Arch Microbiol (1993) 160: 279–283

Archives of
Microbiology

Cloning and sequencing of *curA* encoding curvacin A, the bacteriocin produced by *Lactobacillus curvatus* LTH1174

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Received: 1 April 1993/Accepted: 11 May 1993

Abstract. Curvacin A is a bacteriocin produced by *Lactobacillus curvatus* LTH1174 which is a potential starter organism for the production of fermented dry sausages. This peptide inhibits the growth of the opportunistic food pathogens *Listeria monocytogenes* and *Enterococcus faecalis* and thus, curvacin A may enable better performance of a starter and improvement of the hygienic status of meat products. Oligonucleotides were constructed deduced from the peptide sequence and used for the identification of the curvacin A structural gene *curA* on a 60 kb plasmid of *L. curvatus* LTH1174. Plasmid-cured derivatives of this strain were unable to produce curvacin A but were still resistant to the bacteriocin. *CurA* was cloned into *Escherichia coli* NM554 and its nucleotide sequence was determined. Sequencing revealed the presence of an additional open reading frame of 51 amino acids with unknown function. A promoter was detected upstream of *curA* by primer extension. Both reading frames form a single transcript. Curvacin A is synthesised as a prepeptide of 59 amino acids which is proteolytically processed to the mature bacteriocin of 41 amino acids.

Key words: Bacteriocins – Curvacin A – Cloning – Sequence – *Lactobacillus curvatus* – Food hygiene – Meat products

Lactic acid bacteria produce a variety of substances with antimicrobial activity (Hammes et al. 1990; Schillinger and Lücke 1989). Some of the compounds responsible for antagonistic activity are of a proteinaceous nature and were termed bacteriocins (Klaenhammer 1988). The potential applications of bacteriocins or bacteriocin-producing lactic acid bacteria for food preservation has stimulated interest in the comprehensive characterisation of these substances. On the basis of the protein structure of lactic acid bacteria bacteriocins several groups can be distinguished in *(i)* unmodified peptides e. g. pediocin PA-1 (Marugg et al. 1992), curvacin A and sakacin P (Tichaczek et al. 1992), *(ii)* lantibiotics containing lanthionin bridges e. g. nisin (Kaletta and Entian 1989) and the more recently described lactocin S (Mørtvedt et al. 1991). *(iii)* two-component bacteriocins, whose activity depends on the complementary action of two distinct peptides (Nissen-Meyer et al. 1992), *(iv)* protein bacteriocins e. g. helveticin J (Joerger and Klaenhammer 1990), and *(v)* bacteriocins of glycoprotein nature e. g. leuconocin S (Lewus et al. 1992).

Despite numerous reports on the production, detection and biochemical characterisation of lactic acid bacteria bacteriocins the knowledge about the genes involved in synthesis, processing, secretion and regulation is limited. A number of bacteriocin determinants have recently been cloned including the structural genes of helveticin J (Joerger and Klaenhammer 1990), lactacin F (Muriana and Klaenhammer 1991), lactococcin A (Holo et al. 1991) and leucocin A-UAL 187 (Hastings et al. 1991). The genes were cloned and sequenced which are involved in bacteriocin production and immunity of three different bacteriocins referred to as lactococcins from *Lactococcus lactis* subsp. *cremoris* 9B4 (van Belkum et al. 1991, 1992). The genes for the synthesis of pediocin PA-1 are organised in an operon, where other open reading frames (*orf*s) are located which are required for bacteriocin production and immunity (Marugg et al. 1992).

The characterisation of the genes involved in bacteriocin production and immunity provides the basic knowledge required for practical applications. Bacteriocin genes could be transferred to lactobacilli used as starter cultures in food fermentations. The expression of a bacteriocin gene could improve the performance of starter cultures and the hygienic status of the product by inhibition of food spoiling bacteria (Foegeding et al. 1992; Schillinger and Lücke 1989).

