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An Application in Cheddar Cheese Manufacture for a Strain of *Lactococcus lactis* Producing a Novel Broad-Spectrum Bacteriocin, Lacticin 3147

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***Lactococcus lactis* DPC3147, a strain isolated from an Irish kefir grain, produces a bacteriocin with a broad spectrum of inhibition. The bacteriocin produced is heat stable, particularly at a low pH, and inhibits nisin-producing (Nip$^+$) lactococci. On the basis of the observation that the nisin structural gene (*nisA*) does not hybridize to DPC3147 genomic DNA, the bacteriocin produced was considered novel and designated lacticin 3147. The genetic determinants which encode lacticin 3147 are contained on a 63-kb plasmid, which was conjugally mobilized to a commercial cheese starter, *L. lactis* subsp. *cremoris* DPC4268. The resultant transconjugant, DPC4275, both produces and is immune to lacticin 3147. The ability of lacticin 3147-producing lactococci to perform as cheddar cheese starters was subsequently investigated in cheesemaking trials. Bacteriocin-producing starters (which included the transconjugant strain DPC4275) produced acid at rates similar to those of commercial strains. The level of lacticin 3147 produced in cheese remained constant over 6 months of ripening and correlated with a significant reduction in the levels of nonstarter lactic acid bacteria. Such results suggest that these starters provide a means of controlling developing microflora in ripened fermented products.**

Many lactic acid bacteria (LAB) produce bacteriocins inhibitory to other, usually related, bacteria (16). The aim of using these proteinaceous inhibitors to improve the microbial quality and safety of food has stimulated intensive research efforts in recent years (6, 14, 16). The best characterized inhibitor to date is nisin, a bacteriocin produced by certain strains of *Lactococcus lactis* which is active against a wide range of gram-positive bacteria. Nisin is effective against gram-negative bacteria only if used in combination with chelating agents (33) and has not been shown to inhibit fungi. It is a permitted preservative in 48 countries, in which it is used in a variety of products, including processed cheese, canned foods, and cured meats (5). Since lactococci are the principal starters in a variety of fermented products, some investigators have attempted to use nisin and/or nisin-producing (Nip$^+$) strains to improve product quality. The incorporation of bacteriocin-producing lactococci in foods provides a very attractive alternative to the addition of purified bacteriocin, which would be considered a food additive. A major disadvantage associated with Nip$^+$ lactococci, however, is their reported poor performance as cheese starters. They generally produce acid at slower rates and have lower heat resistance and reduced proteolytic activity compared with those of commercial starters (18, 19). In addition, Nip$^+$ lactococci have been reported to be more sensitive to bacteriophage attack than are strains which do not produce the antibiotic (19). For this reason, Nip$^+$ lactococci have been combined with nisin-resistant starters to ensure adequate performance of the cheese starter. This system is effective only if the resistance mechanism does not result in nisin inactivation (32). Such an approach has been successful in controlling butyric swelling in Edam- and Gouda-type cheeses (18).

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Recently, a Nip$^+$ starter culture system suitable for the manufacture of cheddar cheese was described (32). This involved the use of two Nip$^+$, lactose-positive, proteinase-positive lactococci, one of which is a transconjugant strain. Cheddar cheese manufactured with this starter combination contained approximately 700 IU of nisin per g. A similar level of nisin was observed in Camembert cheese manufactured with a Nip$^+$ starter by Maisnier-Patin et al. (20). The production of nisin in this cheese was sufficient to cause a 3-log reduction in *Listeria* numbers. However, the starter used in this case did have a markedly slower acidification rate than those of Nip$^-$ strains used in control cheeses.

Because of the problems associated with using Nip$^+$ strains, the potential of a number of alternative bacteriocinogenic cultures has been assessed in several food systems. For example, *Pediococcus* species which produce pediocin PA-1 and pediocin PAC 1.0 were found to have antilisterial activity in fresh beef (26) and fermented sausage (8), respectively. Separate studies have reported inhibitory activities for *Pediococcus acidilactici* JD1-23 against *Listeria monocytogenes* in fermented semidry sausage (3) and fully cooked frankfurters (2). There remains considerable potential for a *Lactococcus* strain which produces a broad-host-range bacteriocin but is suitable for inclusion in a starter system.

In a previous study, a number of lactococci exhibiting antimicrobial activities were isolated from kefir grains, traditionally used in Ireland to make buttermilk (30). The inhibitor produced by one of these strains exhibits a broad spectrum of inhibition, similar to that of nisin. In contrast to most Nip$^+$ strains, the *Lactococcus* strain which produces this novel bacteriocin, designated lacticin 3147, can acidify milk at sufficient rates to allow commercial manufacture of cheddar cheese. Significantly, lacticin 3147 production and immunity are plasmid-encoded traits and can be conjugally transferred to commercial starters. Cheeses made with lacticin 3147-producing starters exhibited significantly lower levels of nonstarter LAB

(NSLAB) during a 6-month ripening period. Since lacticin 3147 is also an effective inhibitor of many gram-positive food pathogens and spoilage microorganisms, these starters may provide a very useful means of controlling the proliferation of undesirable microorganisms during cheese manufacture.

