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Use of a Genetically Enhanced, Pediocin-Producing Starter Culture, *Lactococcus lactis* subsp. *lactis* MM217, To Control *Listeria monocytogenes* in Cheddar Cheese

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Cheddar cheese was prepared with *Lactococcus lactis* subsp. *lactis* MM217, a starter culture which contains pMC117 coding for pediocin PA-1. About 75 liters of pasteurized milk (containing ca. 3.6% fat) was inoculated with strain MM217 (ca. 10^6 CFU per ml) and a mixture of three *Listeria monocytogenes* strains (ca. 10^3 CFU per ml). The viability of the pathogen and the activity of pediocin in the cheese were monitored at appropriate intervals throughout the manufacturing process and during ripening at 8°C for 6 months. In control cheese made with the isogenic, non-pediocin-producing starter culture *L. lactis* subsp. *lactis* MM210, the counts of the pathogen increased to about 10^7 CFU per g after 2 weeks of ripening and then gradually decreased to about 10^3 CFU per g after 6 months. In the experimental cheese made with strain MM217, the counts of *L. monocytogenes* decreased to 10^2 CFU per g within 1 week of ripening and then decreased to about 10 CFU per g within 3 months. The average titer of pediocin in the experimental cheese decreased from approximately 64,000 arbitrary units (AU) per g after 1 day to 2,000 AU per g after 6 months. No pediocin activity (<200 AU per g) was detected in the control cheese. Also, the presence of pMC117 in strain MM217 did not alter the cheese-making quality of the starter culture, as the rates of acid production, the pH values, and the levels of moisture, NaCl, and fat of the control cheese and the experimental cheese were similar. Our data revealed that pediocin-producing starter cultures have significant potential for protecting natural cheese against *L. monocytogenes*.

Advances in genetic technologies have made it possible to develop lactic acid bacteria (LAB) starter cultures or adjuncts with enhanced fermentation characteristics, such as increased phage resistance, improved proteolytic activity, and faster acid production (16, 25). Genetically enhanced dairy cultures may also extend the shelf life and/or improve the safety of the resulting cheese through the production of various antimicrobials, notably bacteriocins. At present, nisin, which is produced by *Lactococcus lactis* subsp. *lactis*, is the only bacteriocin approved for direct incorporation into cheese (6). However, compared to non-nisin-producing starter cultures, some nisin-producing starter cultures display slower lactose metabolism, less proteolytic activity, lower heat resistance, and greater sensitivity to phage attacks (18–20). Nisin and other bacteriocins may also be antagonistic towards mesophilic lactococci (non-bacteriocin-producing strains) used in mixed-strain starters or towards nonstarter lactic acid bacteria (NSLAB) important in flavor development. Thus, there has been continued interest in developing commercial starter cultures with the ability to produce bacteriocins to ensure adequate performance of the starter and enhance the quality and safety of the resulting cheese.

Several investigators have evaluated the potential of bacteriocin-producing starters or starter adjuncts, especially lactococci, enterococci, and lactobacilli, to enhance performance and/or control undesirable microbes in cheese. Cheddar cheese made with a paired nisin-producing *Lactococcus* starter system has been used successfully as an antimicrobial agent in pasteurized process cheese and cheese spreads to control *Clostridium sporogenes*, *Listeria monocytogenes*, and *Staphylococcus aureus* (34). Sulzer and Busse (31) used lactococci, enterococci, or lactobacilli that were inhibitory to *L. monocytogenes*, alone or in combination with commercial starter cultures, to control this pathogen in Camembert cheese. Listeriae were suppressed only when the inhibitory strain was the sole starter and when contamination with *Listeria* spp. occurred early during ripening. Appreciable inhibition of *L. monocytogenes* in Camembert cheese was also observed when a nisin-producing paired lactococcal starter system was used (20). In the study of Ryan et al. (28), sufficient lacticin 3147 was produced during cheese manufacturing and ripening to have an impact on the resident microflora; however, the full potential of the bacteriocin was not substantiated against a food-borne pathogen, such as *L. monocytogenes*, in cheese.

