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Bacteriolytic Activity Caused by the Presence of a Novel Lactococcal Plasmid Encoding Lactococcins A, B, and M

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***Lactococcus lactis* subsp. *lactis* biovar diacetylactis DPC938 was identified as a bacteriocin-producing strain which exhibited a bacteriolytic effect on other lactococci. Lysis of such target strains was associated with decreases in optical density and release of the intracellular enzyme lactate dehydrogenase. DPC938 exhibits cross-immunity to *L. lactis* subsp. *cremoris* 9B4 (M. J. van Belkum, B. J. Hayema, A. Geis, J. Kok, and G. Venema, Appl. Environ. Microbiol. 55:1187–1191, 1989), a strain which produces the bacteriocins lactococcins A, B, and M. Genetic analyses revealed that a 15.5-kb region of DNA encoding these bacteriocins is highly conserved in 9B4, DPC938, and DPC3286, an overproducing derivative of DPC938. This region is located on a 72- and a 78-kb nonmobilizable plasmid in DPC938 and DPC3286, respectively. The bacteriolytic effect exhibited by DPC938 and DPC3286 on sensitive cultures is most probably due to the concerted action of all three bacteriocins. Since these cultures exhibit a lytic effect on lactococci, they have a potential application in the dairy industry as accelerators of starter lysis and hence accelerators of cheese ripening.**

Bacteriocin production is a common phenomenon among members of the lactic acid bacteria and has been described for lactococci, pediococci, lactobacilli, and *Leuconostoc* spp. (7). Probably the best-known bacteriocin of lactic acid bacteria origin is nisin, which is produced by certain strains of *Lactococcus lactis*. Nisin exhibits a broad spectrum of inhibition and, in some respects, does not fit neatly within the classical definitions of a bacteriocin as described by Tagg et al. (28). Nisin and other bacteriocins that undergo unusual posttranslational modifications (resulting in a peptide chain containing lanthionine or methyllanthionine) are referred to as peptide antibiotics or lantibiotics. Other lantibiotics include lactocin S (20), lacticin 481 (24), and carnocin UI49 (26). A number of nonlantibiotic bacteriocins of lactic acid bacteria origin have been described in detail. In some instances, production and immunity genes have been shown to be plasmid linked. The nucleotide sequences are available for a number of bacteriocins, including lactacin F (21), helveticin J (16), leucocin A-UAL (13), sakacin A (14), pediocin PA-1 (17), and three lactococcal bacteriocins, lactococcins A, M, and B (32, 33).

The mechanism of action of some bacteriocins has been identified, but for many others only a general description of whether the effect is bacteriostatic or bactericidal has been reported. Van Belkum et al. (33) has reported that bacteriocin activity in *L. lactis* subsp. *cremoris* 9B4 is due to the action of at least three independent bacteriocins, lactococcins A, B, and M. The mechanism of action for the lactococcin A and B components has been reported (34, 35). The effect of lactococcin A or B on sensitive cells is bactericidal. Electron microscopy of cells treated with lactococcin A (34) and the lack of a decline in optical density of cells treated with lactococcin B (35) indicates that neither of the lactococcins is capable of causing cell lysis. Some strains of *Leuconostoc mesenteroides* were reported to lyse after the addition of pediocin AcH, an antibacterial substance produced by *Pediococcus acidilactici* H (4). To date, no lactococcal bacteriocins have been documented as having a bacteriolytic mode of action.

Unlike the broad-spectrum bacteriocins, which have applications in the food industry as inhibitors of spoilage and pathogenic organisms, applications for narrow-spectrum bacteriocins are not immediately obvious. A narrow-spectrum bacteriocin exhibiting a lytic effect on sensitive cells could have a role in the ripening of Cheddar cheese. Cheddar cheese has a long maturation time, of approximately 6 months, which enables the gradual autolysis of starter bacteria to occur. Starter lysis is thought to contribute significantly to flavor development, as a consequence of the release of intracellular enzymes into the cheese matrix. The liberated proteases and peptidases can hydrolyze casein to produce small peptides and amino acids which are important to the development of flavor (2, 18). Theoretically, the addition of a strain exhibiting a lytic effect on the starter culture could cause an increase in the release of intracellular enzymes and accelerate the subsequent reactions important in the development of Cheddar cheese flavor.