MATERIALS AND METHODS

Bacterial strains, media, and cultivation conditions. The strains, growth media, and temperatures of incubation used in this study are listed in Table 1. Both *L. lactis* DPC3147 and DPC3220 were isolated from kefir grains, whereas Nip^+ *L. lactis* NCDO496 and NCDO497 were obtained from the National Collection of Dairy Organisms (NCDO), Agricultural and Food Research Council, Institute of Food Research, Reading Laboratory, Reading, Berkshire, United Kingdom. The source of each indicator organism tested is also listed in Table 1. *L. lactis* cells were routinely propagated at 30°C in M17 (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% glucose (GM17) or lactose (LM17). As indicated in Table 1, other media used in this study included MRS (Difco Laboratories), BHI (Oxoid Ltd., Basingstoke, Hampshire, England), TYP (tryptone at 16 g/liter, yeast extract at 16 g/liter, NaCl at 2.5 g/liter, K_2HPO_4 at 2.5 g/liter), TYT30 (27), TSA (Becton Dickinson Microbiology Systems, Cockeysville, Md.), UBA (Difco Laboratories), Baird-Parker, (Merck, Darmstadt, Germany), LBS (Becton Dickinson Microbiology Systems), VRBA (Oxoid Ltd.), KAA (Oxoid Ltd.), 10% reconstituted skim milk (RSM), and pasteurized whole milk.

To identify lactose-metabolizing bacteria, lactose indicator agar was used (22). Sucrose indicator agar plates were prepared in a similar fashion, except that sucrose was substituted for lactose as the carbon source.

Bacteriocin sensitivity assays. To test the sensitivity of a strain to a bacteriocin, 10-μl aliquots of a fresh overnight culture of each producer to be tested were first spotted on GM17 agar plates and incubated overnight at 30°C. These plates were then overlaid with 3 ml of soft agar seeded with 100 μl of the indicator strain (approximately 10^7 stationary-phase cells). The sensitivity of a strain to each bacteriocin producer was scored according to the diameter of the zone of inhibition surrounding that producer. All strains were stocked in 40% glycerol and stored at −80°C. Working cultures were stored at 5°C and transferred periodically.

Selective plates incorporating bacteriocin were prepared as follows. An overnight culture of *L. lactis* DPC3147 (grown in GM17) was centrifuged at 8,000 × *g* for 10 min, and then the resulting supernatant was sterilized by filtering with Millipore HVLP filters (pore size, 0.45 μm) and tempered to 45°C. Then an equal volume was added to double-strength GM17 held at 45°C, and the plates were poured. When required, streptomycin was added to agar at a concentration of 500 μg/ml.

Bacteriocin assay. Bacteriocin activity was estimated by an agar well diffusion assay, essentially as described by Parente and Hill (27), as follows. Molten agar (48°C) was first seeded with the indicator organism (200 μl of overnight culture per 25 ml of agar, i.e., $\sim 2 \times 10^7$ cells). The inoculated medium was rapidly dispensed in sterile petri dishes and, after solidification, dried for 30 min under a laminar flow hood. Wells of uniform diameter (4.6 mm) were bored in the agar and sealed with 15 μl of tempered soft agar. Aliquots (50 μl) of the bacteriocin solution were dispensed in wells, and plates were incubated overnight at 30°C. Bacteriocin activity was expressed as the area of inhibition surrounding each agar well. Alternatively, serial dilutions of this bacteriocin were prepared and dispensed in wells, and bacteriocin activity was expressed as arbitrary units (AU). To obtain the AU per milliliter, the reciprocal of the highest dilution which gave a definite zone was multiplied by the conversion factor (20 when 50 μl was used). In each case, the AU per milliliter were calculated by using *L. lactis* subsp. *cremoris* HP as the indicator organism, except for the identification of the bacteriocin in cheddar cheese, when the more sensitive strain *L. lactis* subsp. *cremoris* AM2 was used.

Protease sensitivity assay. The following enzymes were dissolved in sterile distilled water to final concentrations of 50 mg/ml: trypsin (type II; Sigma Chemical Co., Poole, Dorset, England), α-chymotrypsin (type II; Sigma), proteinase K (Sigma), pronase E (type XIV; Sigma), and catalase (Sigma). Pepsin was dissolved in 0.02 N HCl to a final concentration of 50 mg/ml. All enzyme solutions were filter sterilized with disposable filters (Rotrand/Red; rim diameter, 0.2 μm; Schleicher & Schuell). Twenty-microliter aliquots of filtered cell-free bacteriocin solution and 20 μl of each enzyme were spotted 1 cm apart on GM17 agar plates and dried for 30 min. Plates were subsequently overlaid with an indicator organism. All plates were incubated overnight at 30°C. Protease sensitivity was observed as a half-moon-shaped zone of inhibition.

Isolation and modification of DNA. Plasmid DNAs were isolated from lactococci by the method of Anderson and McKay (1). Genomic DNAs were extracted from lactococci by a modification of the method of Hoffman and Winston (13) for the isolation of yeast plasmids, which uses shearing with glass beads to lyse cells, as follows: overnight cells were collected by centrifuging 2-ml volumes of cultures for 5 s. The supernatant was discarded, and the remaining pellet was vortexed briefly. Cells were resuspended in 0.2 ml of sterile extraction solution containing 2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris (pH 8), and 1 mM EDTA. Then phenol-chloroform (0.2 ml) was added, with the subsequent addition of 0.3 g of acid-washed glass beads (diameter, 0.45 to 0.52 mm) (Sigma). The suspension was vigorously vortexed for 2 min and then microcentrifuged for 5 min. The resulting upper aqueous phase was gently transferred into a sterile microcentrifuge tube to which 20 μl of 3 M sodium acetate was added. After a gentle vortex, 600 μl of absolute ethanol (−20°C) was added and the mixed suspension was centrifuged for 10 min. The pellet was washed in 70% alcohol, dried, and finally resuspended in 50 μl of sterile distilled water. To estimate DNA concentration, a 5-μl sample was electrophoresed on a 0.7% agarose gel with ethidium bromide staining. Restriction enzyme digestion was performed in Cuts-All buffer containing 20 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 100 mM KCl, and 2 mM β-mercaptoethanol.