In studies of dairy applications of other bacteriocinogenic cultures, Eppert et al. (8) used a linocin-producing *Brevibacterium linens* strain to obtain a 1- to 2-$\log_{10}$ reduction in the amount of *L. monocytogenes* in soft, smear-ripened cheese, and Joosten and Nunez (15) used bacteriocin-producing enterococci and nisin-producing *L. lactis* strains separately to inhibit the growth and production of histamine by *Lactobacillus buchneri* St2A in Manchego cheese. In Taleggio cheese, an enterocin-producing starter adjunct resulted in an appreciable decrease in the number of *L. monocytogenes* cells compared to control cheese (11), while an enterocin-producing adjunct culture, *Enterococcus faecalis* INIA 4, accelerated flavor formation in semihard cheese (10). It should be noted, however, that enterococci may not be desirable for routine application in cheese making because some isolates (i) may produce tyramine, (ii) may be responsible for clinical infections, (iii) may

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inhibit NSLAB involved in cheese ripening, and/or (iv) may be used as indicators of fecal contamination (1).

Uljas and Luchansky (32) reported that lacticin 99, which was produced by the starter adjunct *Lactobacillus plantarum* JBL2132, inhibited NSLAB that form calcium lactate crystals in Cheddar cheese. Other investigators reported that using a commercial bacteriocinogenic mixture of *L. plantarum* strains resulted in a 2-$\log_{10}$ reduction in the number of *S. aureus* cells in 10 days during Montasio cheese ripening at 12°C compared to the control, in which the number of pathogen cells increased slightly (30). In addition, isolation from cheese of *L. plantarum* WHE 92, which produces a pediocin, may provide additional opportunities to use bacteriocinogenic lactobacilli as starters or starter adjuncts in cheese (7). Lactobacilli can play a significant role in flavor development in cheese, and unlike hemolytic or cytolytic enterococci, lactobacilli do not pose a significant threat to human health. Thus, identification of bacteriocinogenic lactobacilli and optimization of these organisms as adjunct cultures could have an appreciable impact on the quality and safety of several varieties of natural cheese.

With the possible exceptions of nisin and lactococci, pediocins and/or pediococci have been used more extensively to control undesirable microbes in foods than any other biopreservatives (23, 29). *Pediococcus* spp. typically ferment lactose poorly and are not known for their proteolytic ability in milk; thus, they are used primarily as starter cultures for meat fermentations (4). The genes coding for pediocin PA-1 (also known as pediocin AcH), a class II, heat-labile, small, peptide bacteriocin produced by *Pediococcus acidilactici*, have been sequenced and subcloned into vectors suitable for expression in other bacterial hosts, including lactococci (3, 5, 14, 22, 33). Pediocin PA-1 was expressed under the control of the strong lactococcal promoter P32 on pMC117 by *L. lactis* in microbiological media (5). To more fully exploit the commercial potential of a pediocin-producing lactococcal starter culture, in the present study we evaluated the efficacy of *L. lactis* subsp. *lactis* MM217 (containing pMC117) for controlling *L. monocytogenes* during the manufacture and ripening of Cheddar cheese.

MATERIALS AND METHODS

Bacterial strains. *L. lactis* subsp. *lactis* MM210 (Lac^+ Prt^+; Rhone-Poulenc Marschall Products, Madison, Wis.) was passaged twice in 11% nonfat dry milk (Carnation; Nestle Food Co., Glendale, Calif.) overnight at 32°C prior to inoculation into M17 (Difco Laboratories Inc., Detroit, Mich.) broth containing 0.5% lactose (LM17 broth). *L. lactis* LL108 (Lac^- Prt^- $RepA^+$) (17), the source of plasmid pMC117 (Em^r; 7.1 kb) carrying the pediocin PA-1 operon (*ped*) (5), was passaged twice in M17 broth containing 0.5% glucose and 5 μg of erythromycin (Sigma Chemical Co., St. Louis, Mo.) per ml at 30°C overnight prior to use. *L. monocytogenes* JBL1003 (= 103M) (serotype 1/2c; meat isolate), JBL1181 (= Ohio) (serotype 4b; cheese isolate), and JBL1180 (= California) (serotype 4b; cheese isolate), which were obtained from the University of Wisconsin Food Research Institute, were each passaged twice in brain heart infusion (Difco) broth at 37°C overnight prior to use. To prepare a mixture of the three *L. monocytogenes* strains, 1-ml portions of the three freshly grown *L. monocytogenes* strains were mixed thoroughly in a test tube.

