APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1996, p. 1283–1286 Vol. 62, No. 4
0099-2240/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Nucleotide Sequence and Taxonomical Distribution of the Bacteriocin Gene *lin* Cloned from *Brevibacterium linens* M18

NATALIA VALDES-STAUBER AND SIEGFRIED SCHERER*

Institut für Mikrobiologie, Forschungszentrum für Milch und Lebensmittel Weihenstephan, Technische Universität München, D-85350 Freising, Germany

Received 29 November 1995/Accepted 10 January 1996

Linocin M18 is an antilisterial bacteriocin produced by the red smear cheese bacterium *Brevibacterium linens* M18. Oligonucleotide probes based on the N-terminal amino acid sequence were used to locate its single copy gene, *lin*, on the chromosomal DNA. The amino acid composition, N-terminal sequence, and molecular mass derived from the nucleotide sequence of an open reading frame of 798 nucleotides coding for 266 amino acids found on a 3-kb *Bam*HI restriction fragment correspond closely to those obtained from the purified protein (N. Valdés-Stauber and S. Scherer, Appl. Environ. Microbiol. 60:3809–3814, 1994). No sequence homology to any protein or nucleotide sequences deposited in databases was found. Comparison of the nucleotide sequence and the N-terminal amino acid sequence derived from the protein suggests that *B. linens* M18 produces an *N*-formyl-methionyl-CAC tRNA. A wide taxonomical distribution of the gene within coryneform bacteria has been demonstrated by PCR amplification. The structural gene from linocin M18 is present at least in three *Brevibacterium* species, five *Arthrobacter* species, and five *Corynebacterium* species.

The last decade has seen the description and characterization of a variety of bacteriocins of gram-positive bacteria (9). Most of them are produced by lactic acid bacteria with activity spectrums usually restricted to closely related strains (8, 12). The extraordinary interest in bacteriocins is based primarily on their potential to inhibit food-borne pathogens such as *Listeria monocytogenes* (7). However, detailed knowledge about bacteriocins from coryneform bacteria in general and *Brevibacterium linens* in particular is almost nonexistent. Because of its proteolytic activity and the production of flavor components, this bacterium plays an important role during the surface ripening of smear cheeses. Because there is a risk for these products to be contaminated with *L. monocytogenes*, considerable interest in developing ripening cultures with antilisterial potential exists. It has been demonstrated that *B. linens* strains exhibit antagonistic action against *L. monocytogenes* (15, 17). A bacteriolytic substance from *B. linens* OC2 with a molecular mass of 2.4 kDa and an unusual stability towards proteases, heat, pH, and organic solvents was not classified as a bacteriocin (14). We have recently reported the isolation and characterization of non-lanthionine-containing linocin M18 from the red smear cheese bacterium *B. linens* M18 (18), which is the only bacteriocin isolated so far from coryneform bacteria. Its native molecular mass is larger than 2,000 kDa, and the bacteriocin has an unusually wide activity spectrum, inhibiting strains of several genera of gram-positive bacteria, including *L. monocytogenes*, *Bacillus cereus* (18), and *Staphylococcus aureus* (2a). In this paper, we report its nucleotide sequence as well as its occurrence in other corynebacteria.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. *B. linens* M18 and other coryneform bacteria were grown in plate count broth supplemented with 3% NaCl (PC^+-broth) as described previously (18). For cloning, plasmid pBluescript II SK^+ (Stratagene Cloning Systems, La Jolla, Calif.), *Escherichia coli* DH5α competent cells (Gibco-BRL Inc., Eggenstein, Germany), and standard protocols according to Sambrook et al. (16) were used.

DNA isolation. Plasmid isolation from *B. linens* M18 and total DNA isolation from other brevibacteria was done by the alkaline-sodium dodecyl sulfate (SDS) method described by Sambrook et al. (16). Chromosomal DNA extraction from *B. linens* M18 was done according to the method of Flamm et al. (3), with the following modifications (final concentrations are given): mutanolysin (1.5 μg/ml) and lysozyme (2 μg/ml) were used in the lysis buffer, proteinase K (0.2 mg/ml) was used instead of pronase E, and RNase A (10 μg/ml; United States Biochemical Corp.) was used.