Amplification of lactococcal DNA was performed by the following method with a Perkin-Elmer DNA Thermal Cycler. PCR mixtures of 100 μl which contained a 1/10 volume of 10× buffer (Bioline), 5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 1 μM primer(s), and 1.25 U of *Taq* DNA polymerase were set up. After overlaying each tube with 100 μl of paraffin oil, 1 μl of DNA (isolated as described above) was added to the reaction. *Taq* DNA polymerase was added during the first temperature cycle (Hot Start), and DNA was amplified for 35 cycles. Each cycle involved 1-min denaturation at 93°C, followed by an annealing step at 55°C for 1 min and an extension step of 72°C for 1 min. Of the final reaction mixture, 10% was analyzed on 1.8% (wt/vol) agarose (Sigma) gels with ethidium bromide staining. The *nisA*-specific primers used were 5′-ATGAGTACAAAAGATTTTAAC-3′ and 5′-CTTACGTGAATACTA CAATG-3′.

DNA-DNA hybridization. After electrophoresis, restricted DNA was transferred to a Hybond N^+ nylon membrane by the capillary blotting method described by Maniatis et al. (21).

The DNA probe was prepared and DNA hybridization was carried out by the method of Morgan et al. (25).

Conjugation. A conjugal mating was set up by using *L. lactis* DPC3147 (lactose-utilizing [Lac^+] and bacteriocin-producing [Bac^+]) as the donor strain and the plasmid-free strain MG1614 (Lac^- Bac^-) as the recipient. Both strains were grown to mid-log phase (optical density at 600 nm, 0.5 to 1). Strain DPC3147 was cultivated in GM17 containing pronase E (50 mg/ml), while MG1614 was grown in GM17 supplemented with streptomycin (500 μg/ml). One-milliliter aliquots of these cultures were centrifuged in a microcentrifuge for 30 s, and the resultant pellets were washed once in 1-ml volumes of GM17. The pellets obtained were resuspended in 25 μl of GM17, mixed, and spotted in the center of a nonselective GM17 agar plate. Donor and recipient controls were prepared in a similar manner. After overnight incubation at 30°C, cultures were harvested from the surface of the agar plate with a sterile loop and resuspended in GM17 broth supplemented with 40% glycerol (so that mating mixes could later be frozen). A serial dilution was carried out on an aliquot of the mating mix, which was then plated on selective media. The selective medium consisted of GM17 containing streptomycin (500 μg/ml) and lacticin 3147 (400 AU/ml). The conjugation frequency was estimated by dividing the number of transconjugants (appearing on selection plates) by the number of donor cells. Putative transconjugants were checked for lactose-metabolizing activity by streaking on lactose indicator agar plates and for the ability to produce this bacteriocin. One of the resulting transconjugants (MG1614 containing pMRC01) was subsequently used as a donor to construct the lacticin 3147-producing starter *L. lactis* DPC4275 (4a).

Cheesemaking. For cheesemaking, bulk starters were cultivated in 10% RSM which had been heat treated at 90°C for 30 min and cooled to 21°C before inoculation. Two cheesemaking trials were performed. Where more than one strain was used in the starter, cultures were grown separately at 21°C for 16 h, mixed in equal proportions, and inoculated at the following levels. In trial 1, *L. lactis* DPC4268, a commercial cheesemaking strain used as a control, was inoculated at 1%, given that higher inoculation rates can lead to bitterness in cheese with this strain; strains DPC3147, DPC3204, and DPC3256 were inoculated at 0.7% each. In trial 2, DPC4268 and its derivative DPC4275 were both inoculated at 1%.

Milk was pasteurized (72°C, 15 s) and cooled to 30°C. Cheese was made in circular jacketed stainless steel 500-liter vats. Filter-sterilized rennet (C. Hansens Laboratories; 31 ml, diluted in 500 ml of sterile distilled water) was added 30 min after the addition of starter, and the coagulum was cut approximately 40 min later. Curds and whey were cooked at 38°C and pitched at pH 6.2. Cheddared curds were milled at approximately pH 5.2, salted at a level of 27 g/kg, and pressed in 18-kg molds overnight at approximately 412 kPa. Cheeses were vacuum packed and ripened for 6 to 9 months at 8°C.

Analysis of cheese. For bacteriological analyses, cheeses were aseptically sampled at regular intervals. Starter cells were enumerated on LM17 agar after incubation at 30°C for 3 days, NSLAB (lactobacilli) were enumerated on LBS agar after 5 days at 30°C, enterococci were enumerated on KAA after 24 h at 37°C, and coliforms were enumerated on VRBA after 24 h at 30°C. Microbiological analyses were single estimations at each sampling time, with the exception of the *Lactobacillus* counts for trial 2, which were performed in triplicate.

The composition (pH, fat, protein, salt, and moisture) and proteolysis of cheese were analyzed by the method of Guinee et al. (12).

Bacteriocin activity in cheese was determined as follows. Cheese samples were initially macerated in equal volumes of distilled water in a stomacher (Labblender 400) for 15 min and heated to 80°C for 10 min. Then aliquots of 50 μl were dispensed in wells, and bacteriocin activity was assayed as outlined above.

