APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1995, p. 2802–2805 Vol. 61, No. 7
0099-2240/95/$04.00+0

PCR Detection of the Lactocin S Structural Gene in Bacteriocin-Producing Lactobacilli from Meat

JUAN M. RODRÍGUEZ,* LUIS M. CINTAS, PILAR CASAUS, ANA SUÁREZ, AND PABLO E. HERNÁNDEZ

Departamento de Nutrición y Bromatología III, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

Received 30 January 1995/Accepted 29 April 1995

The lactocin S structural gene (*lasA*) in seven bacteriocinogenic lactobacilli isolated from fermented sausages was studied. Two degenerate primers were synthesized to amplify a 75-bp fragment of the gene. Three strains amplified the fragment from their plasmid DNA, and hybridization analysis confirmed these results.

Bacteriocins are proteinaceous compounds with a bactericidal or bacteriostatic mode of action against bacteria usually closely related to the producer organism (15, 35). Initially, the studies of bacteriocinogenicity within lactic acid bacteria (LAB) were focused on isolates associated with dairy products; however, the recent and increasing interest in LAB from other sources has resulted in the recognition of bacteriocinogenic activity of strains from meat (21). Bacteriocin production seems to be a common phenotype within LAB isolated from highly competitive environments such as fermented sausages (10). It has even been suggested that in mixed natural populations, and under adequate circumstances, the incidence of bacteriocin producer strains among fresh isolates of any gram-positive species may approach 100% (34).

The high potential of LAB to produce bacteriocins may imply the ability of different strains to produce identical bacteriocins rather than the existence of an almost unlimited number of bacteriocins still not identified. In this context, Cintas et al. (4) have recently recognized that sakacin M, a bacteriocin produced by *Lactobacillus sake* 148 (32), is identical to the well-characterized lactocin S, a lantibiotic produced by *L. sake* L45 (19, 20). The fact that the two bacteriocinogenic strains were originally isolated from Spanish and Norwegian dry fermented sausages, respectively, suggests that the genes responsible for the production of certain bacteriocins are conserved and widespread.

The purpose of this work was to determine, by basic molecular biology techniques, the prevalence of lactocin S-producing lactobacilli among bacteriocinogenic isolates from Spanish dry fermented sausages. Moreover, we also found it desirable to test the usefulness of the PCR technique to rapidly identify lactobacilli that may produce well-characterized bacteriocins, instead of relying on the use of complex biochemical techniques, which are usually required for the identification and characterization of such bacteriocins (13, 20, 36).

Bacterial strains, culture conditions, and bacteriocin assays. A total of 24 Spanish dry fermented sausages manufactured without added starter cultures were used as a source of LAB. Each sausage was produced and collected in a different region of Spain to ensure that there was no connection between them. The LAB were isolated from the sausages by following the procedure described by Sobrino et al. (33). Initially, 4,608 colonies, 192 per sausage, were randomly selected among those growing on double-layer plates of MRS agar (Oxoid Ltd., Basingstoke, United Kingdom). When screened for their direct antimicrobial antagonism (33) against *Pediococcus acidilactici* 347, a pediocin PA-1-producing strain (our own collection), *Lactobacillus plantarum* CECT285, and *Listeria monocytogenes* ScottA, around 30% of the isolates produced visible halos on the indicator organisms. The 21 isolates displaying the largest zones of inhibition were examined microscopically and tested for catalase production (33). These isolates comprised the 7 lactobacilli used in this work and the 14 cocci stored for further studies. The bacteriocin activity of the lactobacillus supernatants was checked by a well diffusion assay (24). All selected lactobacilli displayed activity against a number of spoilage and pathogenic microorganisms of concern in the food industry (Table 1).

All of the LAB used in this study were propagated and maintained in MRS medium, the propionibacterial strains were propagated and maintained in glucose yeast extract Lab Lemco medium (composition per liter of distilled water: peptone, 10 g; meat extract, 10 g; NaCl, 5 g; D-glucose, 5 g; yeast extract, 3 g; pH adjusted to 7.0), the clostridia were propagated and maintained in reinforced clostridial medium (Oxoid), and the rest of the bacteria were propagated and maintained in brain heart infusion broth (Oxoid). These media were supplemented with agar (1.5%) when needed for the antimicrobial assays. *L. sake* 148 was previously isolated and identified in our laboratory (33), while *L. sake* L45 was supplied by I. F. Nes, Laboratory of Microbial Gene Technology, As, Norway.