Here we report on the cloning and nucleotide sequencing of the DNA region containing *curA*, the gene encoding for curvacin A, a bacteriocin produced by *Lactobacillus curvatus* LTH1174 (Tichaczek et al. 1992). This bacteriocin is produced in the late exponential growth phase. It induces lysis of lactobacilli sharing the

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habitat meat, and is active against the opportunistic food pathogens *Listeria monocytogenes* and *Enterococcus faecalis*. Thus, it is a good candidate for hygiene improvement of meat products.

Materials and methods

Bacterial strains, plasmids, and media

Lactobacillus curvatus LTH1174 was isolated from fermented sausage. *E. coli* HB102 harbouring pRB473 was kindly provided by R. Brückner, Tübingen, Germany. *Escherichia coli* NM 554 and the plasmid vector pBluescript SK+ (Stratagene, La Jolla, Calif., USA) were used for cloning experiments. *L. curvatus* was grown in MRS-medium (de Man et al. 1960) at 30 °C. *E. coli* strains were propagated at 37 °C in SOB-broth while shaking (180 rpm) or on SOB-agar-plates (Hanahan 1983). Selective media contained ampicillin at a final concentration of 100 µg/ml (SOB(amp)).

Bacteriocin production and detection

Bacteriocin production by *L. curvatus* LTH1174 and assays by using direct or deferred methods have been described previously (Tichaczek et al. 1992).

DNA technology

Total DNA of *L. curvatus* LTH1174 was isolated as described by Obst et al. (1992). Plasmid DNA of lactobacilli and *E. coli* was isolated as described by Anderson and McKay (1983) or by using Quiagen columns (Diagen, Hilden, Germany) according to the manufacturer, respectively. Enzymes for DNA manipulations were used as recommended by the supplier.

Construction and amplification of the plasmid library

Plasmid DNA of *L. curvatus* LTH1174 was cleaved with *BclI*/*Eco*RI. Fragments of approximately 1.0 kb in length were isolated from preparative 0.8% agarose gels by the method of Dretzen et al. (1981). The fragments were ligated into the *Bam*HI/*Eco*RI-digested pBluescript SK+ vector with T4 DNA ligase (Boehringer, Mannheim, Germany). The ligation mix was used to transform competent *E. coli* NM 554 cells by the method of Hanahan (1983). After being cultured, the transformants were washed off the agar plates and collected by centrifugation. The cells were resuspended in a total volume of 1 ml of SOB(amp) medium and mixed with an equal volume of sterile 60% glycerol. The cells containing the gene library were stored at −85 °C. For amplification of the gene library, 10 ml of SOB(amp)-broth were inoculated with 50 µl of the stock culture and incubated overnight.

Detection of curA

For the detection of *curA* in Southern hybridisations DNA of *L. curvatus* LTH1174 was digested with various restriction enzymes and the fragments were separated in 0.8% agarose gels according to their size. *Hin*dIII fragments of bacteriophage λ DNA (Boehringer) were used as molecular weight markers.

A curvacin A-specific probe for DNA hybridizations was deduced from the amino acid sequence of the peptide. The degeneration of this 26-mer oligonucleotide was 64-fold. The sequence was 3′ ATA(G) ACA(G) TTA(G) TTA(G) TTT(C) TTT(C) AGI ACC CA5′. The probe was end labelled with ^{32}P-γ-ATP (Amersham, Braunschweig, Germany) by using a 5′terminus labelling system (Pharmacia, Freiburg, Germany). Prehybridisation and hybridisation with the probe was performed at 45 °C for 1–2 h and overnight, respectively, in plaque-screen-buffer containing in 50 ml: 2.5 ml Tris-HCl (2 mol/l, pH 7.5), 10 ml 10× Denhardts solution; 10 ml NaCl (5 mol/l), 1.8 ml Na_2HPO_4 (0.2 mol/l), 5 ml SDS (100 g/l).