Isolation, transfer, and digestion of plasmid DNA. Plasmid DNA was isolated from lactococci by the method of O'Sullivan and Klaenhammer (24). Restriction enzymes were purchased from Promega Corporation (Madison, Wis.) and were used as recommended by the manufacturer. Electrotransformation of lactococci was performed essentially as described by Holo and Nes (13). The electroporated cells (40 μl) were immediately mixed with 960 μl of ice-cold expression broth (LM17 containing 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$) and then incubated at 25°C for 1 to 3 h. Cells were spread plated onto selective streptococcal regeneration medium (13) agar plates containing 5 μg of erythromycin per ml and incubated at 32°C. Erythromycin-resistant electrotransformants were transferred with sterile toothpicks onto LM17 agar plates without erythromycin and incubated overnight at 32°C, and then the resulting colonies were overlaid with 8 ml of tryptose phosphate (Difco) soft agar (containing 0.8% agar) seeded with 3 μl of the three-strain *L. monocytogenes* mixture per ml. After overnight incubation at 37°C, colonies exhibiting a zone of inhibition were streak plated onto erythromycin-containing LM17 agar plates to obtain pure colonies. A representative isolate, designated *L. lactis* subsp. *lactis* MM217, was grown overnight at 32°C in LM17 broth containing 5 μg of erythromycin per ml and was used for cheese-making experiments.

Cheddar cheese manufacturing. A standard protocol, obtained from Mark E. Johnson (University of Wisconsin), was used for Cheddar cheese manufacturing. Briefly, about 75 liters (78 kg) of pasteurized cheese milk (containing ca. 3.6% fat) in a 300-liter stainless steel vat (Nu-Vat; Meyer-Blanke Co., St. Louis, Mo.) surrounded by a steam-controlled water jacket was inoculated with about 6.6×10^6 CFU of *L. lactis* subsp. *lactis* MM210 or MM217 previously grown in LM17 broth per ml. About 10 min after the addition of the starter culture, the cheese milk was also inoculated with about 10^3 CFU of the *L. monocytogenes* mixture per ml. After 5 min of constant stirring with a long-handled plastic paddle, a 10-ml sample of the cheese milk was obtained and used for microbiological and pediocin activity analyses. Drained whey at the cooking stage was also analyzed for pediocin activity. The resulting curds (about 10.5 kg [13.5% yield] per 75 liters of cheese milk) were pressed (25 lb/in^2) at room temperature (25°C) for 5 to 6 h, placed into oxygen-impermeable bags (Liquiflex grade 8226-I; Curwood, Oshkosh, Wis.), vacuum packaged (Multivac type AGW; KOCH, Kansas City, Mo.), and then ripened at 8°C for up to 6 months. The cheese was made in the pathogen-compatible food processing laboratory of the University of Wisconsin Food Research Institute. All materials and equipment were autoclaved before and after cheese making. The whey was cooked at 100°C for several hours, and the remaining curds and cheese were autoclaved before they were discarded to eliminate residual bacterial cells.

Bacteriocin assay. Trypticase soy (Difco) agar plates were overlaid with 8 ml of Trypticase soy soft agar (containing 0.8% agar) seeded with 24 μl of freshly grown indicator cells (*L. monocytogenes* mixture). Ten grams of cheese in a stomacher bag (Seward Medical, London, United Kingdom) containing 90 ml of a warm (45°C) 2% sodium citrate solution was macerated for 3 min with a model 400 stomacher (Tekmar Co., Cincinnati, Ohio). Serial twofold dilutions in sterile double-distilled H_2O were analyzed for pediocin activity essentially as described by Pucci et al. (26) by using the *L. monocytogenes* strains in the mixture separately as indicators. Activity was expressed in arbitrary units (AU) (the inverse of the highest dilution that produced a discernible inhibition zone) (26).

Analyses of cheese. Cheese samples were obtained daily for 7 days and then weekly for 7 weeks and after 3 and 6 months. With a sterile kitchen knife, a cube (6.0 by 6.0 by 0.5 cm) was randomly cut from each of six sides of a cheese block. In addition, a core sample (2.0 by 2.0 by 3.0 cm) was removed from the interior of the cheese block. The cheese cubes and core (total weight, approximately 200 g) were placed into a stomacher bag and minced with a sterile knife. The resulting pieces were mixed thoroughly, and then a 10-g portion was removed and placed into another stomacher bag and macerated. Serial dilutions of the homogenized mixture in 0.1% peptone water were spread plated onto listeria enrichment (Difco) agar for enumeration of *L. monocytogenes* or onto LM17 agar and LM17 agar containing 5 μg of erythromycin per ml for enumeration of *L. lactis* subsp. *lactis* MM210 and MM217, respectively. The listeria enrichment agar plates were incubated at 37°C for 48 h, and the LM17 agar plates were incubated at 32°C for 18 to 24 h before colonies were counted. The macerated cheese samples were also tested for pediocin activity. The pH and the moisture, salt, and fat levels of the Cheddar cheese were determined as described in *Standard Methods for the Examination of Dairy Products* (21).