In this report, we describe the characterization of an inhibitory activity from *L. lactis* subsp. *lactis* biovar diacetylactis DPC938. We show that the inhibitory activity of DPC938 is due to the concerted action of at least three bacteriocins, lactococcins A, B, and M. The effect of the bacteriocins in concert on sensitive cells is bacteriolytic, as shown by a reduction in the viable count, a decrease in the optical density, and the release of intracellular enzymes from sensitive cells.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and culture conditions. The bacterial strains (apart from EC target strains) used in this study are listed in Table 1. *L. lactis* subsp. *lactis* biovar diacetylactis DPC938 was isolated from an Irish cheese factory. Lactococcal strains were routinely grown in M17 (Difco, Detroit, Mich.) containing 0.5% (wt/vol) glucose (GM17). A group of strains designated EC target strains was provided by B. Ten Brink, TNO, Utrecht, The Netherlands, K. Venema, University of Groningen, Groningen, The Netherlands, and P. J. Warner, Cranfield Institute of Technology, Cranfield, Bedford, United Kingdom. These strains are listed in Table 2. Solid medium was prepared by the addition of 1.5% agar. For purification of inhibitory activity, cultures were grown in TY broth, a modified version of TYT30 broth (23). The modification to TYT30 broth was the omission of Tween 80.

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TABLE 1. Bacterial strains used in this study

Strain	Remarks	Zone of inhibition (mm)[a]	Source or reference
L. lactis subsp. *lactis* biovar diacetylactis			
DPC938	Lactococcin D producer	0	DPC culture collection
DPC3286	Lactococcin D overproducer	0	This study
DPC3287, DPC3288, DPC3289	Cured derivatives of DPC938	29	This study
DPC3350	Cured derivative of DPC3286	29	This study
DPC2616	Sensitive indicator	38	This study
DPC220		18	12
DRC3	Plasmid size markers	27	19
L. lactis subsp. *lactis*			
DPC3351		29	DPC culture collection
C2		27	DPC culture collection
DPC990		19	DPC culture collection
IL1403	Plasmid-free strain	29	6
MG1614	Plasmid-free strain	27	9
Bu2-61	Lactococcin A, B, and M producer	0	31
IL1403/pMB225	Lactococcin M producer	33	32
IL1403/pMB553	Lactococcin A producer	28	32
IL1403/pMB580	Lactococcin B producer	32	32
L. lactis subsp. *cremoris*			
LMG2130	Lactococcin A producer	0	15
CNRZ117		37	B. Ten Brink, TNO
AM2	Cheese-making strain	36	DPC culture collection
HP	Cheese-making strain	31	DPC culture collection
KH	Cheese-making strain	30	DPC culture collection

[a] Diameter of the zone of inhibition produced by spotting 10 μl of DPC3286 culture on agar plates overlaid with the test culture.

A bacteriophage homologous for DPC938 was isolated from a phage cocktail containing a large number of phages isolated in the Irish dairy industry. Phage ϕ938 formed large, clear plaques on soft overlays (0.7% agar) seeded with strain DPC938. Bacteriophage typing of strains was carried out as described previously (29). All strains and phages are held in the DPC culture collection at Moorepark, Fermoy, Ireland.

TABLE 2. EC target strains used in this study

Strain
Lactobacillus acidophilus ATCC 4356
Lactobacillus bulgaricus ATCC 11842
Lactobacillus casei ATCC 334
Lactobacillus curvatus NCFB 2739
Lactobacillus fermentum ATCC 9338
Lactobacillus helveticus ATCC 15009
Lactobacillus plantarum NCDO 1193
Lactobacillus ruteri DSM 20016
Lactobacillus sake NCFB 2714
Lactobacillus salivarius NCFB 2747
Lactococcus lactis subsp. *cremoris* CNRZ117[a]
Pediococcus pentosaceus FBB63
Pediococcus pentosaceus PC1
Leuconostoc cremoris DB 1275
Streptococcus thermophilus ST 20
Streptococcus hermophilus ST112
Enterococcus faecalis EF 1
Staphylococcus carnosus MC 1
Bacillus cereus ATCC 9139
Listeria innocua BL 86/26
Clostridium sporogenes CS
Clostridium tyrobutyricum CT
Clostridium tyrobutyricum NCDO 1754
Propionibacterium sp. strain P-4
Propionibacterium sp. strain P-6
Propionibacterium acidipropionici NCDO 563
Bacillus subtilis OG1
Bacillus subtilis BD630

[a] This is the only strain which exhibited sensitivity to DPC938 and DPC3286.

Bacteriocin assay and activity determination. Bacteriocin production was initially detected by deferred antagonism (8). The producer strain was toothpicked or plated at low dilution onto the surface of a GM17 plate and allowed to form colonies overnight at 30°C. The colonies were exposed to chloroform vapor for 15 min and aerated for 30 min to remove traces of chloroform. The surface of the plate was subsequently flooded with 3 ml of 0.7% GM17 agar containing 100 μl of an overnight culture of the indicator organism. Bacteriocin production was detected by the formation of a clear zone of inhibition in the indicator lawn after further overnight incubation at 30°C. Bacteriocin activity was determined by the serial dilution method. Serial twofold dilutions of a bacteriocin solution were spotted (10 μl) on a plate which was subsequently flooded with the indicator organism. The bacteriocin activity was calculated as the inverse of the last dilution which gave a clear zone of inhibition after overnight incubation at 30°C. The activity units (AU) were expressed per milliliter (1/dilution × 100). When a clear inhibition zone was followed by a turbid zone, the critical dilution was taken to be the average of these dilutions. To compare the sensitivity of different indicator strains, activity was assayed by measuring the diameter of the zone of inhibition (in millimeters) that occurred upon overlaying 10 μl of an overnight culture with the appropriate indicator strain.