TABLE 1. Inhibition spectra of *L. lactis* DPC3147, *L. lactis* NCDO496, and *L. lactis* DPC3220

Strain or species	Medium	Sensitivity[a]			Source[b]
		DPC3147	NCDO496	DPC3220	
Acetobacter aceti DPC3697	UBA[c]	+	++	NZ	DPC
Acetobacter suboxydons DPC3698	UBA[c]	NZ	NZ	NZ	DPC
Bacillus cereus ATCC9139	GM17[d]	+	+	NZ	DPC
Bacillus subtilis BD630	TYP[d]	+	+	NZ	DPC
Clostridium sporogenes NCFB1791	RCM[e]	+++	+++	NZ	DPC
Clostridium tyrobutyricum NCFB1755	RCM[e]	+++	+++	NZ	DPC
Enterococcus faecium NCDO942	GM17[d]	++	++	NZ	NCDO
Enterococcus faecalis NCDO610	GM17[d]	++	+	NZ	DPC
Enterococcus faecalis NCDO581	GM17[d]	+++	+	NZ	DPC
Enterococcus faecalis 10C1	GM17[d]	++	+	NZ	DPC
Lactobacillus acidophilus ATCC4356	MRS[d,f]	++	++	NZ	ATCC
Lactobacillus bulgaricus ATCC11842	MRS[f,g]	++	++	NZ	ATCC
Lactobacillus casei ATCC334	MRS[d]	++	++	NZ	ATCC
Lactobacillus curvatus CNRZ117	MRS[d]	+	++	NZ	CNRZ
Lactobacillus fermenticum ATCC9338	MRS[d,f]	+++	+++	NZ	ATCC
Lactobacillus helveticus NCDO257	MRS[d,f]	+++	++	NZ	NCDO
Lactobacillus helveticus NCDO1209	MRS[d,f]	+++	++	NZ	NCDO
Lactobacillus helveticus NCDO1244	MRS[d,f]	+++	+++	NZ	NCDO
Lactobacillus helveticus ATCC15009	MRS[d]	++	++	NZ	ATCC
Lactobacillus kefir NCFB2737	MRS[e]	+++	++	NZ	NCFB
Lactobacillus leichmannii NCDO299	MRS[d,f]	+++	++	NZ	NCDO
Lactobacillus leichmannii NCDO302	MRS[d,f]	+++	+++	NZ	NCDO
Lactobacillus reuteri DSM20016	MRS[d]	++	++	NZ	DPC
Lactobacillus sake NCFB2714	MRS[e,f]	+++	+++	NZ	NCFB
L. lactis DPC3147	GM17[e]	NZ	+	++	DPC
L. lactis NCDO496	GM17[e]	++	NZ	++	NCDO
L. lactis NCDO497	GM17[e]	+++	NZ	++	NCDO
L. lactis DPC3220	GM17[e]	+++	+	NZ	DPC
L. lactis DPC33(1)	GM17[e]	+++	+	NZ	DPC
L. lactis DPC2949	GM17[e]	+	+++	++	DPC
L. lactis CNRZ481	GM17[e]	+++	+++	+++	CNRZ
L. lactis 9B4	GM17[e]	+++	+++	++	DPC
L. lactis AM2	GM17[e]	+++	+++	+++	DPC
L. lactis DPC4268	GM17[e]	++	++	++	DPC
L. lactis HP	GM17[e]	+++	+++	+++	DPC
L. lactis 938	GM17[e]	+++	+++	+	DPC
L. lactis DPC147	GM17[e]	+++	+++	+++	DPC
L. lactis DPC712	GM17[e]	+++	+++	+++	DPC
L. lactis IL1403	GM17[e]	+++	+++	+++	DPC
L. lactis SK11G	GM17[e]	+++	+++	+++	DPC
L. lactis 290P	GM17[e]	+++	++	++	DPC
L. lactis DRC3	GM17[e]	+++	++	++	DPC
Leuconostoc strain CNRZ1091	MRS[d,f]	++	+++	NZ	CNRZ
Listeria innocua BD86/26	GM17[e]	++	++	NZ	DPC
L. monocytogenes NCTC5348	GM17[d]	+	+	NZ	DPC
Pediococcus pentriceans NCDO992	GM17[e]	++	++	NZ	NCDO
Pediococcus pentriceans NCDO1850	GM17[e]	+	++	NZ	NCDO
Pediococcus pentosaceus FBB63	GM17[e]	++	++	NZ	DPC
Staphylococcus aureus ATCC25923	TYP[d]	+	+	NZ	DPC
Streptococcus thermophilus HA	GM17[g]	++	+	NZ	DPC
Streptococcus thermophilus ST112	GM17[g]	++	++	NZ	DPC
Salmonella typhi ATCC14025	TSA[d]	NZ	NZ	NZ	DPC
Escherichia coli	GM17[e]	NZ	NZ	NZ	DPC
Pseudomonas aeruginosa NCFB1369	GM17[c]	NZ	NZ	NZ	DPC

[a] NZ, no zone; +, 1 to 5 mm; ++, 5 to 15 mm; +++, 15 mm and over.
[b] DPC, National Dairy Products Research Centre, Moorepark, Fermoy, County Cork, Ireland; ATCC, American Type Culture Collection, Rockville, Md.; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; NCFB, National Collection of Food Bacteria, Institute of Food Research, Reading, United Kingdom.
[c] Incubated at 21°C.
[d] Incubated at 37°C.
[e] Incubated at 30°C.
[f] Incubated under anaerobic conditions.
[g] Incubated at 42°C.

RESULTS

Characterization of bacteriocin-producing lactococci. A number of lactococci which exhibited antimicrobial activities were isolated from Irish kefir grains. Protease sensitivity assays demonstrated that these antimicrobial agents were bacteriocins since they could readily be degraded by proteinase K. Cross-sensitivity assays were used to identify two groups of bacteriocin producers based on biological activity and immu-

nity. One such group contained six strains from four separate grains, while the second group contained two strains from two grains. The plasmid profiles of the six isolates in group one exhibiting cross-immunity appeared to be identical for four of these strains (DPC3147, DPC3153, DPC3215, and DPC3254), whereas strains DPC3204 and DPC3244 contained an extra plasmid of approximately 45 kb. In view of these results, DPC3147 was chosen as the representative of this group of bacteriocin producers. Group two consists of strains DPC3220 and DPC33(1), which exhibit cross-immunity but are sensitive to the bacteriocin produced by DPC3147. DPC3220 was chosen as the representative of this second group.