Cloning and DNA sequencing. Positive restriction fragments were detected by Southern hybridization of digoxigenin-dUTP-labelled (3′-end labelling kit; Boehringer) oligonucleotide probes derived from the N-terminal amino acid sequence of linocin M18 (18). The following probes were constructed: oligonucleotide L (ATGAAYAAYYTITAYMGIGAR), based on amino acid positions 1 to 7, and oligonucleotides W (GCWCCWATTCCWGGHCCWGCWGCWG CWGAAATTGAA) and I (GCICCIATICCIGGICCIGCIGCIGCIGAIATIG AA), based on amino acid positions 9 to 20 (where H is A, C, or T; I is inosin; M is A or C; R is A or G; W is A or T; and Y is C or T). The probes were allowed to hybridize for 14 h at various temperatures. Membranes were washed twice for 10 min each at room temperature with 2× SSC (0.75 M NaCl, 75 mM trisodium citrate [pH 7])–0.1% SDS and finally for 5 min with 0.1× SSC–0.1% SDS at the hybridization temperature. Chemiluminescent detection was done according to the manufacturer's instructions (Boehringer). For cloning and DNA sequencing, standard procedures were used throughout (with the Sequenase Version 2.0 DNA Sequencing Kit [United States Biochemical Corp.] and α-^{35}S-dATP).

DNA sequences were analyzed by the programs MacMolly Tetra (Soft Gene, Berlin, Germany) and DNASIS/PROSIS (Hitachi, Ardon, Germany) and the databases GenBank R81.0, February 1994; EMBL R38, March 1994; PIR R40.0, March 1994; and SWISS-Prot R28, January 1994.

Gene amplifications. PCR was carried out with *Taq* DNA polymerase (Boehringer) and a Perkin-Elmer Cetus DNA thermocycler, with 35 cycles (1 min at 94°C, 45 s at 68°C, and 1 min at 72°C) at final concentrations of 0.2 mM each nucleotide, 1.5 mM $MgCl_2$, 0.5 ng of chromosomal DNA (or 0.5 μg of total DNA when plasmid isolation protocols were performed), 0.05 U of *Taq* DNA polymerase per μl, and 25 pmol (each) of the primers 5′-CGACGACAGCCTCG GCATC-3′, its 3′ end being located 23 nucleotides (nt) upstream of *lin* in an open reading frame (ORF) of unknown function, and 5′-GGCGGAGAAGCTG TCCTGG-3′, its 3′ end being located 286 nt downstream of the stop codons of the structural gene of linocin M18 in another ORF of unknown function. Amplification products with the expected size of 1,151 nt were detected by agarose gel electrophoresis and subjected to dot blot hybridization at high stringency conditions (70°C, 0.1× SSC) to a 827-nt *Sac*I restriction fragment containing most of the *lin* sequence, except 123 nt of the 3′ end. The *Sac*I fragment was prepared from agarose gels by agarase treatment and labelled with digoxigenin by random priming (Random Primed DNA Labelling Kit; Boehringer).

Nucleotide sequence accession number. The nucleotide sequence of the *lin* gene of *B. linens* M18 described in this study has been assigned accession no. X93588 in the EMBL database.

* Corresponding author. Phone: 49-8161-713516. Fax: 49-8161-714512. Electronic mail address: 100424.2340@compuserve.com.

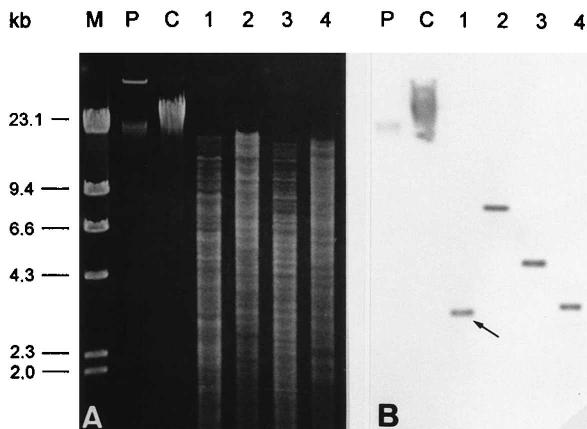

FIG. 1. (A) Agarose gel electrophoresis of extrachromosomal DNA (lane P), undigested chromosomal DNA (lane C), and *Bam*HI (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3), and *Cla*I (lane 4) restriction enzyme-digested chromosomal DNA preparations from *B. linens* M18. High-molecular-weight extrachromosomal DNA is visible when the preparation is assayed according to the plasmid isolation protocol. Lane M, molecular size standards (with sizes indicated on the left). (B) Southern hybridization of extrachromosomal (lane P), undigested (lane C), and restriction enzyme-digested (lanes 1 to 4, as described for panel A) and agarose gel-fractionated chromosomal DNA of *B. linens* M18 with the *Sac*I-*Sac*I random-labelled restriction fragment containing most of the *lin* gene at 65°C; hybridization to oligonucleotide D gave identical results. The arrow indicates the fragment that has been cloned.

