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NOTES

Cloning, Expression, and Nucleotide Sequence of Genes Involved in Production of Lactococcin DR, a Bacteriocin from *Lactococcus lactis* subsp. *lactis*

A. RINCE,[1] A. DUFOUR,[1]† S. LE POGAM,[1] D. THUAULT,[2] C. M. BOURGEOIS,[2] AND J. P. LE PENNEC[1]*

Laboratoire de Génétique Moléculaire, CNRS URA 256, Université de Rennes I, Campus de Beaulieu, 35042 Rennes cédex,[1] and ADRIA, 29334 Quimper cédex,[2] France

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The partial nucleotide sequence of a *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30 bacteriocin-producing operon was determined. The first two open reading frames of the operon are necessary to get bacteriocin expression in *L. lactis* IL1403R.

Bacteriocins are proteinaceous bactericidal compounds that are produced by some microorganisms (1, 17), including lactic acid bacteria (10). Many bacteriocins have been described, but only a few genetic determinants for these substances have been cloned and sequenced. The lactic acid bacterium bacteriocins whose genes are known are generally small peptides that can be divided into two families. The lantibiotic family is composed of posttranslationally modified peptides harboring unusual amino acids termed lanthionines, while the second bacteriocin family does not contain lanthionines. These bacteriocins are synthesized from precursor molecules that include N-terminal extensions. The structural genes of these compounds are members of gene clusters. Additional open reading frames (ORFs) are related to bacteriocin activity, to maturation or excretion functions, and to bacteriocin immunity.

Lactococcus lactis subsp. *lactis* ADRIA 85LO30 (Table 1) produces a bacteriocin of industrial interest that has *Clostridium tyrobutyricum* in its spectrum of inhibitory activity (18). In a previous report (2), this bacteriocin, designated lactococcin DR in this paper to prevent confusion with other lactococcins, was purified and determined to be a 2.3- to 2.4-kDa peptide. The results of curing experiments suggested that genetic determinants for lactococcin DR production are localized on a DNA region that is about 10 kb long (del10) and is carried by a 70-kb plasmid (pOS5). A 14-kb DNA fragment that included 4.1 kb of the del10 region was cloned into bacteriophage lambda 2001 DNA (Table 1) (lambda E23) and subsequently subcloned into lactococcal vector pIL253 to obtain recombinant plasmid pEB23 (Fig. 1A). *L. lactis* IL1403R transformed by pEB23 inhibited the growth of the bacteriocin indicator strain. The nature of the inhibitor produced was shown to be proteinaceous since addition of proteinase K prevented inhibitory activity. This indicated that the cloned fragment encoded all of the genetic information required for lactococcin DR expression in this host. Deletion of 0.9-kb *Hpa*II-*Eco*RI fragment Hp-E_2 from pEB23 resulted in the loss of the lactococcin DR-positive (LcnDR$^+$) phenotype (Fig. 1A, pEB24), indicating that part of the bacteriocin determinant is located at the del10 extremity of this insert. In addition, 4.8-kb *Xba*I-*Eco*RI fragment X_1-E_2, which contained all of the del10 part of pEB23 (4.1 kb), was subcloned into pIL253 as described by

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid(s), or phage	Description	Reference
L. lactis subsp. *lactis* strains		
ADRIA 85LO30	Wild-type strain, LcnDR$^+$	18
IL1403	Plasmid free, Bac$^-$ Imm$^-$	7
IL1403R	Plasmid free, Bac$^-$ Bac^r	This study
Plasmids and phages		
λ2001		9
pIL253	Em^r, 4.9 kb	15
pBluescript (Stratagene)	*lacZ* Ap^r, 2.9 kb	
pES2	LcnDR$^+$, 74 kb	2
λE23	λ2001 carrying 14.5-kb *Eco*RI fragment of pES2	
pEB23	pIL253 carrying 14.5-kb insert of λE23, Em^r LcnDR$^+$	
pEB24	pIL253 carrying 13.6-kb *Eco*RI-*Hpa*II fragment of pEB23, Em^r LcnDR$^-$	
pEB56	pIL253::pBluescript carrying 4.8-kb *Xba*I-*Eco*RI fragment, Ap^r Em^r LcnDR$^+$	This study
pEB57, pEB58, pEB59, pEB60, pEB61, pEB62	pIL253::pBluescript carrying various exonuclease III-deleted fragments of pEB56	
pEMB57	pEB57 with a frameshift mutation in the lcnDR1 gene	