Southern hybridisation of the fractionated DNA bound to nitro-cellulose (nc) was performed as described by Sambrook et al. (1989) and included a depurination step (Wahl et al. 1979).

For the detection of clones containing the bacteriocin gene cells of the gene library were plated on SOB(amp)-agar and subjected to colony hybridisation. The nc-filters were prepared as described by Sambrook et al. (1989). The conditions of prehybridisation and hybridsation were the same as described above. Labelled DNA hybrids were detected by autoradiography using a Kodak x-omat film (Eastman Kodak Company, Rochester, N.Y., USA).

Clones containing hybridising sequences were cultured overnight in 10 ml SOB(amp)-broth, plasmids were isolated from the culture and digested with *XbaI*/*Eco*RI. Inserts were analysed by Southern hybridisation as described above.

DNA sequencing and analysis

Double-stranded DNA sequencing was performed with ^{35}S-α-ATP (Amersham) by using the dideoxy-chain-termination method of Sanger et al. (1977) and the Sequenase 2.0 DNA sequencing kit (Renner, Dannstadt, Germany) as recommended by the supplier. The primers used for initiating DNA sequencing from the pBluescript SK + were the T3 and M13–20 (reverse) primers (Pharmacia). Site-specific 17-mer primers for sequencing within the cloned fragment were synthesized by Roth (Karlsruhe, Germany). Computer analyses of nucleotide and amino acid sequences were performed with a microcomputer system and DNAsis software (Pharmacia). The predicted amino acids sequences encoded by the open reading frames and the nucleotide sequences were compared by the HUSAR (Heidelberg Unix Sequence Analysis Resources) datasystem.

Analyses of the mRNA

The site for transcription initiation was determined by primer extension with reverse transcriptase (BRL, Eggenstein, Germany). As primers two oligonucleotides were used which were targeted to the 5′region of the mRNA (curpr4: 3′TCTTAATTCATACTGTC 5′ and curpr6: 3′AATGTTTGTTAATGGCC5′). For northern hybridisation ^{32}P-end-labelled curpr4 was used as probe. Primer extension and northern hybridisation were essentially performed as described by Sambrook et al. (1989).

Results

Detection of curA

The structural gene encoding curvacin A was identified in Southern hybridisations by using a 26-mer oligonucleotide probe based on the amino acid sequence of curvacin A. One strong signal was detected when total DNA of *Lactobacillus curvatus* LTH1174 was bound to the membranes. To investigate the presence of the curvacin A structural gene on one of the plasmids harboured by *L. curvatus* LTH1174 additional hybridisations with purified plasmid DNA were performed. Again, one signal was observed indicating that the curvacin A structural gene is located on a plasmid of approximately 60 kb. Plasmid DNA of *L. curvatus* LTH1174 was subjected to single and double restriction enzyme digestions and analysed in Southern hybridisations. The curvacin A structural gene was located on a 1.2-kb *Acc*I/*Eco*RI fragment.

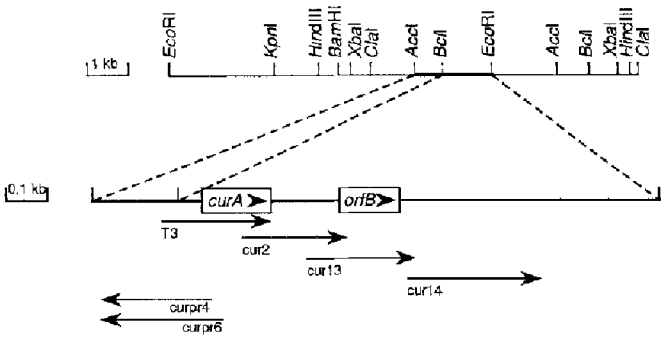

Fig. 1. Restriction map of *L. curvatus* LTH1174 plasmid DNA surrounding the *curA* region. The sequencing strategy and the open reading frames are indicated by arrows or boxes, respectively. T3, cur2, cur13 and cur14 indicate primers used for sequencing. curpr4 and curpr6 were used in the primer extension experiment (compare Fig. 3)