RESULTS

Expression of the pediocin operon in *L. lactis* subsp. *lactis* MM210. *L. lactis* subsp. *lactis* MM210 was electrotransformed with pMC117 to generate a pediocin-producing lactococcal starter culture. When the resulting Em^r cells of strain MM210 were overlaid with the three-strain *L. monocytogenes* mixture, inhibition zones were observed around some of the colonies. Inhibition zones were not observed when 5 μl of proteinase K (0.1 mg/ml) was spotted next to the colonies, indicating that the inhibitory substance was proteinaceous. Also, inhibition zones were not observed when untransformed colonies of *L. lactis* subsp. *lactis* MM210 were overlaid with the three-strain *L. monocytogenes* mixture. Analyses of plasmid DNA isolated from the Em^r electrotransformants that exhibited inhibition zones revealed the presence of a 7.1-kb plasmid which contained a 3.5-kb *Sma*I-*Bam*HI fragment, as expected for pMC117 (data not shown). A representative electrotransformant, designated *L. lactis* subsp. *lactis* MM217, was used for further analyses.

Survival of *L. monocytogenes* in Cheddar cheese made with *L. lactis* subsp. *lactis* MM210 or MM217. In experimental cheese made with *L. lactis* subsp. *lactis* MM217, the number of

TABLE 1. Survival of *L. monocytogenes* and pediocin activities in Cheddar cheese made with the bacteriocinogenic starter culture *L. lactis* subsp. *lactis* MM217 (experimental cheese; $n = 3$) or the isogenic nonbacteriocinogenic starter culture *L. lactis* subsp. *lactis* MM210 (control cheese; $n = 1$)

Cheese-making procedure	Day	Survival of *L. monocytogenes* in control cheese[a]	Exptl cheese	
			Pediocin activity[b]	Survival of *L. monocytogenes*[a]
Manufacturing				
Milk		3.22	200	3.51 ± 0.26[c]
Curd after cooking		3.92	NS	4.01 ± 0.52
Whey when drained		NS[d]	3,200	NS
Curd after pressing		4.41	NS	3.65 ± 0.33
Ripening	1	4.75	64,000	3.22 ± 0.80
	7	7.47	64,000	1.90 ± 0.09
	14	5.65	64,000	1.88 ± 0.12
	21	5.3	32,000	1.73 ± 0.29
	28	5.61	32,000	1.63 ± 0.37
	92	3.83	8,000	0.82 ± 0.10
	184	4.1	2,000	0.92 ± 0.88

[a] $\log_{10}$ CFU per milliliter or $\log_{10}$ CFU per gram.
[b] AU per milliliter or AU per gram.
[c] Mean ± standard deviation.
[d] NS, no sample was obtained.

L. monocytogenes cells steadily decreased from the initial level, 3.5 $\log_{10}$ CFU per g, to about 2.0 $\log_{10}$ CFU per g within 7 days and to about 1.0 $\log_{10}$ CFU per g after 184 days of ripening (Table 1). In contrast, in cheese made with the isogenic nonbacteriocinogenic starter culture, *L. lactis* subsp. *lactis* MM210, the number of *L. monocytogenes* cells increased in the first 7 days of ripening, after which the number of pathogen cells gradually decreased to about 4.0 $\log_{10}$ CFU per g after 184 days of ripening. In general, after 184 days of ripening at 8°C, the number of *L. monocytogenes* cells in the experimental cheese made with the bacteriocinogenic starter culture was at least 3.0 $\log_{10}$ lower than the number of such cells in control cheese made with the nonbacteriocinogenic starter culture. There was no difference between the number of LAB on M17 agar plates (all LAB, including strain MM210 or MM217) and the number of LAB on M17 agar containing erythromycin, indicating that the inhibitory activity produced by strain MM217 was not self-inhibitory or inhibitory to other NSLAB (data not shown).