* Corresponding author. Mailing address: Université de Rennes I, CNRS URA 256, Laboratoire de Génétique Moléculaire, Avenue du Général Leclerc, 35042 Rennes, France. Phone: (33).99.28.61.36. Fax: (33).99.28.67.94. Electronic mail address: Jean-Paul.Lepennec@univ-rennes1.fr.

† Present address: Department of Microbiology, University of Texas Health Science Center, San Antonio, TX 78284-7758.

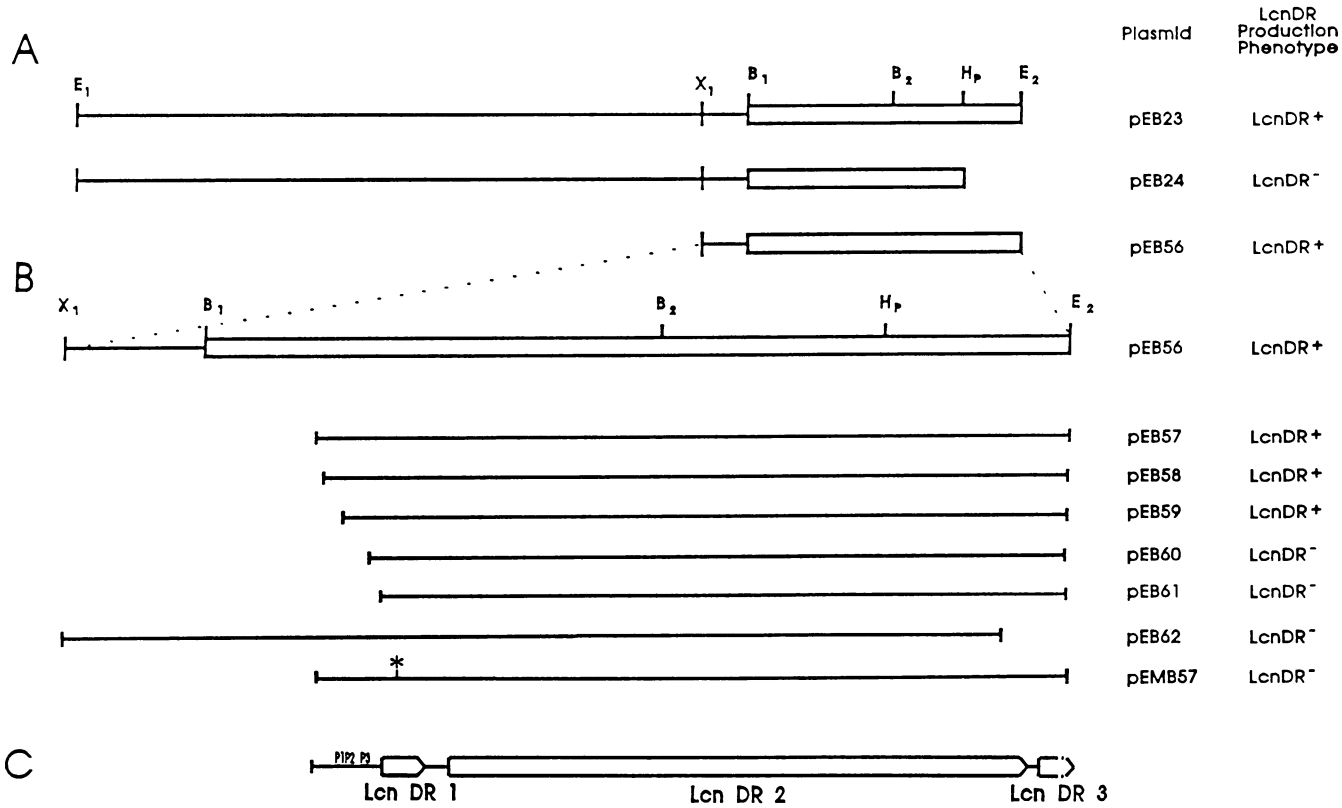

FIG. 1. (A) Restriction enzyme map and deletion analysis of the pEB23 insert. (B) Restriction enzyme map and deletion and mutation derivatives of the pEB56 insert. The open boxes indicate the del10 parts present in the cloned DNA fragments. *Eco*RI (E) and *Bam*HI (B) sites were numbered in order to permit unambiguous designation of the DNA restriction fragments (see text). The positions of unique *Hpa*II (Hp) and *Xba*I (X_1) sites are indicated. The lactococcin DR production phenotypes of *L. lactis* IL1403R derivatives harboring different fragments are indicated. The asterisk indicates the position of the mutation in ORF lcnDR1. (C) Genetic organization of the pEB57 insert. The ORFs are designated Lcn DR 1, Lcn DR 2, and Lcn DR 3. P1, P2, and P3 are the putative promoters.

Holo et al. (6) to form pEB56 (Fig. 1A). *L. lactis* IL1403R transformed by pEB56 had a $LcnDR^+$ phenotype. Subsequent deletion experiments were performed by digesting pEB56 with exonuclease III. Figure 1B shows the extent of digestion as determined by sequencing and the resulting phenotypes of *L. lactis* IL1403R derivatives transformed with the deleted fragments. Deletions larger than 1.3 kb from the *Xba*I site (compare pEB60 and pEB61 with pEB57, pEB58, and pEB59) or a deletion from the *Eco*RI site as small as 0.4 kb (pEB62) resulted in a loss of the Bac^+ phenotype. The 3.5-kb insert of pEB59 is thus the smallest fragment conferring the $LcnDR^+$ phenotype on *L. lactis* IL1403R.