Measuring cell lysis. Cell lysis was measured by incubating MG1614 (mid-log phase of growth) in broth containing 800 AU of inhibitory activity from DPC3286 per ml. Samples (1 ml) were taken immediately before addition and at regular intervals thereafter. Each sample was measured spectrophotometrically at 600 nm, and lactate dehydrogenase (LDH) assays were performed to estimate the degree of cell lysis. LDH has been shown by Booth et al. (5) to be confined to the cell cytoplasm and hence to be an excellent indicator of cell integrity. LDH was assayed with sodium pyruvate as a substrate by the method described by Wittenberger and Angelo (36). The oxidation of NADH was measured by monitoring the A_{340} of the assay mixture in a Philips (PYE Unicam PU 8610) spectrophotometer. Activity is expressed as units per milliliter, where 1 U is the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per min per ml. To determine the total level of LDH present in the indicator cells, an excess of homologous phage was added to the strain and incubated until total lysis was observed.

Protein purification and electrophoresis. DPC3286 (Bac^+, Imm^+) and DPC3289 (Bac^-, Imm^-) were grown overnight in TY broth which had been previously filtered through Millipore type HA filters (to remove any proteins that bind to the filters). Supernatant from the overnight cultures was filtered through Millipore type HV filters (to remove cells). Cell-free supernatant of both cultures was then filtered through Millipore type HA filters (50 ml per filter). No activity could be detected in the filtrate, implying that the inhibitory activity of DPC3286 was retained by the HA filters. The bacteriocin activity retained by the HA filters

was recovered by dissolving the filters in acetone. The acetone was removed by evaporation under vacuum, and the protein was resuspended in 0.2 M phosphate buffer (pH 7.0) (referred to as acetone-derived protein). A total of 80% of the inhibitory activity was recovered.

The size of the inhibitory peptide was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Swank and Munkres (27) gels. Bacteriocin activity was detected by overlaying the gel with a sensitive indicator strain as described by Bhunia et al. (3). Gels were silver stained by the method of Giulian et al. (11).

Protein blotting and sequencing. The acetone-derived preparation (outlined above) was boiled for 5 min in sample buffer (27) and then loaded on a Sephadex G-50 column and eluted with 0.2 M phosphate buffer–0.1% SDS (pH 7.0). The most active fractions were pooled and run on a 13.8% polyacrylamide gel. This gel was subsequently electroblotted onto an Immobilon-P transfer membrane (Millipore) with a Sartoblot 11 Electro-Blotter at 150 mA for 5 min. The Immobilon-P transfer membrane was stained with Coomassie blue stain. The protein band which corresponded to bacteriocin activity was sequenced with an Applied Biosystems 477A protein sequencer.

DNA manipulations. Plasmid DNA was isolated from lactococcal strains by the method of Anderson and McKay (1). Plasmids were separated on 0.65% vertical agarose gels and sized with the plasmids of known molecular weight present in *L. lactis* subsp. *lactis* DRC3 (19).

Filter matings were performed as outlined by Neve et al. (22) on GM17 plates. The donor strains were washed to remove bacteriocin activity and treated with proteinase K to inactivate any bacteriocin that might be produced during incubation at 30°C overnight. Transconjugants were selected on GM17 agar with bacteriocin supernatant and streptomycin.

Mobilization of plasmids was attempted with pAMβ1, a plasmid which is suitable for conjugal mobility of lactococcal plasmids (10). pAMβ1 (Ery^r) was transferred into DPC938 (Bac^+) and then from DPC938 to IL1403 (Str^r). Transconjugants were selected on GM17 agar with bacteriocin (800 AU/ml), erythromycin (50 μg/ml), and streptomycin (500 μg/ml).