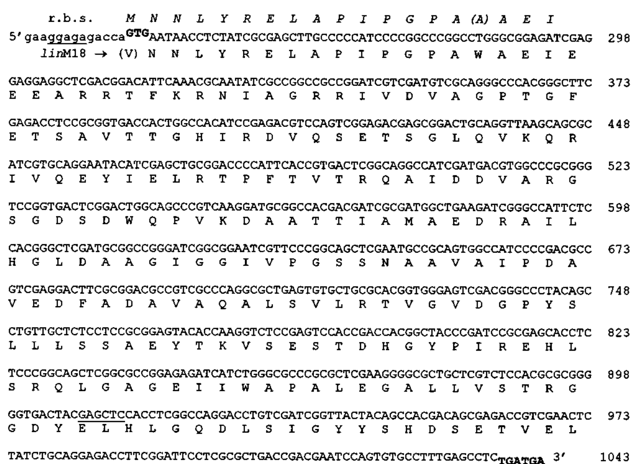

FIG. 2. Nucleotide and derived amino acid sequences of *lin*, the structural gene of linocin M18. The putative ribosome binding site (r.b.s.) as well as a *Sac*I cleaving site at position 908 used to produce a fragment for hybridization to amplified *lin* is underlined. Putative start and stop codons are indicated by bold type. The N-terminal amino acid sequence obtained by Edman degradation (18) is given in italics.

RESULTS AND DISCUSSION

Nucleotide sequence of linocin M18 gene. Oligonucleotide probes were hybridized to Southern blots of restriction endonuclease-digested chromosomal DNA from *B. linens* M18. No specific signals were found with probes W and I. Positive signals were found with unspecific oligonucleotide L but only at a low hybridization temperature of 32°C, and there was always more than one signal per lane of restricted DNA (data not shown). A 6-kb *Pst*I restriction fragment found by probe L was cloned and completely sequenced. The gene coding for linocin M18 was not found, but instead, the DNA sequence which hybridized to probe L was determined. Since there are no known nucleotide sequences from *B. linens* and because the failure to obtain specific hybridization of the probes used could have been due to the unknown codon usage of this bacterium, we searched the 6-kb *Pst*I fragment for ORFs and derived a hypothetical codon usage to obtain probe D (ATGAAYAA YCTNTAYCGNGAR, where Y is C or T; N is G, A, T, or C; and R is A or G), which yielded clear single positive signals when hybridized to chromosomal DNA (Fig. 1B). A 3-kb *Bam*HI restriction fragment detected by probe D was cloned, and both DNA strands were sequenced. An ORF of 798 nt was preceded by a potential ribosome binding site (Fig. 2), coding for a protein of 266 amino acids. The calculated amino acid composition is in agreement with that obtained from protein analysis (18). Since in both cases no cysteine residues and only one methionine were detected, intramolecular sulfur bridges were not expected to occur in the tertiary structure. Hydrophobicity analysis showed no strongly defined domains. No signal peptide structures were found in the 235 nt upstream from *lin*. Moreover, no homology to cleavage site amino acid motifs described for other bacteriocins was found (12). The molecular mass for linocin M18 detected by SDS-polyacrylamide gel electrophoresis (PAGE) is 29 to 31 kDa, and the one derived from the ORF is 28.5 kDa. The difference may be due to SDS-PAGE error or to covalently bound residues leading to decreased electrophoretic mobility.