The complete nucleotide sequence of the pEB57 insert plus the 2-kb fragment downstream from *Eco*RI site E_2 was determined on both strands by the dideoxy chain method of Sanger et al. (14). A total of 5,546 bp was sequenced (Fig. 2). This sequence contained three complete ORFs, designated lcnDR1 through lcnDR3 (Fig. 1C and 2), which encoded putative proteins that had 51, 922, and 691 amino acid residues, respectively. Potential ribosome binding sites were located upstream from each ORF. Three putative promoter sequences, designated P1 to P3, were found upstream from lcnDR1. As recombinant plasmid pEB59 lacked the −35 box of the putative P1 promoter and yet conferred a Bac^+ phenotype on strain IL1403R, P1 did not seem to be the only active promoter.

In primer extension experiments performed with primer PE_1 (GTTCTTTCATTATTGTTCAC) complementary to positions 366 to 348 (Fig. 2), we identified at least four double bands which indicated transcription initiation sites starting with a guanosine, which is typical for procaryotic mRNA. The largest extended product (Fig. 3B) started at position 201 (guanosine) (Fig. 2) and should have corresponded to a transcript initiated by the P1 promoter. The three other doublets observed (Fig. 3A) should have corresponded to transcripts initiated at positions 310 (guanosine), 314 (guanosine), and 319 (guanosine) (Fig. 2) by the P3 promoter. In Northern blots we detected two bands, a major band at 260 nucleotides and a minor band at approximately 400 nucleotides (Fig. 3C). Assuming that the inverse repeat (Fig. 2) was used as the ORF 1 terminator, the observed bands should have corresponded to RNA initiated at weak promoter P1 and at stronger promoter P3. RNAs corresponding to ORFs 2 and 3 were not visualized with the probe used.