Inhibitory spectra. The relative sensitivities of 54 strains from a number of different genera to *L. lactis* DPC3147, NCDO496 (included for comparison), and DPC3220 are presented in Table 1. All three producers inhibited other lactococci, including a number of commercially used cheesemaking strains. Both DPC3147 and DPC3220 inhibit previously characterized bacteriocin producers, including *L. lactis* CNRZ481, the producer of lacticin 481 (29); Nip^+ strains *L. lactis* NCDO496 and NCDO497; and *L. lactis* subsp. *cremoris* 9B4, which produces lactococcins A, B, and M. In addition, known bacteriocin producers inhibited both DPC3147 and DPC3220. On the basis of these observations, it appeared that neither strain DPC3147 nor DPC3220 produced nisin, lacticin 481, or lactococcin A, B, or M.

The bacteriocin produced by *L. lactis* DPC3220 inhibits only other lactococci and, as such, can be characterized as having a narrow spectrum of inhibition. In contrast, the bacteriocin produced by strain DPC3147 has a very broad spectrum of inhibition which closely resembles that of the nisin producer NCDO496. Without exception, all gram-positive indicator bacteria tested, including lactococci, lactobacilli, enterococci, bacilli, leuconostocs, pediococci, clostridia, listeriae, staphylococci, and streptococci, were sensitive to both nisin and the 3147 bacteriocin. For this reason, subsequent studies concentrated on the characterization and use of the 3147 producer.

***L. lactis* DPC3147 is not a nisin producer.** Even though cross-sensitivity studies suggest that strain DPC3147 is not a nisin producer, its biological activities against other strains appeared to be remarkably similar to those of Nip^+ strains. Consequently, a number of experiments were performed to investigate any possible relationship between lacticin 3147 and nisin. Protease sensitivity assays revealed that the 3147 bacteriocin is sensitive to trypsin, α-chymotrypsin, proteinase K, and pronase E but is not sensitive to pepsin. In contrast, nisin is not sensitive to trypsin but is degraded by α-chymotrypsin, pancreatin, and subtilopeptidase. In addition, *L. lactis* DPC3147 was incapable of fermenting sucrose, which was confirmed by plating this strain on sucrose-metabolizing indicator agar. This provides additional indirect evidence that lacticin 3147 is not closely related to nisin, since the gene encoding nisin (*nisA*) is invariably linked to genes responsible for sucrose catabolism on the nisin-sucrose transposon, Tn*5276* (15). By using the published sequence of the nisin structural gene (7), two primers which are complementary to sequences occurring proximal to the 3′ and 5′ ends of the *nisA* gene were synthesized. In PCRs, these should amplify a product of 166 bp from *nisA*-containing template DNA. Indeed, an amplified product of approximately that size was consistently amplified from genomic DNA isolated from the NCDO496 and NCDO497 strains (data not shown). In contrast, no amplified product was observed when genomic DNA from DPC3147 or DPC3220 was used. The DNA amplified from *L. lactis* NCDO496, representing a significant portion of the *nisA* gene, was used as a gene probe for DNA isolated from lactococcal strains NCDO496, DPC3147, and DPC3220. As shown in Fig. 1, the *nisA* gene probe hybridized to a 3.5-kb *Hin*dIII fragment on the NCDO496 genome. In contrast, no hybridizing DNA was observed in DNA isolated from strain MG1614 or DPC3147. This suggests that the bacteriocin produced by DPC3147 is not a close homolog of nisin. We therefore concluded that lacticin 3147 is a novel broad-spectrum bacteriocin that is genetically and biologically distinct from nisin.

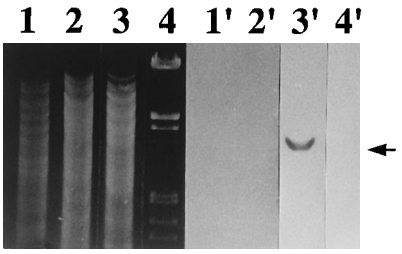

FIG. 1. DNA hybridization. *Hin*dIII-restricted genomic DNAs from *L. lactis* DPC3147, MG1614, and NCDO496 are shown in lanes 1, 2, and 3, respectively. Lane 4, DNA molecular weight markers. The *nisA* gene probe hybridized to a 3.5-kb fragment (position indicated by the arrow on the right) on the NCDO496 genome (lane 3′). No hybridizing DNA is observed in DNA isolated from DPC3147 (lane 1′) or MG1614 (lane 2′).

Characterization of lacticin 3147. The growth of *L. lactis* DPC3147 and the production of lacticin 3147 in GM17 and TYT30 were monitored over 24 h. In both media, this bacteriocin is produced during the exponential phase and peaks during the early stationary phase (data not shown). Subsequently, bacteriocin activity declines gradually during the stationary phase. Production in a variety of media, including MRS, BHI, TYP, RSM, and whole milk, was also determined. Activity (in square millimeters) was measured from the filtered supernatant of an overnight culture. Under these conditions, production was found to be greatest in MRS, with an activity of 170 mm^2. The activities in RSM and whole milk were 90 and 73% of that found in MRS, respectively.