PCR amplification. Oligonucleotide primers for PCR were synthesized on an Applied Biosystems DNA synthesizer. The primer sequences were based on the known sequence of lactococcins A, B, and M (32, 33). The reaction mixture of 50 μl consisted of 34 μl of Tris-EDTA (TE) buffer, 5 μl of 10× Taq buffer, 2 μl of each deoxynucleoside triphosphate (A, T, C, and G each at 1.25 mM), 1 μl of template DNA (10 ng/μl), 1 μl of primer DNA (1 μM), and 0.5 μl of *Taq* DNA polymerase. Each reaction mixture was covered with mineral oil. Amplification reactions were performed in a Perkin-Elmer thermal cycler with the following cycle times: denaturation at 92°C for 1 min, primer annealing at 37°C for 1 min, and primer extension at 72°C for 1 min. Thirty cycles were carried out. The resulting amplified DNA was electrophoresed on a 1.5% agarose gel and analyzed after ethidium bromide staining. The sequences of the primers used are as follows. The primer 5′-TAAAAATAAGGAGATTATTATG-3′ was based on the common 5′ region upstream of the three operons and was used in all reactions. Three primers specific for lactococcins A, B, and M were 5′-TTAGA ATAGTCGTCCCCACG-3′ (lactococcin A primer), 5′-TATCAAACCTACC ATCCAGG-3′ (lactococcin B primer), and 5′-ACTACTGTCATGAATATAC C-3′ (lactococcin C primer).

Hybridizations. The PCR products were run on a 1.5% agarose gel, extracted, and purified with a GeneClean kit (Bio 101, La Jolla, Calif.). Approximately 50 ng of this DNA was used to probe plasmid DNA isolated from DPC938, DPC3286, and 9B4. Hybridizations were performed with the enhanced chemiluminescence random-prime labelling and detection system (Amersham) and Hybond N membranes (Amersham).

RESULTS

Inhibitory activity of DPC938. By using the deferred-antagonism test, it was shown that DPC938 inhibits the growth of the plasmid-free indicator strain *L. lactis* subsp. *lactis* MG1614. Clear zones of inhibition were observed surrounding colonies of the producer strain. In addition, the cell-free supernatant of an overnight culture of DPC938 was shown to inhibit the growth of MG1614. Care had to be taken in selecting the filter membranes used to filter sterilize the active supernatant. No activity was detected in the filtrates when Millipore GS 0.22-μm, HA 0.45-μm, or Millex PF 0.8-μm (Millipore Ireland, Cork, Ireland) filters were used, presumably because of the binding of the bacteriocin. Large zones were detected when the used filters were placed on a plate and overlaid with a sensitive strain, indicating that the active fraction had been retained by the filter. The inhibitor could be recovered after dissolving the spent filters with acetone. However, other filters, including Millipore HV 0.45-μm, Millex HV 0.45-μm, and Gelman low-protein-binding 0.2-μm (Gelman Sciences, Dublin, Ireland) filters did not prevent the passage of the active fraction, and these filters were subsequently used in further experiments, except where otherwise indicated.

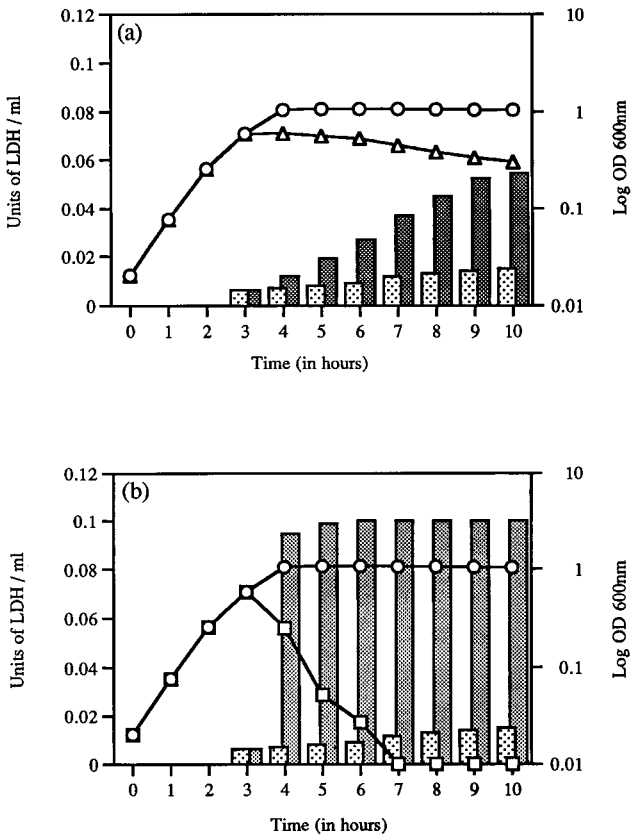

FIG. 1. Effect of the inhibitory activity produced by DPC3286 (a) and bacteriophage on MG1614 (b), decline in OD_{600}, and release of LDH.

The host range of the inhibitory substance was determined with a number of strains from the National Dairy Products Research Centre culture collection. All lactococcal strains with the exception of LMG2130 and Bu2-61 proved sensitive to the DPC938 supernatant (Table 1). By using the size of the inhibitory zone as an indicator of sensitivity, it was determined that the most sensitive lactococcal strain was DPC2616 (Table 1). With the exception of *L. lactis* subsp. *cremoris* CNRZ117, no sensitivity was detected among the group of strains designated as EC target strains (Table 2). In all subsequent experiments, either MG1614 or IL1403 was used as the indicator. These strains were chosen because of their intermediate sensitivity to the inhibitor and their common use among laboratories engaged in lactococcal research.