Two lantibiotics, lacticin 481 (13) and streptococcin A-FF22 (7), exhibit high levels of homology with the lcnDR1-encoded peptide. The seven previously described N-terminal residues of lacticin 481 are identical to seven internal amino acid residues of the lcnDR1-encoded peptide (Fig. 2). Streptococcin A-FF22 (7) is approximately 50% homologous to the lcnDR1-encoded peptide. These levels of homology strongly suggest that lcnDR1 is the structural lactococcin DR gene, that the product of this ORF is a precursor which contains a peptide leader, and that lactococcin DR is a lantibiotic. The resulting compound,

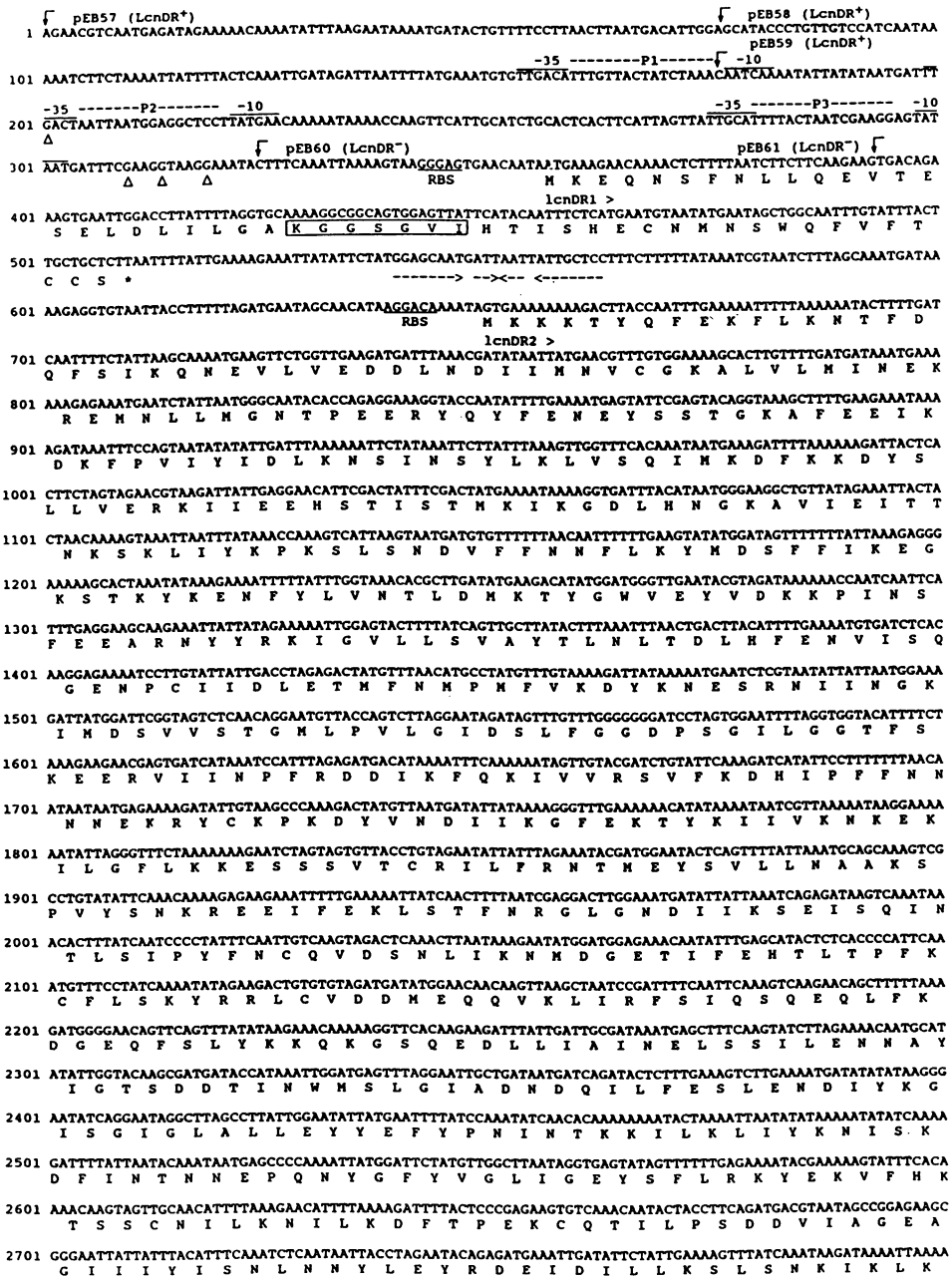

FIG. 2. Nucleotide sequence containing ORFs lcnDR1, lcnDR2, and lcnDR3. The three putative −35 and −10 promoters designated P1 to P3 are overlined. The putative ribosome binding sites (RBS) are underlined. The deduced amino acid sequences are given in single-letter code. The position of the termination codon is indicated by an asterisk. The residues enclosed in a box are the seven amino acid residues that are identical to the residues in the N-terminal sequence of mature lacticin 481 (13). The arrows below the nucleotide sequence indicate the positions of inverted repeats. The positions of deletion mutants pEB57 to pEB62 are indicated along with their corresponding phenotypes. The triangles indicate the positions of transcription start sites.

lactococcin DR, should be 27 amino acids long and have a calculated molecular mass (3.4 kDa) that is in good agreement with the molecular mass estimated by using the purified bacteriocin (2). The results of a mutational experiment performed with ORF lcnDR1 confirmed the importance of this ORF for bacteriocin expression (compare pEMB57 and pEB57 in Fig. 1).

lcnDR2 encodes a putative 922-amino-acid protein with a calculated molecular mass of 108.1 kDa which exhibits no significant level of homology to previously described sequences. The parent strain, *L. lactis* IL1403, was transformed with plasmid pEB57. The resulting recombinant produced lactococcin DR but was not immune to lactococcin DR, indicating that lcnDR2 is not able by itself to confer the Imm^+ phenotype. Expression studies revealed that only one additional ORF, lcnDR2, is required to obtain lactococcin DR