The effects of pH and temperature on the stability of lacticin 3147 were investigated (Fig. 2). Three samples of bacteriocin were brought to pHs of 5, 7, and 9, and aliquots of each were

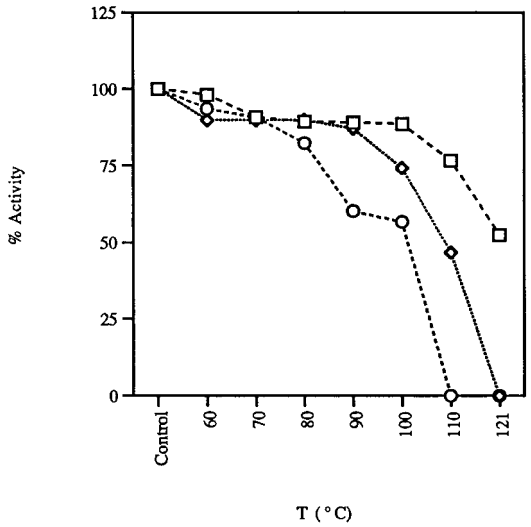

FIG. 2. Effect of heating at 60 to 121°C for 10 min on the stability of lacticin 3147 at pH 5 (□), pH 7 (◇), and pH 9 (○). The activity at pH 5, 7, or 9 with no heating (control) was taken as 100% activity for that pH. T, temperature.

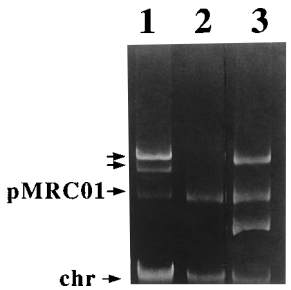

FIG. 3. Analysis of the plasmid complements of a transconjugant (lane 2) obtained from a mating between *L. lactis* DPC3147 (lane 1) and the plasmid-free strain MG1614. Transconjugants contain a 63-kDa plasmid, pMRC01, acquired from the DPC3147 donor, which is not evident in the plasmid-free MG1614 strain. The chromosomal band (chr) is indicated, as are two extra plasmids contained in strain DPC3147 (arrows). The plasmid profile of *L. lactis* subsp. *diacetylactis* DRC3 is included (lane 3) as a molecular weight marker (78, 60, and 51 kb).

subsequently heated to 60, 70, 80, 90, 100, 110, and 121°C for 10 min. The activity at each pH prior to heat treatment was measured and taken to be 100% for that pH. Like many other LAB bacteriocins, lacticin 3147 is heat stable, particularly at an acid pH.

Lacticin 3147 production and immunity are plasmid encoded. Preliminary experiments attempted to establish if bacteriocin production by DPC3147 is a conjugally transmissable characteristic. Conjugal matings were performed, with DPC3147 as the donor strain and the streptomycin-resistant (Sm^r), plasmid-free strain *L. lactis* subsp. *lactis* MG1614 (10) as the recipient. The selection of transconjugants from such matings was achieved by using lacticin 3147 immunity and Sm^r as selectable markers. This was done by incorporating lacticin 3147 into selective media. Even when plated at a high cell density (up to 10^8 to 10^9 CFU/ml), *L. lactis* subsp. *lactis* MG1614 failed to produce colonies on such media, indicating a very low level of spontaneous resistance to lacticin 3147. In matings involving strains MG1614 and DPC3147, putative transconjugants capable of forming colonies on selective plates were isolated at a frequency of 10^{-3} per donor cell. These isolates were subsequently scored as lactose deficient (a trait associated with the recipient) and had acquired the ability to produce bacteriocin. As expected, these putative transconjugants exhibited cross-immunity to DPC3147 and could inhibit a similarly wide range of gram-positive bacteria. Evidence that these isolates were true transconjugants was obtained by analyzing their plasmid complements. In all cases, Bac^+ Imm^+ Sm^r cells had acquired a 63-kDa plasmid, pMRC01. Interestingly, a similarly sized plasmid is clearly evident in plasmid profiles of strain DPC3147 (Fig. 3). One such transconjugant was subsequently used as the donor in a conjugal mating with the commercial cheesemaking strain *L. lactis* DPC4268. The resulting transconjugant, *L. lactis* DPC4275, was found to have acquired Bac^+ Imm^+ properties in addition to maintaining its original cheesemaking characteristics. The amount of lacticin 3147 produced by this transconjugant was found to be similar to that produced by the parent strain, *L. lactis* DPC3147.

Cheesemaking with lacticin 3147 producers. Two cheddar cheese trials were performed to test the abilities of lacticin 3147-producing strains to act as cheese starters. In trial 1, the starter consisted of a combination of three natural kefir isolates, all of which produce lacticin 3147 (DPC3147, DPC3204, and DPC3256). As illustrated in Fig. 4A, bacteriocin-producing starter strains produced lactic acid in milk at rates similar to that of the fast acid commercial strain DPC4268 during cheesemaking. In this regard, the three-strain mixture would be acceptable in commercial practice. This was borne out in trial 1, in which sufficient acid was produced and cheese was manufactured within the same time frame as the control cheese manufactured simultaneously with the commercial starter.

During extended cheddar cheese ripening times, NSLAB can reach levels exceeding 10^7 CFU/g. These NSLAB are mainly lactobacilli which we have previously shown to be sensitive to lacticin 3147. The floras in both test and control cheeses were monitored for 6 months, a critical time in the ripening of cheddar cheese. No NSLAB were detected in cheese made with Bac^+ strains at any stage throughout ripening (Fig. 4B). In contrast, NSLAB reached levels of $10^{7.5}$ CFU/g after approximately 4 months in control cheese. The presence of lacticin 3147 in test cheese was confirmed (by using *L. lactis* AM2 as the indicator strain) at a level of approximately 1,280 AU/ml and remained constant over the 28-week sampling period (Fig. 5). In a separate experiment, this first trial was repeated; as before, no NSLAB were evident in test cheese after 6 months (data not shown).