Bactericidal action. The viability of MG1614 after exposure to 800 AU of inhibitory activity per ml was assessed by plate counts. A log-phase culture of MG1614 contained a total of 1×10^8 CFU/ml. The posttreatment count after 10 min was estimated at 2×10^3 CFU/ml, indicating greater than 99.98% killing. Further reductions in cell counts were observed after increasing time intervals, with only 53 CFU/ml remaining after 120 min of exposure.

Bacteriolytic action. The lytic effect of addition of 800 AU of inhibitory activity per ml on the sensitive indicator strain MG1614 was measured in two different ways. First, the inhibitor caused a decrease in optical density (OD) over time (Fig. 1). After 24 h, the sample had almost completely cleared and

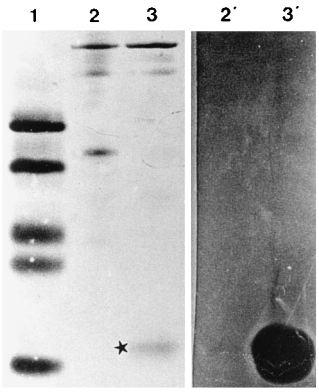

FIG. 2. SDS-PAGE of acetone-derived protein from DPC3289 (Bac$^-$) and DPC3286 (Bac$^+$), silver stained in lanes 2 and 3, and overlaid with MG1614 in lanes 2′ and 3′. The star indicates the position of a 3.16-kDa peptide found only in lane 3, which comigrates with activity in lane 3′. Lane 1 contains myoglobin molecular mass standards: 17.201, 14.632, 8.235, 6.383, and 2.556 kDa.

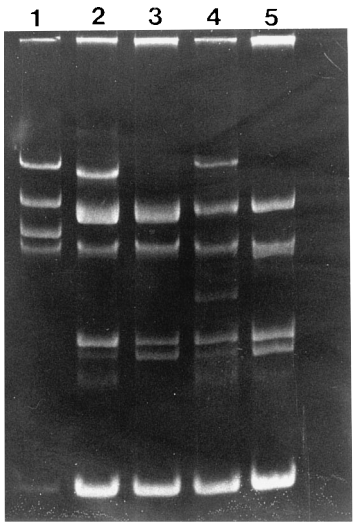

FIG. 3. Agarose gel electrophoresis of DNA from lactococcal strains. Lanes: 1, molecular size markers from DRC3 (78, 50, and 40 kb) and chromosomal DNA (8.2 kb); 2, DPC938 (Bac$^+$); 3, DPC3289 (Bac$^-$ derivative of DPC938); 4, DPC3286 (Bac$^+$); 5, DPC3350 (Bac$^-$ derivative of DPC3286).

the OD was reduced to 0.07. This decrease in OD suggested that cell lysis had occurred. This was confirmed by assaying for the intracellular marker enzyme LDH as a second indicator of cell lysis. A marked increase in the LDH level was observed after treatment with the inhibitor (Fig. 1). The control culture continued to grow normally until stationary phase was reached, and little LDH activity was detected, indicating an essentially unlysed cell population and confirming the effectiveness of LDH as a marker enzyme of cell lysis of this strain. To estimate the total possible release of LDH in a completely lysed population, an excess of MG1614 homologous phage (ϕc2) was added to cells at the same time point. The OD decreased rapidly, indicating complete lysis of the cells (Fig. 1). The LDH activity increased rapidly, mirroring the fall in OD. On the basis of the LDH levels released after the addition of ϕc2, we estimate that 57% of the total enzyme is liberated by the action of the bacteriocins (at a level of 800 AU/ml^{-1}) within 10 h. The total LDH released was stable over the time course of the assay but was unstable on overnight incubation, and so a final level of LDH release from the bacteriocin-treated cells could not be determined. However, on the basis of an OD of 0.07 after overnight incubation, it appears that lysis is virtually complete.

Partial purification and size estimation. Size estimations were performed by three SDS-PAGE determinations to give an average size of 3.16 kDa. Interestingly, a protein band of 15.8 kDa appears in the DPC3289 (Bac$^-$) lane which is not present in the DPC3286 (Bac$^+$) lane; we have no explanation for the origin of this band (Fig. 2).

To determine the sequence of the protein band corresponding to bacteriocin activity, a gel containing acetone-derived protein which had been passed through a Sephadex G-50 column was blotted onto an Immobilon-P membrane. Sequencing of the blotted protein band revealed the first 10 N-terminal amino acids. This sequence is identical to the first 10 amino acids (aa) of the lactococcin A peptide (32). The N-terminal amino acid sequence determined for lactococcin A is shown below (with the known sequence from 9B4 shown on top and the peptide sequence from DPC938 below):

Lys-Leu-Thr-Phe-Ile-Gln-Ser-Thr-Ala-Ala-Gly-Asp-42 aa
NH-Lys-Leu-Thr-Phe-Ile-Gln-Ser-Thr-Ala-Ala-.....