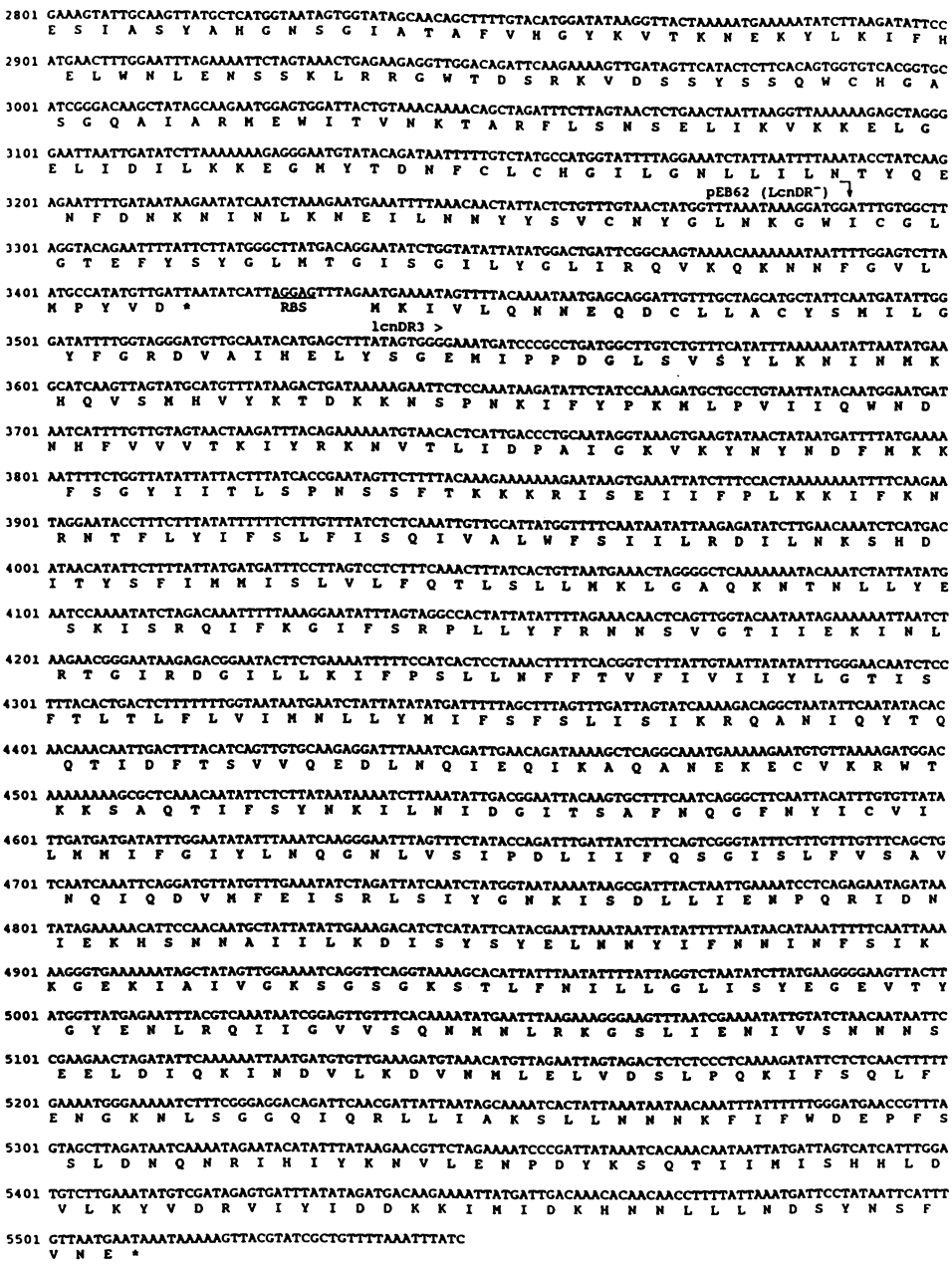

FIG. 2—*Continued.*

production in IL1403R. In contrast, Klein et al. (11) showed that two ORFs (*spaB* and *spaC*), in addition to the subtilin structural gene, *spaS*, are essential for subtilin biosynthesis. Genes homologous to *spaB* and *spaC* were found in both nisin and epidermin operons (3), suggesting that subtilin, nisin, and epidermin have similar biosynthetic pathways that are probably different from the lactococcin DR biosynthetic pathway. As lcnDR2 is essential to lactococcin DR production, it is probably involved in either lactococcin maturation or export.

The amino acid sequence deduced from lcnDR3 exhibits high degrees of homology with the sequences of ATP-dependent transport proteins. The highest level of homology was found with CylB (68.2% of conserved amino acids with 26.9% identity), an *Enterococcus faecalis* protein required for the expression of the cytolysin (4). Noticeably, in the C-terminal part of the protein, an ATP-binding motif (GEKIAIVGKSGSGKSTLFN) at positions 490 to 508 is highly conserved, as is a region unique to proteins of the active transport group (5). Comparable levels of similarity to the CylB protein were observed with HlyB, an *Escherichia coli* membrane protein required for the export of hemolysin A (8), SpaT, a *Bacillus subtilis* protein involved in the production of subtilin (11), and NisT, a *L. lactis* protein involved in the production of nisin (3). The entire lcnDR3 ORF seems not to be essential for bacteriocin expression in *L. lactis* IL1403R. However, we have not excluded the possibility that the function of lcnDR3 is provided by a protein encoded by the genome of *L. lactis* subsp. *lactis* IL1403R. This type of complementation by strain IL1403 has