Recovery of pediocin activity from Cheddar cheese. When the bacteriocinogenic starter *L. lactis* subsp. *lactis* MM217 was added to cheese milk, the level of inhibitory activity detected was 200 AU per ml. Although some inhibitory activity was lost in the whey, a considerable amount remained with the cheese after 1 day of ripening (Table 1). The level of inhibitory activity remained at 64,000 AU per g during the first 14 days of ripening and then gradually decreased. The decrease in the level of recoverable inhibitory activity corresponded to the decrease in the pathogen counts. In all cases, the level of activity decreased when proteinase K (0.1 mg/ml) was added to the test mixture, indicating that the inhibitory substance was proteinaceous. As inhibitory activity was not detected (the detection limit was 200 AU per ml or 200 AU per g) during the manufacturing or ripening of the control cheese and as the strains used in this study are isogenic (that is, they differ only in the presence of the *ped* operon), we concluded that pediocin was the inhibitory substance.

Analyses of Cheddar cheese composition. Proximate analyses of each cheese within 1 week after ripening at 8°C showed that it conformed to the standard of identity for Cheddar cheese (2) and that there were no appreciable differences between the composition of the control cheese (32% moisture, 36% fat, 1.3% salt, pH 5.2; $n = 1$) and the composition of the experimental cheese (31.3% ± 1.25% moisture, 36.3% ± 0.94% fat, 1.26% ± 0.05% salt, pH 5.1 ± 0.05; $n = 3$).

DISCUSSION

L. monocytogenes has caused numerous outbreaks and sporadic cases of listeriosis worldwide, and some of these have been attributed to dairy products (9). A national survey conducted in England and Wales showed that although most soft-ripened, unripened, or hard cheeses made from pasteurized or unpasteurized milk contained <10 CFU of *Listeria* spp. per g, 12% of the samples contained $>10^3$ *L. monocytogenes* CFU per g (12). The threat of listeriosis may be increased by the ability of *L. monocytogenes* to persist in certain cheeses, sometimes for extended periods of time (9). Thus, the purpose of the present study was to construct a pediocin-producing lactococcal starter culture and evaluate its antilisterial potential in Cheddar cheese. Advantageous characteristics, such as heat stability, as well as retention of activity at pH 5.1 and/or at refrigeration temperatures, extend the utility of pediocin as a biopreservative for cheese.

The pediocin-producing plasmid pMC117 was originally cloned into *L. lactis* LL108; however, this strain is both proteinase negative and lactose negative and is incapable of adequate growth and acid production in milk (5). In the present study, *L. lactis* subsp. *lactis* MM210 was selected as an alternate host for pMC117, as this strain is well characterized genetically, has a weakened but active host restriction and modification system, and has previously been used in Cheddar cheese manufacturing (27). The electrotransformed derivative of strain MM210 containing pMC117, designated *L. lactis* subsp. *lactis* MM217, had the same growth characteristics and acid-producing capability as the isogenic parental strain, *L. lactis* subsp. *lactis* MM210 (data not shown). Also, the behavior of strain MM217 was physiologically similar to the behavior of strain MM210 during cheese manufacturing, indicating that the presence of pMC117 did not alter the cheese-making properties of the original starter strain. More important, the level of survival of *L. monocytogenes* in Cheddar cheese made with *L. lactis* subsp. *lactis* MM217 was considerably lower than the level of survival of the pathogen in the control cheese. Since no inhibitory activity was detected in the control cheese, in situ production of pediocin by strain MM217 in the experimental cheese was responsible for the appreciable decline in the number of *L. monocytogenes* cells. Although other investigators established that preformed pediocin added to foods, including cheese, was inhibitory to *L. monocytogenes* (23, 29), inhibition of *L. monocytogenes* by in situ production of pediocin in Cheddar cheese by a lactococcal starter culture has not been reported previously.

After 6 months of ripening at 8°C, the level of recoverable pediocin activity decreased from 64,000 to 2,000 AU per g. Garde et al. (10) also described a decrease in the recovery of enterocin activity from a Hispanico type of cheese after 15 days of ripening at 14°C. In contrast, Ryan et al. (28) reported that the levels of lacticin 3147 remained relatively constant during 6 months of ripening at 8°C. The primary cause of the decline in the pediocin titer after 6 months of ripening was probably the sensitivity of pediocin to proteinases, in addition to autolysis of the starter and release of peptidases. However, other factors, including depletion of carbon and energy sources and binding of pediocin to cheese components, may also have contributed to the decrease in pediocin production and/or activity.