In trial 2, cheddar cheese was manufactured by using the pMRC01 transconjugant *L. lactis* DPC4275 (Bac^+ Imm^+) as a single-strain starter. The parent strain, *L. lactis* DPC4268, a commercial cheesemaking strain, was again used as the control. The results demonstrate that transconjugant DPC4275 performed satisfactorily as a starter during cheese manufacture, although it did produce acid at a slightly lower rate than that of the control (Fig. 4C). In this trial, NSLAB were detected in test cheese, but at significantly lower levels (in excess of 100-fold) than those in control cheese (Fig. 4D). As in trial 1, the amount of bacteriocin detected in cheese in trial 2 was approximately 1,280 AU/ml, which remained stable over the entire ripening period.

The gross composition of each cheese was within the parameters set out by Giles and Lawrence (11) for cheddar cheese. There was no significant differences in water-soluble nitrogen, phosphotungstic acid-nitrogen, and free amino acid composition between control cheese and cheese made with bacteriocin producers.

DISCUSSION

L. lactis DPC3147 was isolated from an Irish kefir grain used domestically to produce buttermilk. Complex microbiological environments, such as those found in kefir grains, may provide a suitable ecological niche for the proliferation and evolution of Bac^+ strains. While the actual grain is principally composed of lactobacilli and yeasts, it is lactococci which predominate in milk after fermentation (31). Interestingly, the four apparently identical lactococcal strains identified in this study which produce lacticin 3147 were isolated from grains obtained from different locations in Ireland. To the best of our knowledge, these grains were not recent subcultures of each other and had an oral history of up to 38 years of domestic use. It is tempting to speculate that bacteriocins such as lacticin 3147 play a pivotal role in maintaining the microbial integrity of kefir grains by inhibiting the growth of foreign organisms. It is possible that the solid matrix of kefir grain offers protection against bacteriocin, since a number of lactobacilli isolated from grains were sensitive to lacticin 3147 in liquid culture.

The results of this study demonstrate that strains which produce lacticin 3147 are suitable for cheddar cheese manufacture. In pasteurized milk, they performed in a manner similar to that of commercial starters and reduced the pH to 5.2 within the desired time. In addition, during manufacture they

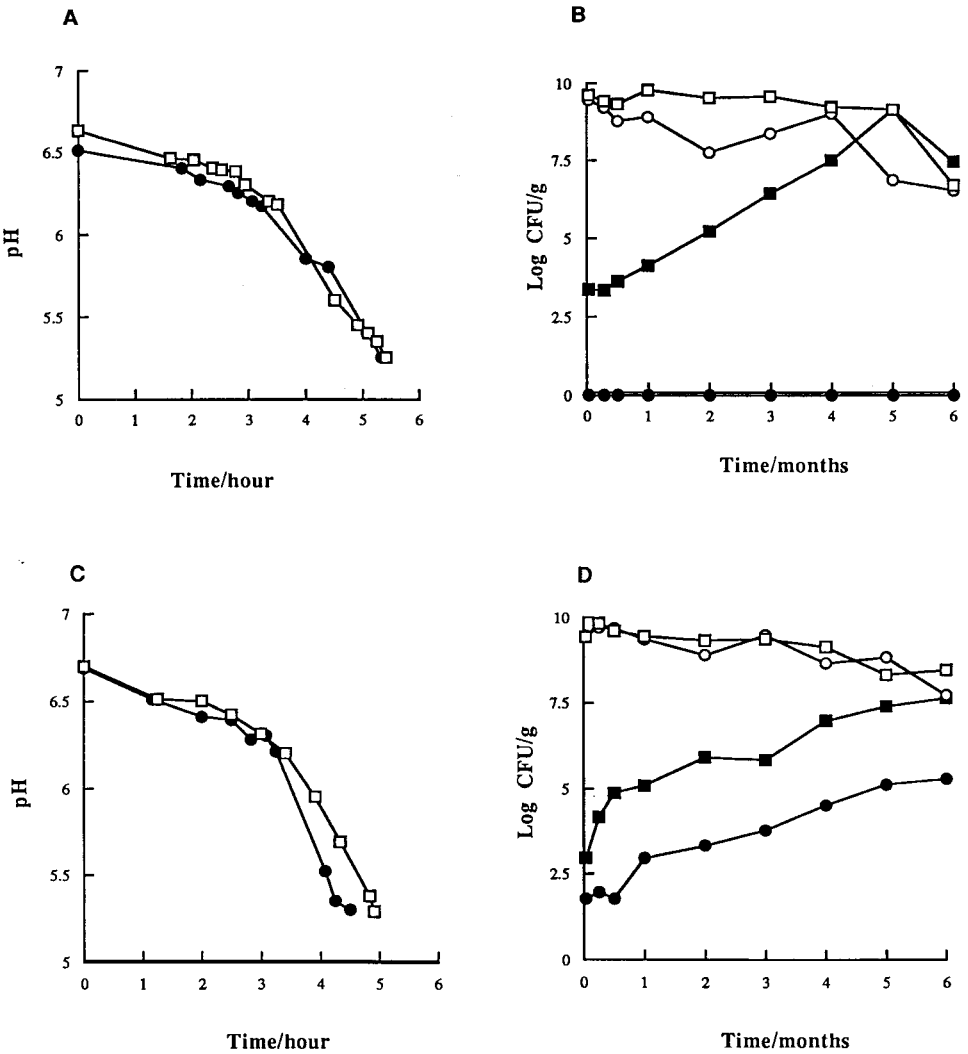

FIG. 4. (A) pH profiles during the manufacture of cheddar cheeses made with control starter DPC4268 (●) and Bac$^+$ starters DPC3147, DPC3256, and DPC3204 (□). (B) Growth of starter (open symbols) and NSLAB (closed symbols) during the ripening of cheddar cheeses made with DPC4268 (squares) and Bac$^+$ strains (circles). (C) pH profiles during the manufacture of cheeses made with control starter DPC4268 (●) and Bac$^+$ transconjugant DPC4275 (□). (D) Growth of starter (open symbols) and NSLAB (closed symbols) during the ripening of cheddar cheeses made with DPC4268 (squares) and Bac$^+$ DPC4275 (circles).