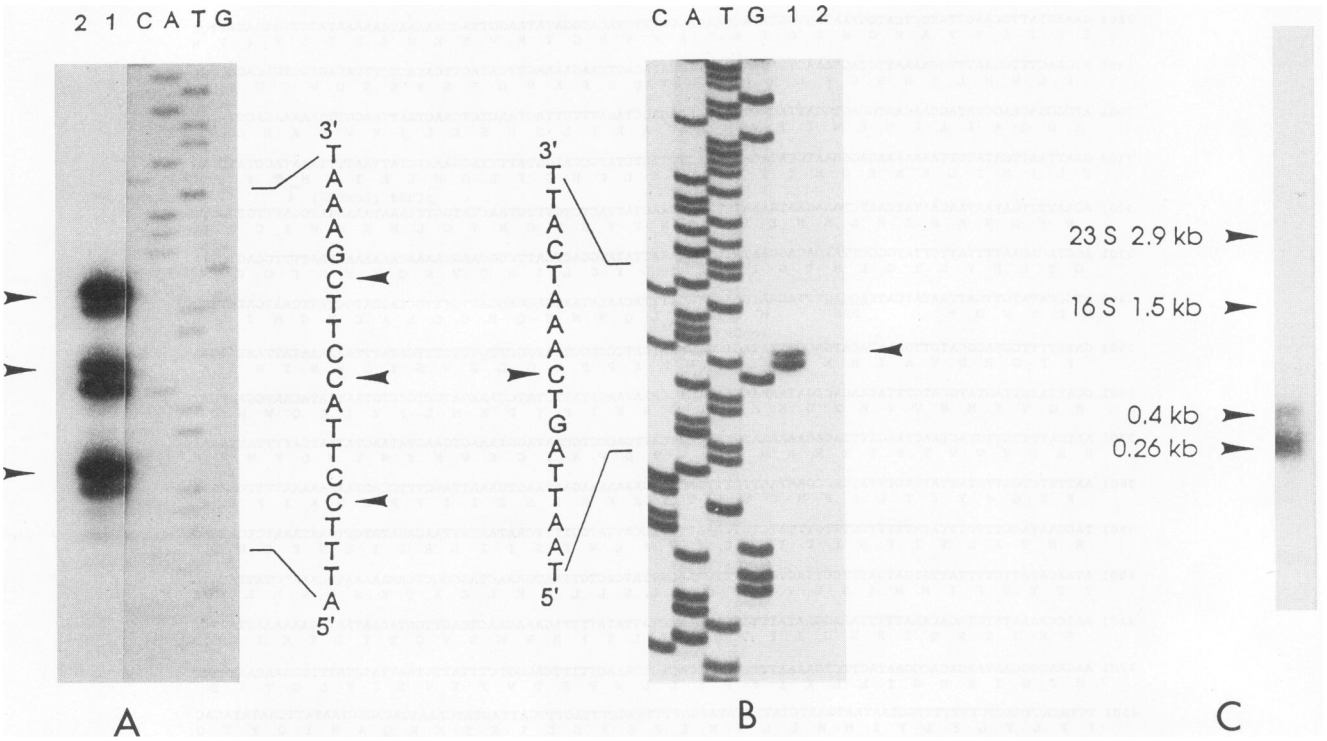

FIG. 3. Primer extension and Northern hybridization experiments. (A and B) Primer extension signals obtained with primer PE_1 when *L. lactis* ADRIA 85LO30 RNA (lanes 1) or trout egg total RNA (lanes 2) was used. Lanes C, A, T, and G contained the products of the sequencing reactions performed with the transcribed DNA strand used for standardization. The arrowheads indicate primer extension signals which may correspond to transcriptional start sites. The migration times were 1.5 h (A) and 4 h (B). (C) Northern hybridization of *L. lactis* ADRIA 85LO30 RNA. Hybridization was performed with a probe corresponding to the first 884 bp of pEB57. The sizes of the transcripts determined with RNA molecular weight markers (Boehringer) are indicated on the left.

been observed to some extent in the production of lactococcin A (16, 19).

The DNA nucleotide sequence determined in this study has been deposited in the EMBL and GenBank data bases under accession number U04057.

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ADDENDUM

After this paper was submitted for publication, a partial DNA sequence of the lacticin 481 operon was published (12). This sequence included lcnDR1 and part of lcnDR2. The sequences of both of these regions were identical to the sequences described in this paper except that one more codon was present in lacticin 481 ORF 2.