The presence of the curvacin A structural gene on a plasmid was further proved by plasmid curing. After electroporation of *L. curvatus* LTH1174 with plasmid pGKV210, derivatives were isolated which had lost their ability to produce curvacin A. These derivatives were devoid of the 60-kb plasmid and gave no signal in Southern hybridisations with the oligonucleotide probe. They were resistant to curvacin A.

Cloning of curA

DNA fragments in the size range of the by hybridising 1.0 kb *Bcl*I/*Eco*RI and 1.2 kb *Acc*I/*Eco*RI, respectively, fragments were ligated into plasmid pBluescript SK+ and cloned in *E. coli* NM554. Clones containing *curA* were detected by colony hybridisation with the oligonucleotide probe. Plasmid DNA was isolated from the hybridising clones and digested with *Eco*RI/*Xba*I. The 1.2-kb *Lactobacillus* DNA insert reacted with the labelled oligonucleotide probe in Southern hybridisations.

Sequencing of the cloned fragment

The restriction map of the cloned fragment and its adjacent regions, and the sequencing strategy are depicted in Fig. 1. Sequencing of the cloned fragment revealed the presence of two open reading frames coding for polypeptides of 59 and 51 amino acids referred to as *orfA* and *orfB* . The nucleotide sequence is shown in Fig. 2. Translation of *orfA* results in a sequence, parts of which are homologous to the amino acid sequence determined for the purified curvacin A peptide (Tichaczek et al. 1992). Thus, *orfA* is the structural gene encoding curvacin A and is subsequently referred to as *curA*. The molecular weight of the mature curvacin A was calculated from the nucleotide sequence to 4309 Da.

Comparison with the HUSAR data bank revealed similarity to pediocin PA-1 (Marugg et al. 1992) and leucocin A-UAL 187 (Hastings et al. 1991). Curvacin A appears to be identical with sakacin A produced by *L. sake* Lb706 (Holck et al. 1992), and further homologies were detected to sakacin P (Tichaczek et al. 1992; Lozano et al. 1992). No homologies to other proteins were detected. No sequence homologies to other proteins were found for a putative protein encoded by *orfB*. The nucleotide accession no. at Genebank is 72223.