Seventeen of 19 amino acids translated from the 5′ end of the ORF were identical to those found by Edman degradation of linocin M18 (18). Exceptions were Trp-16 instead of Ala-16 and the start codon GTG instead of ATG for Met-1 found by Edman degradation. By rechecking the results of the Edman degradation experiment, we found that the occurrence of amino acid Ala-16 was only slightly more likely than that of Trp-16 in the single Edman degradation yielding residues 14 to 19. The misidentification by Valdés-Stauber and Scherer (18) was clearly due to Ala-15 and Ala-17 flanking Trp-16. Methionine at position 1 was found twice in two independent N-terminal sequence analyses, and GTG was clearly established by sequencing both strands of two independent positive clones. There could be two possible explanations. (i) Two genes coding for two highly similar bacteriocin genes exist in *B. linens*. This possibility is most unlikely because only one hybridization signal was seen when probe D was hybridized to different restriction digests of genomic DNA. Identical hybridization patterns were obtained when the labelled *Sac*I-*Sac*I restriction fragment carrying most of the *lin* sequence and the amplified

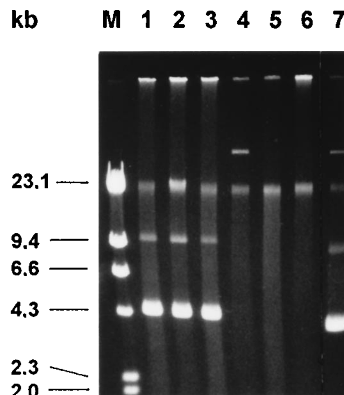

FIG. 3. Total DNA obtained for *lin*-positive *B. linens* strains when a plasmid isolation protocol was used. Lanes: M, molecular size standards (with sizes indicated on the left); 1 to 7, strains IO1, G2, M10, M18, M19, WS 1954, and WS 1942, respectively.

and labelled *lin* gene itself (Fig. 1B) were used as the hybridization probes. We conclude that *lin* is a single copy gene with no close homologs. (ii) Although posttranslational exchange of valine and methionine is also most unlikely, it has been found for other gram-positive bacteria that GTG is frequently used as a start codon (5). Interestingly, two other ORFs of unknown functions downstream of *lin*, including putative ribosome binding sites, also start with GTG (data not shown). We, therefore, suggest that *B. linens* M18 produces an *N*-formyl-methionyl-CAC tRNA.

No homology of the *lin* sequence to any sequence accessible in nucleotide and protein sequence databases was found. We conclude that linocin M18 is a novel bacteriocin.

Localization of bacteriocin genes. Usually, bacteriocin genes produced by gram-positive bacteria are located on plasmids (9), but location on chromosomal DNA has, for example, been reported for lactacin B (1), plantaricin A (2), jenseniin G (4), and helveticin J (10). Genetic determinants for nisin have been found on both plasmid and chromosomal DNA (12). There are several reports dealing with the occurrence of plasmids in *B. linens* (6, 11), but little is known about their physical properties and functions. We prepared DNA from *B. linens* M18 using both plasmid and chromosomal DNA preparation protocols. Our strain harbors high-molecular-weight extrachromosomal DNA whose molecular weight has not been determined (Fig. 1A) because hybridization of the probes to extrachromosomal DNA did not occur (Fig. 1B). Plasmids were also found in some other *B. linens* strains (Fig. 3), but the presence of visible extrachromosomal DNA did not correlate with either the presence of antilisterial activity or the detection of *lin* as shown by PCR. Plasmid-curing experiments with *B. linens* M18 by treatment for 6 h with protoplasting buffer (0.5 M sucrose, 0.01 M Tris-HCl, 2 mg of lysozyme per ml [pH 8]) or ethidium bromide (2 μg/ml), acriflavine (20 μg/ml), or acridine orange (20 μg/ml) or by raising the incubation temperature up to 45°C failed. Since it was not possible to obtain plasmid DNA without contaminating chromosomal DNA (Fig. 1A and 3), it cannot be strictly excluded that plasmid DNA carrying the bacteriocin gene is copurified by the chromosomal DNA preparation protocol and, additionally, comigrates with chromosomal DNA in agarose gels. However, because we were able to amplify the bacteriocin gene from a variety of different, extremely highly diluted brevibacterial chromosomal DNA preparations by PCR (see below), this possibility is considered very unlikely, and we suggest that the bacteriocin gene is located on the chromosome.