REFERENCES

1. **De Graaf, F. K., and B. Oudega.** 1986. Production and release of cloacin DF13 and related colicins. Curr. Top. Microbiol. Immunol. **125:**183–205.
2. **Dufour, A., D. Thuault, A. Boulliou, C. M. Bourgeois, and J. P. Le Pennec.** 1991. Plasmid-encoded determinants for bacteriocin production and immunity in a *Lactococcus lactis* strain and purification of the inhibitory peptide. J. Gen. Microbiol. **137:**2423–2429.
3. **Engelke, G., Z. Gutowski-Eckel, M. Hammelmann, and K. D. Entian.** 1992. Biosynthesis of the lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Appl. Environ. Microbiol. **58:**3730–3743.
4. **Gilmore, M. S., R. A. Segarra, and M. C. Booth.** 1990. An HlyB type function is required for expression of the *Enterococcus faecalis* hemolysin/bacteriocin. Infect. Immun. **58:**3914–3923.
5. **Higgins, C. F., I. D. Hiles, G. P. C. Salmond, D. R. Gill, J. A. Downie, I. J. Evans, I. B. Holland, L. Gray, S. D. Buckel, A. W. Bell, and M. A. Hermodson.** 1986. A family of related ATP-binding subunits coupled to many distinct biological processes in bacteria. Nature (London) **323:**448–450.
6. **Holo, H., O. Nilssen, and I. F. Nes.** 1991. Lactococcin A, a new bacteriocin from *Lactococcus lactis* subsp. *cremoris*: isolation and characterization of the protein and its gene. J. Bacteriol. **173:**3879–3887.
7. **Hynes, W. L., J. J. Ferretti, and J. R. Tagg.** 1993. Cloning of the gene encoding streptococcin A-FF22, a novel lantibiotic produced by *Streptococcus pyogenes*, and determination of its nucleotide sequence. Appl. Environ. Microbiol. **59:**1969–1971.
8. **Juranka, P., F. Zhang, J. Kulpa, J. A. Endicott, M. Blight, I. B. Holland, and V. Ling.** 1992. Characterization of the hemolysin transporter, HlyB, using an epitope insertion. J. Biol. Chem. **267:**3764–3770.
9. **Karn, J., H. W. D. Matthes, M. J. Gait, and S. Brenner.** 1984. A new selective phage cloning vector, λ2001, with sites for *Xba*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Sst*I and *Xho*I. Gene **32:**217–224.
10. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70:**337–349.
11. **Klein, C., C. Kaletta, N. Schnell, and K. D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **58:**132–142.
12. **Piard, J. C., O. P. Kuipers, H. S. Rollema, M. J. Desmazeaud, and W. M. De Vos.** 1993. Structure, organization and expression of the *lct* gene for lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*. J. Biol. Chem. **268:**16361–16368.

13. **Piard, J. C., P. M. Muriana, M. J. Desmazeaud, and T. R. Klaenhammer.** 1992. Purification and partial characterization of lacticin 481, a lanthionine-containing bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481. Appl. Environ. Microbiol. **58:**279–284.
14. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
15. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559–566.
16. **Stoddard, G. W., J. P. Petzel, M. J. van Belkum, J. Kok, and L. L. McKay.** 1992. Molecular analyses of the lactococcin A gene cluster from *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* WM4. Appl. Environ. Microbiol. **58:**1952–1961.
17. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40:**722–756.
18. **Thuault, D., E. Béliard, J. Le Guern, and C. M. Bourgeois.** 1991. Inhibition of *Clostridium tyrobutyricum* by bacteriocin-like substances produced by lactic acid bacteria. J. Dairy Sci. **74:**1145–1150.
19. **van Belkum, M. J., B. J. Hayema, R. E. Jeeninga, J. Kok, and G. Venema.** 1991. Organization and nucleotide sequences of two lactococcal bacteriocin operons. Appl. Environ. Microbiol. **57:** 492–498.