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1          TGTTTTTTTCAATGTAGTTAAGTTTCATTATCAATTAATCTCCTCTCAAAT   50
51 AAAATTACTATGTCATACTTTTCGTTTTTAAGAGAAATAAATTCCTGAACCTTACAGAAA  110
111 TCATTCCTGAATGGTTAAATTGATAATTCTCCCTGTTTAGGAATGATTTCTGTAGGCTTC  170
          -35                    -10       1
171 AAGAAGTTATGCCACGTGATCAAAGAAATCTTGTTATACTTCACTCGTACAAAAAATAAT  230
                                          curA ➤
231 AACAGAGGAGATTCTTAGTT ATG AAT AAT GTA AAA GAA TTA AGT ATG ACA  280
                        M   N   N   V   K   E   L   S   M   T    10
281 GAA TTA CAA ACA ATT ACC GGC GGT GCT AGA TCA TAT GGC AAC GGT  325
    E   L   Q   I   I   T   G   G   A   R   S   Y   G   N   G    25
326 GTT TAC TGT AAT AAT AAA AAA TGT TGG GTA AAT CGG GGT GAA GCA  370
    V   Y   C   N   N   K   K   C   W   V   N   R   G   E   A    40
371 ACG CAA AGT ATT ATT GGT GGT ATG ATT AGC GGC TGG GCT AGT GGT  415
    T   Q   S   I   I   G   G   M   I   S   G   W   A   S   G    55
416 TTA GCT GGA ATG TAA ATAGAGGGTCTAATTTGAAGGCAGATTATAAAAAAATAAA  470
    L   A   G   M   *                                            59
471 TTCAATACTAACTTACACATCTACTGCTTTAAAAAACCCTAAAATTATAAAAGATAAAGA  530
                                                      orfB ➤
531 TTTAGTAGTCTTCTAACTATTATCAAGAAGAAGCCAAACAAAATAGAATCTTTT ATG  587
                                                            M     1
588 ATT ATA AAA GAA AAT TTC GTC CAG CGG TTA CTC GCT TTA CAA TTG  632
    I   I   A   E   N   F   V   Q   R   L   L   A   L   Q   L    16
633 ATA ATA ATT TTG AGA TTC CTG ATT GTT TGG TTA AAC TAC TGT CAG  677
    I   I   I   L   R   F   L   I   V   W   L   N   Y   C   Q    31
678 CTG TTG AAA CAC CTA AGG CGT GGT CTG GAT TTA GTT AGA ATG ATT  722
    L   L   K   K   L   R   R   G   L   D   L   V   R   M   I    46
723 TTT ATA TTG TCG ATA TAA ATAAAATCATTAGGAATTTGGAGTAATCCATTAGAT  776
    F   I   L   S   I   *                                        51
777 AAGCACTATATCTTAACTTTGCAGACGCCAAATGATCCCAGTAAAAAACCACCCGCATGG  836
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Fig. 2. Nucleotide sequence of *curA* and its adjacent regions. The boxed areas indicate *curA* and *orfB*. The shaded box covers the mature curvacin A. The sequences responsible for transcription initiation are marked 1, −10 and −35 The putative ribosome binding site is the boxed AGGAG sequence. Stop codons are marked with as asterisk

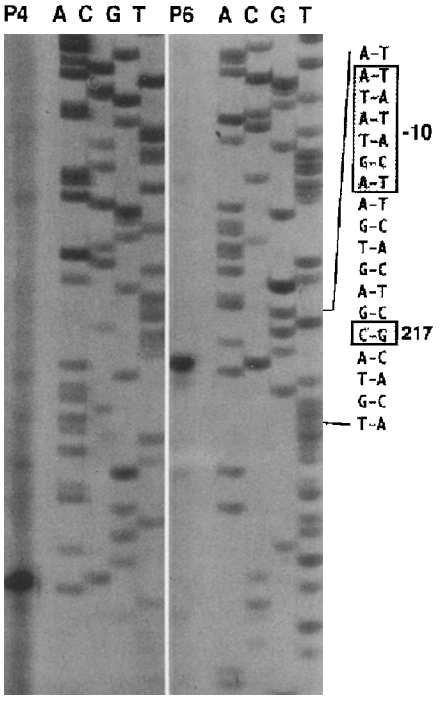

Fig. 3. Determination of the transcription initiation site in *curA*. The result is depicted of a primer extension experiment with reverse transcriptase by using two different oligonucleotides (P4: curpr4; P6: curpr6) directed against *curA* mRNA. Both signals indicate that the Guanine residue 217 is the starting point of transcription. The probable −10 region is boxed. The sequencing lanes are marked for the dideoxy nucleotides used in the Sanger reaction

Analyses of the mRNA

The site for transciption initiation was determined by primer extension the result of which is depicted in Fig. 3. With both primers used the Guanine residue 217 was identified as the 5'terminus of the mRNA. With RNA isolated from *E. coli* NM554 harbouring pPT4000 the same signal was obtained which was much less intense. This indicates weak transcription of *curA* in the *E. coli* transformant which was, on the other hand, not able to inhibit the indicator organism (data no shown). Corresponding −10 and −35 regions where detected upstream of Guanine residue 217. A putative ribosome binding site was present 10 bp upstream of the N-terminal methionine of pre-curvacin A (Fig. 2). In northern hybridisations with RNA isolated from *L. curvatus* LTH1174 one signal corresponding to an mRNA size of 1.2 kb was obtained. This indicates that *curA* and *orfB* are transcribed as a single transcript.