Molecular techniques. Two regions within the known sequence of lactocin S (20) were selected for degenerate primer synthesis. The nucleotide sequences were based on *Lactobacillus* codon usage, taking into account the 11 genes of this species available after searching in the GenBank database, using the Genetics Computer Group program (5). The DNA sequence of the coding strand primer (oligonucleotide 1) was 5′-ATG GAA TT(GA) TT(GA) CC(AG) AC(GT) GC(TC) GC(TC) GT(TC) (TC)T(GA) TA-3′, while that of the complementary strand primer (oligonucleotide 2) was 5′-ATG (AG)TG TTT (AG)GC (ACGT) (GC)(TA) (AG)TA (CT) TT-3′ (Fig. 1). The primers were used in PCR to amplify a 75-bp DNA fragment of the lactocin S structural gene from plasmid and chromosomal DNA of the selected strains.

Plasmid DNA from the lactobacilli was isolated by the sodium dodecyl sulfate (SDS) alkaline lysis method (28), using 20 mg of lysozyme per ml in the lysis buffer (19), and purified by ethidium bromide-cesium chloride density gradient ultracen-

* Corresponding author. Mailing address: Departamento de Nutrición y Bromatología III, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain. Phone: 34-1-3943747. Fax: 34-1-3943743.

TABLE 1. Activity of seven lactobacilli from meat against selected indicator microorganisms

Indicator strain	Origin[b]	Activity of strain[a]: V90 A	V90 B	V20 A	V20 B	B18 A	B18 B	C33 A	C33 B	SV124 A	SV124 B	AA24 A	AA24 B	BB48 A	BB48 B
Lactobacillus casei ATCC 334	ATCC	11.0	13.5	9.3	15.0					8.0	16.0	16.0	18.0		13.0
Lactobacillus fermentum CECT285	CECT		9.3	9.0	13.0		9.0		9.0	11.1	15.7	14.2	15.3		15.7
Lactobacillus plantarum NCDO1193	NCDO				7.6										7.0
Lactobacillus sake NCFB2714	NCFB	10.0	14.4								7.3	10.0	11.4		
Pediococcus pentosaceus FBB63	TNO	9.2	14.6						8.5			10.0	11.4		
Lactococcus cremoris CNRZ117	INRA	7.0	9.1		12.4				7.5		13.3	14.3	16.0		
Enterococcus faecalis EF	TNO		9.3						8.6			7.4	9.3	10.0	11.0
Propionibacterium acidipropionici NCDO573	NCDO		9.6				10.0				7.2	7.5	8.7	8.5	10.0
Propionibacterium acidipropionici P4	TNO		9.1				10.6				7.3	7.7	8.4	8.6	11.0
Clostridium perfringens CECT376	CECT		9.6		13.0				10.8		7.3	13.0	11.0		10.0
Clostridium botulinum CECT551	CECT		13.3		13.4				11.1		7.5	13.0	15.5		9.0
Listeria monocytogenes NCTC7973	NCTC		9.0				8.0					6.1	8.0	8.6	9.0
Listeria monocytogenes LI5sv1/2	FVM		9.0				11.0					6.2	8.6	8.0	14.4
Listeria monocytogenes NCTC5105	NCTC		8.3				9.4		11.3			6.6	9.4	9.5	10.5
Listeria monocytogenes LI1sv4	FVM		10.4				11.2					7.0	8.0	8.0	8.0
Listeria monocytogenes ScottA	FVM		9.0				9.6					7.0	8.3	8.0	9.0
Staphylococcus aureus FRI137	FRI		9.0				9.1					7.0	8.5	8.2	11.0
Staphylococcus aureus FRI 196E	FRI		9.0				9.6		8.0			7.0	8.0	9.0	9.6

[a] Data are diameters of inhibition zones (in millimeters). A, supernatant; B, 20-fold-concentrated supernatant.
[b] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CECT, Colección Española de Cultivos Tipo (Valencia, Spain); FRI, Food Research Institute (Madison, Wis.); FVM, Facultad de Veterinaria (Madrid, Spain); INRA, Institut National de la Recherche Agronomique, Station de Recherches Laitieres (Jou-en-Josas Cedex, France); NCDO, National Collection of Dairy Organisms (Reading, United Kingdom); NCFB, National Collection of Food Bacteria (Reading, United Kingdom); NCTC, National Collection of Type Cultures (London, United Kingdom); TNO, TNO Nutrition and Food Research (Zeist, The Netherlands).