Production and immunity are plasmid encoded. DNA analysis of DPC938 demonstrated the presence of five individual plasmids ranging in size from 8.2 to 72 kb (Fig. 3). No attempt was made to distinguish between different plasmid forms, and each band was sized as if it were a closed circular plasmid, with the plasmids of *L. lactis* subsp. *lactis* DRC3 as size standards (19). Spontaneous nonproducing (Bac$^-$) colonies were never detected ($<10^{-4}$), and growth at 37°C or growth and incubation in unbuffered medium did not cause loss of the production (Bac$^+$) phenotype. Finally, DPC938 was grown in the presence of acridine orange (20 μg/ml) to induce plasmid loss. Three Bac$^-$ derivatives were isolated in three separate experiments. All three strains (designated DPC3287, DPC3288, and DPC3289) were sensitive to the parental phage ϕ938. Plasmid profiles revealed that all were missing a single large plasmid of 72 kb, designated pSM72 (Fig. 3). Nonproducing strains were sensitive to the bacteriocins produced by DPC938 (Table 1), suggesting that loss of pSM72 causes concomitant loss of both production and immunity phenotypes.

A spontaneous derivative of DPC938, designated DPC3286, was observed to overproduce one or all of the bacteriocins. On agar plates, the zones of inhibition around DPC3286 were notably larger than those around DPC938 (data not shown). Plasmid analysis of this derivative revealed that pSM72, the plasmid encoding production and immunity, was replaced by a larger plasmid in DPC3286 (Fig. 3). This plasmid was sized at 78 kb and was designated pSM78. Treatment of DPC3286 with acridine orange (20 μg/ml) to induce plasmid loss resulted in loss of pSM78 and concomitant loss of the bacteriocin production and resistance phenotypes. The Bac$^-$ derivative of DPC3286 was designated DPC3350. Production of the bacteriocins by DPC3286 was detectable at an earlier point in the growth cycle than was production by DPC938, and overnight cultures in broth produced 1,600 AU/ml, significantly more than that produced by DPC938 (800 AU/ml), against the indicator MG1614.

pSM72 and pSM78 contain the lactococcin A, B, and M genes. PCR amplification revealed that the genes encoding lactococcins A, B, and M are present in both DPC938 and DPC3286 (Fig. 4). One primer was based on the common upstream region present in all three operons. Specific primers were used for the downstream region of the three lactococcin genes. The positions of the primers on p9B4-6 (the 60-kb

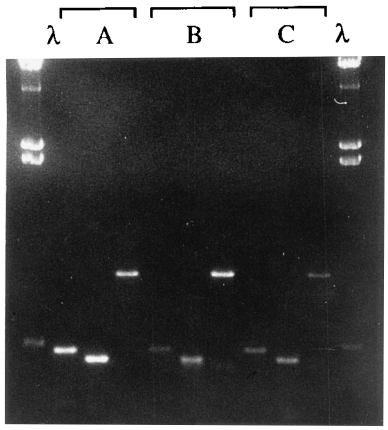

FIG. 4. Amplified DNA from Bu2-61 (lane A), DPC938 (lane B), and DPC3286 (lane C), with primers directed against lactococcins A, B, and M. Lanes marked λ contain λ *Hin*dIII-digested DNA and show molecular size markers of 6.55, 4.36, 2.32, 2.07, and 0.564 kb.

plasmid from *L. lactis* subsp. *cremoris* 9B4, which encodes lactococcin M, A, and B production) are shown in Fig. 5. The PCR products expected for lactococcins A, B, and M are approximately 0.55, 0.5, and 0.9 kb, respectively. Fragments of the correct size were amplified from the plasmids of strain 9B4, confirming the specificity of the primers used. Fragments of identical size were amplified from plasmid preparations from both DPC938 and DPC3286, confirming that all three operons are present in both strains.

The probes generated from the PCRs were used in hybridization experiments against restriction enzyme-digested plasmid DNA from 9B4, DPC938, and DPC3286. The results confirm that a similar genetic organization is shared by p9B4-6, pSM72, and pSM78 (Fig. 5). The only detectable difference was the absence of a *Bsp*HI site in pSM72 and pSM78.

Comparison of DPC938 and 9B4. In DPC938, the plasmid encoding bacteriocin production is 72 kb, whereas a 60-kb plasmid encodes bacteriocin production in 9B4. Mating experiments between DPC938 and IL1403 revealed that the plasmid encoding bacteriocin production is nonconjugative. Control experiments with Bu2-61, the strain containing p9B4-6, from *L. lactis* subsp. *cremoris* 9B4 (Table 1), were successful. In a further attempt to mobilize pSM72 into a plasmid-free background, pAMβ1 was inserted into DPC938 and then transferred out, but pSM72 was not comobilized.