efficiently produced lacticin 3147 which was detectable in cheese for up to 6 months, a critical time for the ripening of cheddar cheese. Of major importance to any application is the fact that this bacteriocin is produced at sufficient levels and is active enough during cheese manufacture and ripening to affect the developing microflora. During cheddar cheese ripening, NSLAB levels normally exceed 10^7 to 10^8 CFU/g (9, 28), which in some instances can be significantly higher than the starter levels themselves in cheese. Their contribution to cheese quality is as yet unclear, although they are thought to play a significant role in proteolysis and flavor development during ripening (23, 24). However, they have also been implicated in the formation of Ca-lactate crystals in cheese, a defect which can result in major economic losses (34). Since NSLAB found in Irish cheeses (17) are predominantly mesophilic lactobacilli, it was expected that cheeses made with lacticin 3147 producers would contain fewer NSLAB, given that strain DPC3147 inhibited all lactobacilli tested (Table 1). Indeed, in both cheese trials described here, the NSLAB counts were greatly reduced when Bac$^+$ cheese starters were used in manufacture. The ability to produce cheeses with reduced NSLAB levels provides a means by which a number of outstanding questions about the role of NSLAB in cheese flavor may be resolved. If, as expected, a significant role in flavor development can be ascribed to NSLAB, then the ability to control that flora will have obvious commercial ramifications. Given that the levels of lacticin 3147 detected in both test cheeses were identical (1,280 AU/ml), we are unable to explain the appearance of NSLAB in trial 2 compared with their complete absence in trial 1. It should be borne in mind that the assays for bacteriocin activity are crude and give only an indication of the actual levels present; it should also be pointed out that some representative NSLAB from trial 2 were examined and proved to be sensitive to lacticin 3147. Therefore, the appearance of this adventitious flora was not due to the appearance of lacticin 3147-resistant strains.

The novel bacteriocin described here, lacticin 3147, is clearly distinct from nisin. The observation that the *nisA* gene probe does not hybridize to genomic DNA isolated from *L. lactis* 3147 would suggest that this bacteriocin is not a close homolog

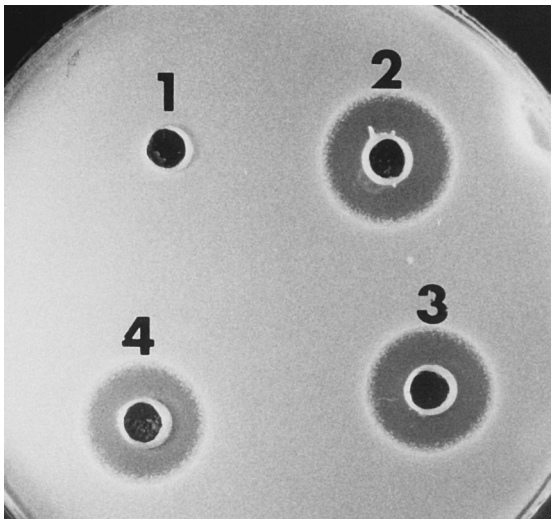

FIG. 5. Residual lacticin 3147 activities in cheddar cheeses made with bacteriocin-producing strains. Wells: 1, control cheese manufactured by using *L. lactis* DPC4268 (non-bacteriocin-producing starter); 2, 3, and 4, cheeses made with bacteriocin producers (DPC3147, DPC3256, and DPC3204) sampled after 2 weeks and 4 and 6 months, respectively.

of nisin, despite similarities in their respective inhibitory spectra. Like nisin, lacticin 3147 is heat stable, particularly at a low pH, and produced efficiently in milk-based and synthetic laboratory media. While we can state with confidence that lacticin 3147 is distinct from nisin, we do not as yet possess sequence data from either the lacticin 3147 peptide or its determinant. This information will be required to distinguish lacticin 3147 from the many other bacteriocins which have been identified worldwide, but a careful search of the literature revealed no previously identified inhibitor with similar biological characteristics, with the possible exception of pediocin L50, which has recently been characterized (4).

We suggest that lacticin 3147 is a suitable alternative for the type of applications that have been identified for nisin as a natural biopreservative in foods. In some key respects, such as in cheese manufacture, lacticin 3147 may indeed offer significant advantages over nisin. One very useful trait of lacticin 3147 is that it is encoded on a 63-kb conjugative plasmid. Transconjugants containing pMRC01 can easily be selected because of their ability to grow on media containing lacticin 3147. Thus, the genetic determinants encoding immunity and production of this bacteriocin may form the basis for a useful food-grade selectable marker. Equally, this plasmid can be readily transferred to strains with industrially important characteristics. Maintenance should be assured, since cells which undergo plasmid loss would rapidly succumb to the bacteriocin produced by cells possessing pMRC01. Presently, lacticin 3147 is being characterized at the molecular level, with a view to determining its structure and establishing its mode of action.

In conclusion, this study has demonstrated the performance and usefulness of starters which produce lacticin 3147 in cheddar cheese manufacture. The influence that these starters exert on the developing microflora of cheese, as evidenced by the reduction in NSLAB numbers, may offer the cheesemaker greater control over the total cheese flora, both from the perspective of greater quality control and as a means of improving product safety.

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