trifugation. Chromosomal DNA was prepared according to the method of Lewington et al. (17). The PCR mixtures were prepared as described previously (7). Amplifications were carried out in an OMNIGENE thermal reactor (Hybaid Ltd., Teddington, United Kingdom), and the reaction parameters were 92°C for 2 min, 47°C for 2 min, and 72°C for 2 min for a total of 25 cycles. The amplified PCR fragments were visualized in 10% polyacrylamide gels. The gels were run at 100 V for 40 min, using the Bethesda Research Laboratories (Gaithersburg, Md.) 100-bp ladder as a molecular weight standard. Only plasmid DNA of the strains BB48, V20, and SV124 generated PCR-amplified bands of the expected size (Fig. 2), indicating that they encoded the *lasA* gene and, therefore, that they probably were lactocin S-producing strains. The three suspected lactocin S-producing strains were submitted to plasmid analysis. To visualize the plasmids, electrophoresis in 0.7% agarose gels (TBE buffer [Tris-borate-EDTA]) was performed at 100 V for 1 h. Plasmids extracted from *L. sake* L45 were used as molecular weight standards. The three strains exhibited plasmid profiles identical to those of *L. sake* L45 and *L. sake* 148, displaying plasmids pCIM1 (50 kbp) and pCIM2 (34 kbp), previously observed in *L. sake* L45 (19) (Fig. 3a). Plasmid DNA was transferred to nylon membranes for hybridization analysis. The two oligomers previously used as primers in the PCRs were labelled, using the 3′-oligolabelling system (Amersham International plc, Amersham, United Kingdom). Hybridizations were carried out at 42°C according to the instructions for the DNA labelling kit. Filters were washed twice in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.4% SDS for 10 min at 52°C and then washed twice in 2× SSC for 5 min at room temperature. The probes hybridized not only with pCIM1, the plasmid responsible for lactocin S production, but also with pCIM1 (Fig. 3b). Mørtvedt and Nes (19) have suggested that pCIM2 constitutes a deleted form of pCIM1. Alternatively, the amplified DNA fragment was employed as a probe, being labelled by the Probe-amp system (Amersham). It hybridized with the same plasmids; however, the signals were weak, probably because of the small size of the PCR fragment.

The isolation of lactobacilli with bacteriocinogenic activity from meat and meat products and their identification (1, 9, 10, 18, 25, 30, 33), as well as their usefulness for inhibiting or reducing the growth of and spoilage caused by pathogenic microorganisms in meat substrates (30), have already been reported. However, only a few bacteriocins produced by lac-

FIG. 1. (A) Amino acid sequence of the C-terminal part of lactocin S. (B) Regions of the known lactocin S sequence selected for oligonucleotide synthesis. 1 and 2, primers 1 and 2, respectively.

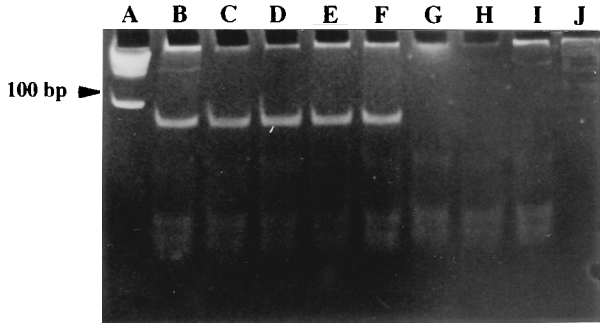

FIG. 2. Polyacrylamide gel electrophoresis of PCR fragments generated with primers 1 and 2 from plasmid DNA of the selected lactobacilli. Lanes: A, 100-bp ladder marker (Bethesda Research Laboratories); B, *L. sake* L45; C, *L. sake* 148; D, strain BB48; E, strain V20; F, strain SV124; G, strain AA24; H, strain B18; I, strain C33; J, strain V90.