Since the bacteriocin gene cluster appears to be highly conserved in DPC938 and 9B4, we investigated the lytic ability of 9B4. A similar experimental approach to that outlined above revealed that 9B4 also caused a decline in the optical density of sensitive lactococcal cultures, with associated LDH release. In parallel studies, lactococcal clones containing either lactococcin M (IL1403/pMB225), lactococcin A (IL1403/pMB553), or lactococcin B (IL1403/pMB580) genes alone failed to induce lysis of target strains. This suggests that the observed bacteriolytic effect necessitates the concerted action of at least two of the bacteriocins.

DISCUSSION

L. lactis subsp. *lactis* biovar diacetylactis DPC938 was found to produce an inhibitory substance. The activity of this substance was shown to comply with the criteria outlined by Tagg et al. (28) for definition as a classical bacteriocin in that it has a narrow spectrum of inhibition, it is proteinaceous, and both production and immunity are plasmid encoded.

The method for partial protein purification outlined in this paper has not, to our knowledge, been reported previously. It is a rapid and simple method, whereby the inhibitory activity is bound to a membrane, the membrane is then dissolved, and the inhibitory activity is resuspended in buffer. By this method, 80% of the activity can be recovered. In the procedure outlined above, acetone was used to dissolve the membranes, but in other experiments the inhibitory activity was removed from the membrane by washing in 60% ethanol. If the growth medium is first filtered through the membranes to which the inhibitory activity binds, any contaminating proteins from the medium which bind will be removed before inoculating.

Initially, it was thought that the inhibitory activity of DPC938 was due to the action of a single bacteriocin, but through a combination of cross-immunity and PCR approaches, it became apparent that the observed activity is probably due to the combination of at least three different bacteriocins, namely, lactococcins A, B, and M. Plasmid p9B4-6, originally described by van Belkum et al. (31), also encodes all three bacteriocin production and resistance operons (lactococcins M, A, and B). The inhibitory activities of strain Bu2-61 and DPC938 are similar, since we have shown that strain Bu2-61 is immune to DPC938 supernatant and vice versa (data not shown). It is probable that pSM72 and p9B4-6 are related, although they are not identical, since p9B4-6 is a 60-kb conjugative plasmid and pSM72 is a 72-kb nonmobilizable plasmid (and pSM72 could not be mobilized with pAMβ1). The size

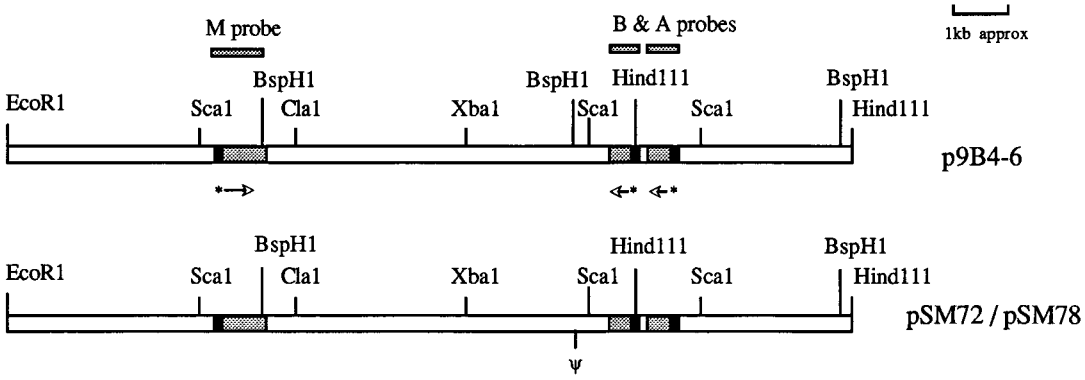

FIG. 5. Map of a 15.5-kb fragment of p9B4-6 and pSM72/pSM78. The amplified fragments of lactococcins A, B, and M are indicated above the map. *, common 5′ primer; ψ, possible loss of a *Bsp*HI site.