Taxonomic distribution of *lin*. Fifty-two isolates of different species of the genera *Brevibacterium*, *Arthrobacter*, and *Corynebacterium* were probed for *lin* by PCR (Table 1). The gene could be amplified from 12 of 26 *B. linens* strains isolated from different cheeses. The 5′ ends of the nucleotide sequences of PCR products of *B. linens* M19, WS 1954, and WS 2165 were determined and found to be identical to the *lin* sequence, including GTG as a presumptive start codon. Only eight of the *lin*-positive *B. linens* strains showed antilisterial activity. Amplification of *lin* without demonstration of antilisterial activity is not surprising because low levels of bacteriocin production are below the detection limit of the assay and the optimal conditions for bacteriocin production are unknown. *B. linens* WS 1976 does not harbor *lin* but clearly exhibits antilisterial activity (Table 1). This discrepancy may be due to the production of any antagonistic substance, perhaps another bacteriocin. A few mutations at the positions of the PCR primers used may also lead to negative amplification of *lin*. Furthermore, amplification of *lin* was achieved with one strain of "*Brevibacterium flavum*," one strain of *Brevibacterium lyticum*, five of six *Arthrobacter* species, and five of nine *Corynebacterium* sp.

TABLE 1. Presence of *lin* in *Brevibacterium*, *Arthrobacter*, and *Corynebacterium* isolates[a]

Species and strain[b]	Antilisterial activity[c]	Amplification of *lin*[d]	Hybridization to *Sac*I probe[e]
A. citreus WS 1749	−	+	+
A. globiformis WS 2074	+	+	+
A. nicotianae WS 1765	+	+	+
A. oxydans WS 1762	+	+	+
A. protophormiae WS 1779	+	+	+
A. variabilis WS 2213	+	−	−
C. fascians WS 1142	−	+	+
C. ammoniagenes			
WS 1973	+	+	+
WS 2211	−	−	−
WS 2212	−	−	−
C. glutamicum			
WS 1462	−	−	−
WS 1492	+	+	+
WS 1795	+	−	−
C. variabilis WS 1646	−	+	+
C. xerosis WS 1600	+	+	+
B. casei			
WS 2124	−	−	−
WS 2125	−	−	−
WS 2127	−	−	−
WS 2128	−	−	−
B. chang-fua WS 1084	−	−	−
B. fermentans WS 1957	−	−	−
"*B. flavum*" WS 1575	+	+	+
B. imperiale WS 1959	−	−	−
"*B. lactofermentum*" WS 1579	−	−	−
B. oxydans WS 1969	−	−	−
B. lyticum WS 1775	+	+	+
B. linens			
G2	+	+	+
D2	−	−	−
D7	−	−	−
IO1	+	+	+
M10	+	+	+
M17	+	+	+
M18	+	+	+
M19	+	+	+
WS 1692	−	−	−
WS 1694	−	−	−
WS 1782	−	−	−
WS 1917	−	−	−
WS 1939	−	−	−
WS 1942	−	+	+
WS 1945	−	+	+
WS 1946	−	−	−
WS 1949	−	−	−
WS 1950	−	−	−
WS 1951	−	−	−
WS 1952	−	+	+
WS 1954	+	+	+
WS 1968	−	−	−
WS 1975	−	−	−
WS 1976	+	−	−
WS 1978	−	+	+
WS 2165	+	+	+

[a] The results of the activity assay, PCR amplification, and hybridization of PCR products to the *Sac*I restriction fragment containing most of the *lin* gene are shown.

[b] Quotation marks indicate an uncertain species status (13). WS, Weihenstephan Collection of Bacteria and Yeasts, Institute for Microbiology, Forschungszentrum für Milch und Lebensmittel Weihenstephan, Freising, Germany.

[c] Antilisterial activity was determined as described previously (18).

[d] Positive gene amplification results indicate that fragments of the expected size were amplified.

[e] For hybridization conditions, see Materials and Methods.

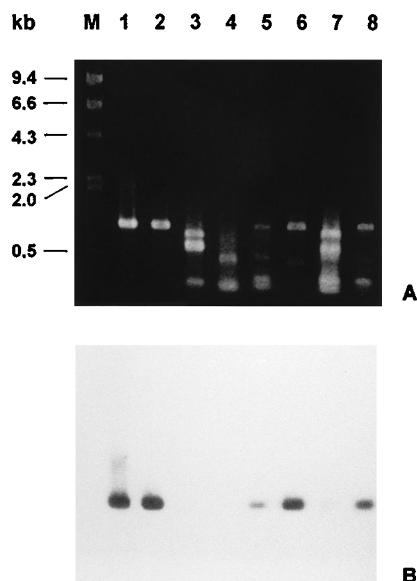

FIG. 4. (A) Agarose gel fractionation of PCR products obtained after amplification of *lin*. Lane M, molecular size standards (with sizes indicated on the left). (B) Southern hybridization of agarose gel-fractionated PCR products to the random digoxygenin-labelled *Sac*I-*Sac*I restriction fragment containing most of the *lin* gene at 70°C. Lanes: 1 to 3, *B. linens* M18, WS 2165, and WS 1968, respectively; 4, *B. imperiale* WS 1959; 5, "*B. flavum*" WS 1575; 6, *A. globiformis* WS 2074; 7, *Arthrobacter variabilis* WS 2213; 8, *C. glutamicum* WS 1492.