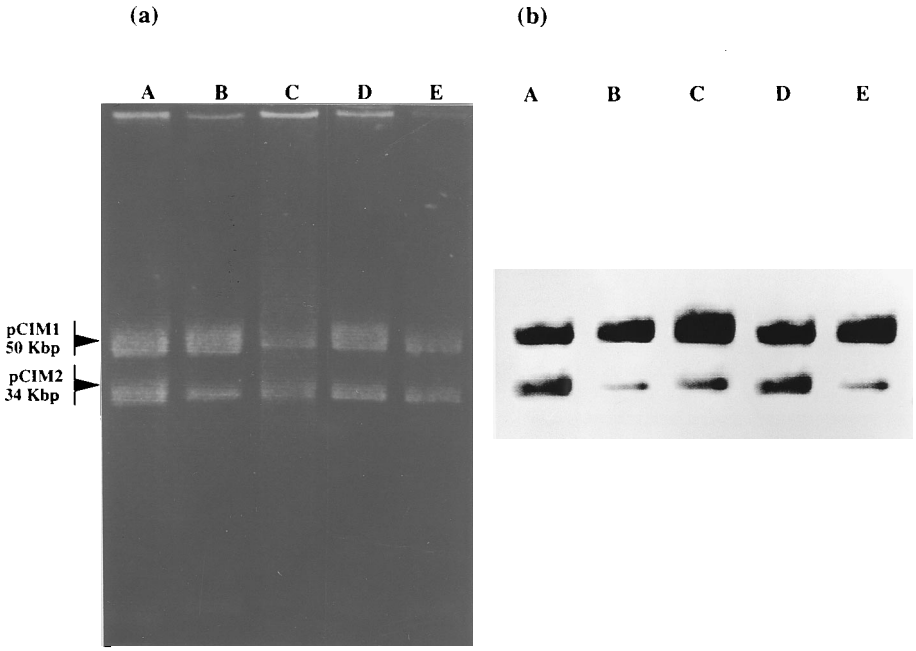

FIG. 3. (a) Agarose gel electrophoresis of plasmid DNA of the selected lactobacilli. (b) Autoradiogram prepared after hybridization with oligonucleotide 3 as a probe. Lanes: A, *L. sake* L45; B, *L. sake* 148; C, strain BB48; D, strain V20; E, strain SV124.

tobacilli from meat have been well characterized. Among them, only lactocin S (19, 20), curvacin A and sakacin P (36), and sakacin A (2, 13) have been completely characterized. Lactocin S was the first bacteriocin from an *L. sake* strain to be purified and characterized to date, and, to date, remains the only known lantibiotic produced by lactobacilli. Although in our study three of the lactobacilli isolated from meat are probably lactocin S producers, their activities against selected indicator microorganisms differ (Table 1). While strains V20 and SV124 have an *L. sake* L45-like activity (19), the antimicrobial activity of BB48 closely resembles that of *L. sake* 148 (33). These differences may be due to the presence of transposable elements that affect lactocin S production or to the existence of other regulatory mechanisms still unknown. Transposable elements affecting lactocin S production in *L. sake* L45 have already been described (31).

One of the characteristics of lantibiotics, their broader antimicrobial spectrum when compared with other classes of bacteriocins (16), along with the excellent adaptation of *L. sake* strains to growth in meat and meat products (11), may help to explain why *L. sake* strains producing lactocin S are commonly found in environments as highly competitive as fermented sausages. At present, only five lantibiotics produced by LAB have been described (16). Nisin has a record of safe use as a food preservative, being the first bacteriocin with practical application in the food industry. In this context, the prospect of a future application of lactocin S in foods should be evaluated regarding its activity against pathogens of concern in the food industry such as *Clostridium* spp., *L. monocytogenes*, *Staphylococcus aureus*, and others (26, 27, 33).

Molecular biology techniques can be of great value for identifying strains with the ability to produce well-characterized bacteriocins. Plasmid analysis, DNA fingerprinting, and RNA typing techniques have been used to confirm strain identities among bacteriocin-producing pediococci (3, 14, 23). The use of genetic probes containing a fragment of the structural gene for nisin has permitted the characterization of nisin-producing strains of *Lactococcus lactis* subsp. *lactis* isolated from vegetables (12), while the amplification of the nisin structural gene by PCR followed by direct sequencing of the amplified product has been used to evaluate the distribution of nisin variants in different lactococcal strains from dairy products and meat (6, 24).

In this work, we have investigated whether a number of independently isolated bacteriocinogenic lactobacilli from meat encode the *lasA* gene. The selection of primers for PCR amplification of the structural genes of bacteriocins has to be done with care, as it is known that bacteriocins produced by LAB may share consensus sequences (8, 22). In initial screenings, the use of reliable molecular biology techniques may facilitate the identification of LAB strains producing already described bacteriocins, thus avoiding the utilization of complex biochemical techniques and the duplication of research efforts involved in the purification and characterization of such bacteriocins.