difference between the two plasmids was confirmed by plasmid analysis. Although plasmids pSM72 and p9B4-6 differ in relation to their size and their conjugative ability, the region encoding the lactococcin M, A, and B genes appears to be highly conserved on the basis of hybridization and restriction analysis. The restriction map constructed for that region shows that the fragments of DNA encoding lactococcin M, A, and B production on pSM72 and p9B4-6 are virtually identical. Holo et al. (15) have described a 55-kb conjugal plasmid in *L. lactis* subsp. *cremoris* LMG2130 which encodes lactococcin A. Nucleotide analysis showed that the lactococcin A operon from LMG2130 is followed by the lactococcin B operon, and inhibition experiments indicate that the genetic determinants for lactococcin M are also present (30). LMG2130 and DPC938 are also mutually resistant. Stoddard et al. (25) reported that *L. lactis* subsp. *lactis* biovar diacetylactis WM4 contains a 131.1-kb plasmid (pNP2) which encodes the genes for lactococcin A production and immunity. Comparison of the restriction map downstream of the lactococcin A operon with that for p9B4-6 indicates that this operon is also followed by the lactococcin B operon (30). It has yet to be revealed whether WM4 also contains the genes encoding lactococcin M production. By PCR, it was revealed that not only did DPC938 contain the lactococcin M, A, and B genes, but the overproducing derivative DPC3286 did also.

DPC938 exhibits a bactericidal effect against the sensitive indicator strain MG1614, with at least 99.98% of cells committed to death within 10 min in the presence of inhibitory activity at a concentration of 800 AU/ml. The mechanism of action is not understood, but the activity leads to a significant disruption of cell integrity. The amount of LDH (140 kDa) released within 10 h of addition of the bacteriocin is 57% of the total amount within the culture as estimated by lysis with an excess of phage. However, the cell death results indicate that >99.98% of cells are nonviable within 10 min. Some structural integrity may be retained by the affected cells, and this may account for the slower decrease in OD and the smaller increase in LDH concentration than would be expected from the viable cell counts. In this instance, the bacteriocin action would not directly cause lysis per se but would trigger a series of events leading to the eventual lysis of the affected cell. The use of LDH as an indicator of cell lysis was based on the observation that 98% of the enzyme is located within the cell (5). This was supported by the results obtained in this study, showing that little LDH was released from control cells whereas phage-lysed cells released LDH at the point of lysis. The observed lytic activity is probably due to the combination of at least three different bacteriocins, namely, lactococcins M, A, and B. Cell-free supernatants from strains 9B4 and Bu2-61 (which encode the three lactococcal bacteriocins) are capable of lysing sensitive cells, but supernatants from clones IL1403/pMB225 (encoding lactococcin M), IL1403/pMB553 (encoding lactococcin A), and IL1403/pMB580 (encoding lactococcin B) give no lysis. The mechanism of action of lactococcins A and B has been determined. The lactococcin A component acts by depolarizing the cell membrane of sensitive cells, leading to an efflux of low-molecular-weight compounds (34). The lactococcin B component acts by dissipating the proton motive force of sensitive cells, causing leakage of intracellular substrates (35). As yet, no information on the mechanism of action of the lactococcin M component is available. Even though the clones producing lactococcin A, B, or M are incapable of causing lysis of sensitive cells, it may be that their concerted action, as expressed in 9B4, Bu2-61, DPC938, and DPC3286, imposes such a degree of membrane damage that sensitive cells eventually lyse.

A spontaneous derivative of pSM72 which overproduces inhibitory activity was isolated. Production is detected earlier and reaches higher levels in this derivative than in the parental strain. Plasmid profile analysis revealed an increase of approximately 6 kb from pSM72 to pSM78. It is unclear whether the increase in plasmid size is involved in the observed overproduction. If an insertion event has caused overproduction, it must have occurred well upstream of either the lactococcin M or A gene, since analysis of hybridization results indicates a high degree of genetic conservation between 9B4 and DPC3286 in the region spanning the lactococcin A, B, and M genes. Overproduction by DPC3286 may be the result of a point mutation unconnected with the increase in plasmid size, leading to an altered inhibitory activity with a more potent effect on MG1614 and other sensitive strains. Construction of plasmid maps of pSM72 and pSM78 has been complicated by the observation that the plasmids are nonconjugative, and attempts to remove companion plasmids were unsuccessful.

It is interesting that both DPC938 and DPC3286 have a lytic effect on sensitive strains, causing the release of large intracellular enzymes. This phenomenon may have an application in developing strategies to accelerate the lysis of cheese starter cultures. In Cheddar cheese manufacture, the release of intracellular enzymes as a result of lysis is normally a slow process and is believed to contribute to the long ripening times. When used in combination with sensitive cheese-making strains, the bacteriolytic effect of DPC938 or DPC3286 could be exploited to cause the premature lysis of the starters, thereby increasing the release of intracellular enzymes and speeding the formation of small peptides and amino acids important to the development of Cheddar cheese flavor. The host range of the inhibitory activity is also particularly suitable in this regard, since all of those strains routinely used in cheese making in Ireland have been found to be sensitive. Furthermore, all those tested for release of intracellular enzymes, including strains HP and AM2, were shown to undergo lysis in the presence of the bacteriocins. In addition, nonstarter lactic acid bacteria, which are also thought to play an important role in Cheddar cheese ripening, are unaffected by all three lactococcins encoded by pSM72.

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