Discussion

Bacteriocins have been investigated from lactic acid bacteria with respect to their biochemical nature, inhibitory spectrum and mode of action. However, only a limited number of genes have been cloned and sequenced which are involved in the biosynthesis of these antimicrobial peptides. As reported recently, *L. curvatus* LTH1174 produces a bacteriocin designated curvacin A (Tichaczek et al. 1992). The bacteriocin has been purified to homogeneity and the sequence of 30 N-terminal amino acids was determined. The *curA* gene appears to encode a precursor from which the N-terminal peptide is proteolytically removed. This was concluded from comparison of the gene-derived amino acid sequence with that one determined by Edman degradation of the purified curvacin A peptide. The gene derived amino acid sequence differed in some amino acids from the sequence obtained by Edman degradation of purified curvacin A. This is probably due to uncertainties commonly occurring during Edman degradation, as the sequence data were unambiguous. Upstream of the GCT codon encoding the N-terminal alanine residue of the mature curvacin A two ATG start codons are present at position 251 and 272 which encode presumable N-terminal methionins of the pre-curvacin A. It is likely that the ATG at position 251 is used as this would result in an 18-amino-acid N-terminal peptide which is approximately the size of leader peptides observed for other bacteriocins (Marugg et al. 1992). Furthermore, this is supported by the location of the putative AGGAG ribosome binding site 10 bp upstream of ATG at position 251. Thus, *curA* encodes a 59 amino acid prepeptide which is processed to the mature curvacin A consisting of 41 amino acids. The leader sequence does not share any homology to conserved regions common in signal peptides which promote transport of proteins, e.g. a hydrophobic α-helix core. The presence of a Gly-Gly sequence adjacent to the N-terminal alanine residue of the mature curvacin A indicates a conserved proteolytic cleavage site which was also found in other bacteriocins (Marugg et al. 1992). This type of cleavage site was not observed in signal peptides (Simonen and Palva 1993; Pugsley 1993). Thus, the leader sequence is not a common secretion signal, but it might function as a chaperone maintaining a specific, inactive conformation of the bacteriocin or as an anchor for modifying enzymes. The latter function is unlikely as no modification was detected of curvacin A.

Downstream of *curA* a second open reading frame *orfB* is located which could encode a protein of 51 amino acids. The function of this putative protein remains unknown. It has been found for other well characterised bacteriocins that genes involved in synthesis, transport and immunity are in close association with the bacteriocin structural gene (Holo et al. 1991; Marugg et al. 1992). No homology of *orfB* was detected to such genes. The analysis of plasmid cured derivatives of *L. curvatus* LTH1174 suggests that immunity determinants are chromosomally encoded or present on one of the residing plasmids of *L. curvatus* LTH1174, as the partially plasmid-cured strain was still resistant to curvacin A. However, the formation of a single transcript from the *curA* operon supports some function of *orfB* in curvacin A biosynthesis, transport or resistance. Studies on the expression of *curA* and immunity to curvacin A will reveal the role of *orfB* and improve the knowledge about the genetic organisation, biosynthesis and transport of bacteriocins in starter organisms. Hence, starter organisms can be developed with an enhanced ability to inhibit food spoiling or poisoning microorganisms. This is especially

valuable for meat starters as the meat substrate is highly susceptible to microbial spoilage or may be contaminated with potential food pathogens.

Acknowledgements. We thank H. J. Knauf (this institute) for his help in constructing plasmid free variants of *L. curvatus*. LTH 1174 This work was supported by the Bundesministerium für Forschung und Technologie grant PBE 0319280B and the Commission of the European Communities in the BRIDGE T-project on Lactic Acid Bacteria Contract BIOT CT91 0263 (SSMA).

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