strains which, in general, were also active against *Listeria* spp. (Table 1). The results of agarose gel fractionation of several PCR products and Southern hybridization with a randomly labelled *Sac*I-*Sac*I restriction fragment containing most of *lin* are shown in Fig. 4. Our results establish a surprisingly wide distribution of the structural gene encoding this linocin M18 within coryneform bacteria isolated from dairy products.

ACKNOWLEDGMENT

This study was supported in part by the Bayerisches Staatsministerium für Ernährung, Landwirtschaft und Forsten.

REFERENCES

1. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **45:**1808–1815.
2. **Diep, D. B., L. S. Håvarstein, J. Nissen-Meyer, and I. F. Nes.** 1994. The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an *agr*-like regulatory system. Appl. Environ. Microbiol. **60:**160–166.

2a. **Eppert, I., N. Valdés-Stauber, and S. Scherer.** Unpublished results.

3. **Flamm, R. K., D. J. Hinrichs, and M. F. Thomashow.** 1984. Introduction of pAMβ1 into *Listeria monocytogenes* by conjugation and homology between native *L. monocytogenes* plasmids. Infect. Immun. **44:**157–161.
4. **Grinstead, D. A., and S. F. Barefoot.** 1992. Jenseniin G, a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. **58:**215–220.
5. **Hager, P. W., and J. C. Rabinowitz.** 1985. Translational specificity in *Bacillus subtilis*, p. 1–29. *In* D. Dubnau (ed.) The molecular biology of bacilli. Academic Press, Inc., New York.
6. **Holz, C., N. Domeyer, and B. Kunz.** 1992. Occurrence and physical properties of plasmids in *Brevibacterium linens*. Milchwissenschaft **47:**705–707.
7. **Holzapfel, W. H., R. Geisen, and U. Schillinger.** 1995. Biological preservation of food with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. **24:**343–362.
8. **Hoover, D. G., and L. R. Steenson (ed.).** 1993. Bacteriocins of lactic acid bacteria. Academic Press Inc., San Diego, Calif.
9. **Jack, R. W., J. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
10. **Joerger, M. C., and T. R. Klaenhammer.** 1990. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. **167:**439–446.
11. **Kato, F., N. Hara, K. Matsuyama, K. Hattori, M. Ishii, and A. Murata.** 1989. Isolation of plasmids from *Brevibacterium*. Agric. Biol. Chem. **53:**879–881.
12. **Klaenhammer, T. R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–86.
13. **Liebl, W., M. Ehrmann, W. Ludwig, and K. H. Schleifer.** 1991. Transfer of *Brevibacterium divaricatum* DSM 20297^T, "*Brevibacterium flavum*" DSM 20411, "*Brevibacterium lactofermentum*" DSM 20412 and DSM 1412, and *Corynebacterium lilium* DSM 20137^T to *Corynebacterium glutamicum* and their distinction by rRNA gene restriction patterns. Int. J. Syst. Bacteriol. **41:**255–260.
14. **Maisnier-Patin, S., and J. Richard.** 1995. Activity and purification of linenscin OC2, an antibacterial substance produced by *Brevibacterium linens* OC2, an orange cheese coryneform bacterium. Appl. Environ. Microbiol. **61:**1847–1852.
15. **Ryser, E. T., S. Maisnier-Patin, J. J. Gratadoux, and J. Richard.** 1994. Isolation and identification of cheese-smear bacteria inhibitory to *Listeria* sp. Int. J. Food Microbiol. **21:**237–246.
16. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Valdés-Stauber, N., H. Götz, and M. Busse.** 1991. Antagonistic effect of coryneform bacteria from red smear cheese against *Listeria* species. Int. J. Food Microbiol. **13:**119–130.
18. **Valdés-Stauber, N., and S. Scherer.** 1994. Isolation and characterization of linocin M18, a bacteriocin produced by *Brevibacterium linens*. Appl. Environ. Microbiol. **60:**3809–3814.