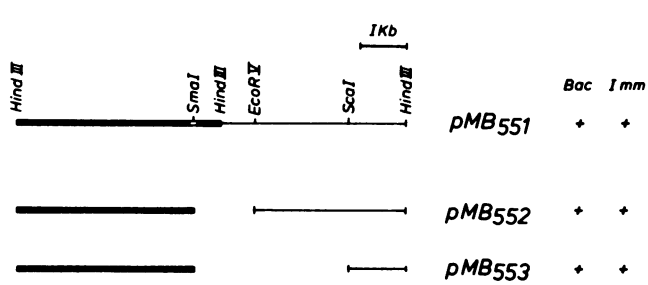

FIG. 5. Phenotype of deletion derivatives of pMB551. Abbreviations and symbols: Bac, bacteriocin production; Imm, immunity; ▬, pGKV210 vector DNA; □, multiple cloning site of pGKV210 of which only the *Sma*I site is shown; ——, p9B4-6 DNA.

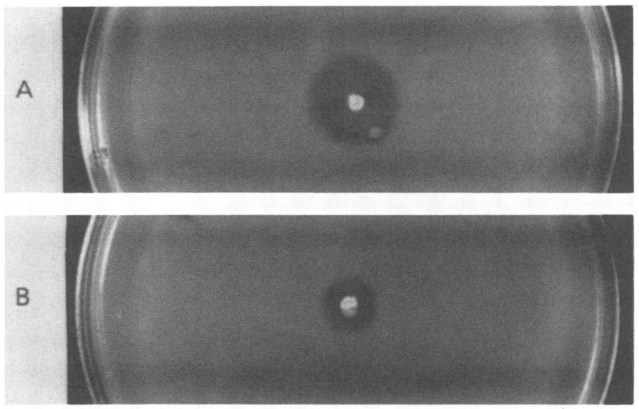

FIG. 6. Difference in specificity of the two bacteriocins. (A) Inhibition of growth of IL1403(pMB200) by IL1403(pMB551). (B) Inhibition of growth of IL1403(pMB551) by IL1403(pMB200).

of *E. coli* containing the various bacteriocin clones. This indicates that *E. coli* does not produce active bacteriocin at a detectable level.

Preliminary results suggest that the two bacteriocins are not exactly the same. First, the size of the halo produced on the Bu2-60 indicator by IL1403 carrying pMB500 was larger than that produced by IL1403(pMB200), although this difference may also be caused by a difference in promotor strength and/or plasmid copy number. The low activity of the bacteriocin gene(s) on the 7.9-kb *Eco*RI-*Bam*HI fragment could not be increased by cloning of the regions flanking this fragment. Second, the specificities of the two bacteriocins seemed to be different: a strain with pMB551 inhibited an indicator containing pMB200 and vice versa (Fig. 6). Since p9B4-6 contains both bacteriocin regions, both clones pMB200 and pMB551 (and also pMB500 which has a higher activity), are sensitive to Bu2-61 (data not shown). Subcloning of the 4-kb *Hin*dIII fragment from the 15-kb *Xba*I-*Sal*I fragment of pMB500 reduced the level of bacteriocin activity. Apparently, some information lacking on the 4-kb fragment is responsible for the high bacteriocin production.

Deletion analysis showed that the two genes encoding the low and high bacteriocin activity could further be localized to a 1.8-kb *Sca*I-*Cla*I fragment and a 1.3-kb *Sca*I-*Hin*dIII fragment, respectively, and that these fragments also conferred immunity to the bacteriocins. Although the bacteriocins seem to have different specificities, hybridization experiments showed a high degree of homology between the two bacteriocin gene regions. Neve et al. (11) observed homology between the bacteriocin plasmids of different lactococcal strains. The cloned bacteriocin genes may be quite useful as specific probes for the detection of bacteriocin genes in lactococcal strains, thus facilitating their cloning and comparison at the molecular level. A better insight into the structure and action of bacteriocins will be of help in the construction and improvement of bacteriocin-producing strains used in the food fermentation and preservation industry. In addition, the isolation of bacteriocin production and immunity genes may be used in a profitable way for the development of food-grade vectors which are selectively retained within a plasmid-bearing population.

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