The skillful technical assistance of all the staff of the Prokaryotic Genetics Group (Genetics and Microbiology Department, AFRC Institute of Food Research, Norwich, United Kingdom) is gratefully acknowledged. We also thank I. F. Nes and H. Holo, Laboratory of Microbial Gene Technology, As (Norway), for their sharing of unpublished biochemical and genetic data on lactocin S, as well as for providing us with *L. sake* L45.

Part of this work has been supported by grant ALI91-0255 from the Comisión Interministerial de Ciencia y Tecnología (CICYT), Spain, and by the Commission of European Communities (Contract BIOT-CT91-0263).

REFERENCES

1. **Ahn, C., and M. E. Stiles.** 1990. Antibacterial activity of lactic acid bacteria isolated from vacuum-packaged meats. J. Appl. Bacteriol. **69:**302–310.
2. **Axelsson, L., A. Holck, S. E. Birkeland, T. Aukrust, and H. Blom.** 1993. Cloning and nucleotide sequence of a gene from *Lactobacillus sake* Lb706 necessary for sakacin A production and immunity. Appl. Environ. Microbiol. **59:**2868–2875.
3. **Bhunia, A. K., T. K. Bhowmik, and M. G. Johnson.** 1994. Determination of bacteriocin-encoding plasmids of *Pediococcus acidilactici* strains by Southern