Although the level of pediocin activity recovered from the experimental cheese after 6 months of ripening at 8°C was lower than the levels recovered earlier, the number of pathogen cells was reduced to <10 CFU per g. As the residual *L. monocytogenes* population did not increase, the persistence of *L. monocytogenes* in an otherwise hostile environment could be attributed in part to individual cells that were entrapped in the casein matrix, which may have sequestered the pathogen somewhat from exposure to pediocin. The residual *L. monocytogenes* population may also have included a subpopulation of pediocin-resistant cells that over a longer time period could grow to levels sufficient to detect. However, it is also possible that the low pH, low water activity, and low ripening temperature provided additional hurdles which suppressed the growth and survival of residual *L. monocytogenes* cells in Cheddar cheese.

In conclusion, a genetically modified starter culture that produced pediocin in situ improved the microbiological safety of Cheddar cheese contaminated with *L. monocytogenes*. The acid-producing quality of the bacteriocinogenic starter culture and the composition of the Cheddar cheese produced with it were not altered by the acquisition of pMC117. In addition, the Standard of Identity of Cheddar Cheese was maintained, as pediocin was produced in situ and, thus, was not considered an additive. The use of genetically enhanced starter cultures that produce bacteriocins in situ may lessen microbiological safety problems with foods that do not undergo high-heat treatment and/or foods in which the use of chemical preservatives is not suitable.