hybridization. Lett. Appl. Microbiol. **18:**168–170.
4. **Cintas, L. M., P. Casaus, J. M. Rodríguez, W. L. Moreira, O. J. Sobrino, M. F. Fernández, and P. E. Hernández.** 1993. Further isolation and biochemical characterization of bacteriocins of lactic acid bacteria of meat origin, abstr. P-57. *In* Abstracts of the 3rd Meeting of Contractors of BRIDGE T-Project on Biotechnology of Lactic Acid Bacteria, Athens.
5. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **De Vos, W. M., J. W. M. Mulders, R. J. Siezen, J. Hugenholtz, and O. P. Kuipers.** 1993. Properties of nisin Z and distribution of its gene, *nisZ*, in *Lactococcus lactis*. Appl. Environ. Microbiol. **59:**213–218.
7. **Dodd, H. M., N. Horn, and M. J. Gasson.** 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. **136:**555–566.
8. **Fremaux, C., C. Ahn, and T. R. Klaenhammer.** 1993. Molecular analysis of the lactacin F operon. Appl. Environ. Microbiol. **59:**3906–3915.
9. **Garriga, M., M. Hugas, T. Aymerich, and J. M. Monfort.** 1993. Bacteriocinogenic activity of lactobacilli from fermented sausages. J. Appl. Bacteriol. **75:**142–148.
10. **Garver, K. I., and P. M. Muriana.** 1993. Detection, identification and characterization of bacteriocin-producing lactic acid bacteria from retail food products. Int. J. Food Microbiol. **19:**241–258.
11. **Hammes, W. P., A. Bantleon, and S. Min.** 1990. Lactic acid bacteria in meat fermentation. FEMS Microbiol. Rev. **87:**165–174.
12. **Harris, L. J., H. P. Fleming, and T. R. Klaenhammer.** 1992. Characterization of two nisin-producing *Lactococcus lactis* subsp. *lactis* strains isolated from commercial sauerkraut fermentation. Appl. Environ. Microbiol. **58:**1477–1483.
13. **Holck, A., L. Axelsson, S. E. Birkeland, T. Aukrust, and H. Blom.** 1992. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb706. J. Gen. Microbiol. **138:**2715–2720.
14. **Jager, K., and S. Harlander.** 1992. Characterization of a bacteriocin from *Pediococcus acidilactici* PC and comparison of bacteriocin-producing strains using molecular typing procedures. Appl. Microbiol. Biotechnol. **37:**631–637.
15. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70:**337–349.
16. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–68.
17. **Lewington, J., S. D. Greenaway, and B. J. Spillane.** 1987. Rapid small scale preparation of bacterial genome DNA suitable for cloning and hybridization analysis. Lett. Appl. Microbiol. **5:**51–53.
18. **Lewus, C. B., A. Kaiser, and T. J. Montville.** 1991. Inhibition of food-borne pathogens by bacteriocins from lactic acid bacteria isolated from meat. Appl. Environ. Microbiol. **57:**1683–1688.
19. **Mørtvedt, C. I., and I. F. Nes.** 1990. Plasmid-associated bacteriocin production by a *Lactobacillus sake* strain. J. Gen. Microbiol. **136:**1601–1607.
20. **Mørtvedt, C. I., J. Nissen-Meyer, K. Sletten, and I. F. Nes.** 1991. Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. Appl. Environ. Microbiol. **57:**1829–1834.
21. **Nettles, C. G., and S. F. Barefoot.** 1993. Biochemical and genetic characteristics of bacteriocins of food associated lactic acid bacteria. J. Food Prot. **56:**338–356.
22. **Nieto Lozano, J. C., J. Nissen-Meyer, K. Sletten, C. Pelaz, and I. F. Nes.** 1992. Purification and amino acid sequence of a bacteriocin produced by *Pediococcus acidilactici*. J. Gen. Microbiol. **138:**1985–1990.
23. **Noerlis, Y., and B. Ray.** 1994. Factors influencing immunity and resistance of *Pediococcus acidilactici* to the bacteriocin, pediocin AcH. Lett. Appl. Microbiol. **18:**138–143.
24. **Rodríguez, J. M., L. M. Cintas, P. Casaus, N. Horn, H. Dodd, P. E. Hernández, and M. J. Gasson.** 1995. Isolation of nisin-producing strains from dry fermented sausages. J. Appl. Bacteriol. **78:**109–115.
25. **Rodríguez, J. M., O. J. Sobrino, M. F. Fernández, P. E. Hernández, and B. Sanz.** 1989. Antimicrobial activity of lactic acid bacteria isolated from Spanish dry fermented sausages, p. 308–312. *In* Proceedings of the 35th International Congress on Food Science Technology, Copenhagen.
26. **Rodríguez, J. M., O. J. Sobrino, W. L. Moreira, L. M. Cintas, P. Casaus, M. F. Fernández, B. Sanz, and P. E. Hernández.** 1994. Inhibition of *Yersinia enterocolitica* by *Lactobacillus sake* strains of meat origin. Meat Sci. **37:**305–313.
27. **Rodríguez, J. M., O. J. Sobrino, W. L. Moreira, L. M. Cintas, P. Casaus, M. F. Fernández, B. Sanz, and P. E. Hernández.** 1994. Inhibition of *Listeria monocytogenes* by *Lactobacillus sake* strains of meat origin. Meat Sci. **38:**17–26.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Schillinger, U., M. Kaya, and F. K. Lücke.** 1991. Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sake*. J. Appl. Bacteriol. **70:**473–478.
30. **Schillinger, U., and F. K. Lücke.** 1989. Antimicrobial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. **55:**1901–1906.
31. **Skaugen, M., and I. F. Nes.** 1994. Transposition in *Lactobacillus sake* and its abolition of lactocin S production by insertion of IS*1163*, a new member of the IS*3* family. Appl. Environ. Microbiol. **60:**2818–2825.
32. **Sobrino, O. J., J. M. Rodriguez, W. L. Moreira, L. M. Cintas, M. F. Fernandez, B. Sanz, and P. E. Hernandez.** 1992. Sakacin M, a bacteriocin-like substance from *Lactobacillus sake* 148. Int. J. Food Microbiol. **16:**215–225.
33. **Sobrino, O. J., J. M. Rodriguez, W. L. Moreira, M. F. Fernandez, B. Sanz, and P. E. Hernandez.** 1991. Antibacterial activity of *Lactobacillus sake* isolated from dry fermented sausages. Int. J. Food Microbiol. **13:**1–10.
34. **Tagg, J. R.** 1992. Bacteriocins of gram-positive bacteria: an opinion regarding their nature, nomenclature and numbers, p. 33–35. *In* R. James, C. Lazdunski, and F. Pattus (ed.), Bacteriocins, microcins and lantibiotics. Springer-Verlag, Berlin.
35. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40:**722–756.
36. **Tichaczek, P. S., J. Nissen-Meyer, I. F. Nes, R. F. Vogel, and W. P. Hammes.** 1992. Characterization of the bacteriocin curvacin A from *Lactobacillus curvatus* LTH1174 and sakacin P from *Lactobacillus sake* LTH673. Syst. Appl. Microbiol. **15:**460–468.