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REFERENCES

1. **Adams, M. R., and P. Marteeau.** 1995. On the safety of lactic acid bacteria from food. Int. J. Food Microbiol. **27:**263–264.
2. **Anonymous.** 1988. Cheeses and cheese products. Definitions and standards under the Federal Food, Drug, and Cosmetic Act, Part 19, Title 21, Code of Federal Regulations. Food and Drug Administration, U.S. Department of Health, Education and Welfare, Washington, D.C.
3. **Bukhtiyarova, M., R. Yang, and B. Ray.** 1994. Analysis of the pediocin AcH gene cluster from plasmid pSMB74 and its expression in a pediocin-negative *Pediococcus acidilactici* strain. Appl. Environ. Microbiol. **60:**3405–3408.
4. **Caldwell, S. L., D. J. McMahon, C. J. Oberg, and J. R. Broadbent.** 1996. Development and characterization of lactose-positive *Pediococcus* species for milk fermentation. Appl. Environ. Microbiol. **62:**936–941.
5. **Chikindas, M. L., K. Venema, A. M. Ledeboer, G. Venema, and J. Kok.** 1995. Expression of lactococcin A and pediocin PA-1 in heterologous hosts. Lett. Appl. Microbiol. **21:**183–189.
6. **Delves-Broughton, J., P. Blackburn, R. J. Evans, and J. Hugenholtz.** 1996. Applications of the bacteriocin, nisin. Antonie Leeuwenhoek **69:**193–202.
7. **Ennahar, S., D. Aoude-Werner, O. Sorokine, A. van Dorsselaer, F. Bringel, J.-C. Hubert, and C. Hasselmann.** 1996. Production of pediocin AcH by *Lactobacillus plantarum* WHE 92 isolated from cheese. Appl. Environ. Microbiol. **62:**4381–4387.
8. **Eppert, I., N. Valdes-Stauber, H. Gotz, M. Busse, and S. Scherer.** 1997. Growth reduction of *Listeria* spp. caused by undefined industrial red smear cheese cultures and bacteriocin-producing *Brevibacterium linens* as evaluated in situ on soft cheese. Appl. Environ. Microbiol. **63:**4812–4817.
9. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a foodborne pathogen. Microbiol. Rev. **55:**476–511.
10. **Garde, S., P. Gaya, M. Medina, and M. Nunez.** 1997. Acceleration of flavour formation in cheese by a bacteriocin-producing adjunct lactic culture. Biotechnol. Lett. **19:**1011–1014.
11. **Giraffa, G., and D. Carminati.** 1997. Control of *Listeria monocytogenes* in the rind of Taleggio, a surface-ripened cheese, by a bacteriocin from *Enterococcus faecium* 7C5. Sci. Aliments **17:**383–391.
12. **Greenwood, M. J., D. Roberts, and P. Burden.** 1991. The occurrence of *Listeria* species in milk and dairy products: a national survey in England and Wales. Int. J. Food Microbiol. **12:**197–206.
13. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
14. **Horn, N., M. I. Martinez, J. M. Martinez, P. E. Hernandez, M. J. Gasson, J. M. Rodriguez, and H. M. Dodd.** 1998. Production of pediocin PA-1 by *Lactococcus lactis* using the lactococcin A secretory apparatus. Appl. Environ. Microbiol. **64:**818–823.
15. **Joosten, H. M. L. J., and M. Nunez.** 1996. Prevention of histamine formation in cheese by bacteriocin-producing lactic acid bacteria. Appl. Environ. Microbiol. **62:**1178–1181.
16. **Kuipers, O. P., P. G. G. A. de Ruyter, M. Kleerebezem, and W. M. de Vos.** 1997. Overproduction of proteins by lactic acid bacteria. Trends Biotechnol. **15:**135–140.
17. **Leenhouts, K., A. Bolhuis, G. Venema, and J. Kok.** 1998. Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl. Microbiol. Biotechnol. **49:**417–423.
18. **Lipinska, E.** 1973. Use of nisin-producing lactic streptococci in cheesemaking. Bull. Int. Dairy Fed. **73:**1–24.
19. **Lipinska, E.** 1977. Nisin and its application, p. 103–130. *In* M. Woodbine (ed.), Antimicrobials and antibiosis in agriculture. Butterworth, London, England.
20. **Maisnier-Patin, S., N. Deschamps, S. R. Tatini, and J. Richard.** 1992. Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter. Lait **72:**249–263.
21. **Marth, E. H.** 1978. Standard methods for the examination of dairy products, 14th ed. American Public Health Association, Inc., Washington, D.C.
22. **Marugg, J. D., C. F. Gonzalez, B. S. Kunka, A. M. Ledeboer, M. J. Pucci, M. Y. Toonen, S. A. Walker, L. C. M. Zoetmulder, and P. A. Vandenbergh.** 1992. Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. Appl. Environ. Microbiol. **58:**2360–2367.
23. **Muriana, P. M.** 1996. Bacteriocins for control of *Listeria* spp. in food. J. Food Prot. Suppl. **1:**54–63.
24. **O'Sullivan, D. J., and T. R. Klaenhammer.** 1993. Rapid mini-prep isolation of high-quality plasmid DNA from *Lactococcus* and *Lactobacillus* spp. Appl. Environ. Microbiol. **59:**2730–2733.
25. **Platteeuw, C., I. van Alen-Boerrigter, S. van Schalkwijk, and W. M. de Vos.** 1996. Food-grade cloning and expression system for *Lactococcus lactis*. Appl. Environ. Microbiol. **62:**1008–1013.
26. **Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vandenbergh.** 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. Appl. Environ. Microbiol. **54:**2349–2353.
27. **Romero, D.** 1998. Personal communication.
28. **Ryan, M. P., M. C. Rea, C. Hill, and R. P. Ross.** 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl. Environ. Microbiol. **62:**612–619.
29. **Schillinger, U., R. Geisen, and W. H. Holzapfel.** 1996. Potential of antagonistic microorganisms and bacteriocins for the biological preservation of foods. Trends Food Sci. Technol. **7:**158–164.
30. **Stecchini, M. L., I. Sarais, and M. Bertoldi.** 1991. The influence of *Lactobacillus plantarum* culture inoculation on the fate of *Staphylococcus aureus* and *Salmonella typhimurium* in Montasio cheese. Int. J. Food Microbiol. **14:**99–110.
31. **Sulzer, G., and M. Busse.** 1991. Growth inhibition of *Listeria* spp. on Camembert cheese by bacteria producing inhibitory substances. Int. J. Food Microbiol. **14:**287–296.
32. **Uljas, H., and J. B. Luchansky.** 1995. Characterization of bacteriocins produced by lactobacilli and their use to control calcium lactate crystal-forming lactic acid bacteria in Cheddar cheese. M.Sc. thesis. University of Wisconsin-Madison, Madison.
33. **Venema, K., J. Kok, J. D. Marugg, M. Y. Toonen, A. M. Ledeboer, G. Venema, and M. L. Chikindas.** 1995. Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0: PedB is the immunity protein and PedD is the precursor processing enzyme. Mol. Microbiol. **17:**515–522.
34. **Zottola, E. A., T. L. Yezzi, D. B. Ajao, and R. F. Roberts.** 1994. Utilization of Cheddar cheese containing nisin as an antimicrobial agent in other foods. Int. J. Food Microbiol. **24:**227–238.