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GENETIC CHARACTERIZATION OF LF221 ACIDOCINS

Andreja ČANŽEK MAJHENIČ[a)] and Irena ROGELJ[b)]

[a)] Univ. of Ljubljana, Biotechnical Fac., Zootechnical Dept., Groblje 3, SI-1230 Domžale, Slovenia, M.Sc., B.Sc.Food Tech.
[b)] Same address, Assos.Prof., Ph.D., M.Sc., B.Sc.Food Tech.

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A B S T R A C T

Acidocin LF221 A and B are at least two different bacteriocins produced by *Lactobacillus acidophilus* LF221. In previous research work the N-terminal amino acid (AA) sequence was determined from the purified peptides and probes for both acidocins were prepared as well. Southern hybridization was used to locate structural genes for acidocin LF221 A and B on the genome. Using as a template digested chromosomal DNA of either LF221 or its bac$^-$ derivative, identical results were obtained. A possible explanation for this is that bac$^-$ mutant still contains the structural genes for both acidocins. When acidocin LF221 A or B were compared to other known bacteriocins, they showed some homology to ThmB peptide of thermophilin 13 or LafX peptide of lactacin F. We assume that LF221 acidocins are novel representatives of the class II bacteriocins.

Key words: microbiology / bacteria / *Lactobacillus acidophilus* / bacteriocins / molecular genetics / cloning / classification

GENSKI OPIS ACIDOCINOV SEVA LF221*

I Z V L E Č E K

Acidocina LF221 A in B sta vsaj dva različna bakteriocina, ki ju proizvaja *Lactobacillus aciodiphilus* LF221. V prejšnjih raziskavah sta bili iz očiščenih peptidov za acidocina ugotovljeni N-terminalni aminokislinski (AK) zaporedji ter pripravljeni sondi. S Southern hibridizacijo smo na genomu lokalizirali strukturne gene za acidocina LF221 A in B. Ko smo kot tarčno DNK uporabili kromosomsko DNK seva LF221 oz. njegovega nebakteriocinogenega mutanta bac$^-$, smo v obeh primerih dobili identične rezultate. Sklepali smo, da bac$^-$ derivat še vedno nosi strukturne gene za acidocina. Pri primerjavi acidocina LF221 A oziroma B z že opisanimi bakteriocini smo ugotovili, da sta delno homologna peptidu ThmB termofilina 13 oz. peptidu LafX laktacina F. Predvidevamo, da sta acidocina seva LF221 nova predstavnika II. skupine bakteriocinov.

Ključne besede: mikrobiologija / bakterije / *Lactobacillus acidophilus* / bakteriocini / molekularna genetika / kloniranje / razvrščanje

INTRODUCTION

The antimicrobial activity of lactic acid bacteria (LAB) has been appreciated for more than 10000 years and has enabled man to extend the shelf life of many foods through fermentation processes. The major preservative effect of LAB is due to their ability to synthesize various inhibitory compounds such as organic acids, hydrogen peroxide and bacteriocins (Lindgren and Dobrogosz, 1990; Piard and Desmazeaud, 1992). For the last few decades, special attention has been focused on the ribosomally synthesized bacteriocins, which are defined as proteins or protein complexes that show bactericidal effect towards species that are closely related to the producer bacteria (Tagg *et al.*, 1976). In fact, the discovery of nisin initiated the investigation of proteinaceous compounds from LAB and a large number of diverse bacteriocins has been described and reported so far. Recent developments and improvements of the biochemical and genetic methods characterized LAB bacteriocins in four different classes: small, membrane active (I) lantibiotics and (II) non-lantibiotics, (III) large, heat labile proteins and (IV) complex bacteriocins, composed of protein and one or more chemical moieties (lipid, carbohydrate). Class II bacteriocins are small, mostly hydrophobic heat-stable and membrane active peptides, characterized by Gly-Gly^{-1} processing site in the bacteriocin precursor (Klaenhammer, 1993). To date, many *Lactobacillus* bacteriocins belonging to this class have been identified and classified, including: curvacin A and sakacin P (Tichaczek *et al*., 1992), sakacin A (Holck *et al*., 1992), lactacin B (Barefoot and Klaenhammer, 1983), lactacin F (Muriana and Klaenhammer, 1991), plantaricin S (Jimenez-Diaz *et al*., 1995), acidocin 8912 (Kanatani *et al*., 1995) and acidocin J1132 (Tahara *et al*., 1996).

Lactobacillus acidophilus LF221, a child's faeces isolate, produces at least two different bacteriocins. The main characteristics of crude and purified acidocins LF221 A and B were already described. LF221 displayed a wide inhibitory activity against numerous genera, including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Listeria*, *Clostridium* and *Bacillus.* Acidocins of LF221 strain were partially purified and, moreover, the N-terminal AA sequences were determined with Edman degradation: 45 AA residues for acidocin LF221 A and 35 for acidocin LF221 B. The biochemical properties of LF221 acidocins resemble most the class II bacteriocins (Bogovič Matijašič *et al*., 1998). Determination of some unidentified AA residues and isolation of LF221 non-bacteriocinogenic derivative was reported as well (Čanžek Majhenič and Rogelj, 1998).

The classification of LF221 acidocins and partial characterization of their structural genes is shown herewith.

MATERIAL AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are shown in Table 1. Cultures were maintained at –20 °C in 20 % glycerol. *Lactobacillus acidophilus* LF221 and its non-bacteriocinogenic derivative LF221-1 were propagated in MRS broth (deMan *et al*., 1960) at 37 °C. *Escherichia coli* JM 110 (Yanisch-Perron *et al*., 1985) were grown in LB broth or on LB agar (Sambrook *et al*., 1989) where kanamycin (50 µg/ml; Sigma) was added to LB media for growth and selection of *E. coli* JM 110 transformants.

Isolation of plasmid and chromosomal DNA

Chromosomal DNAs from *L. acidophilus* LF221 and its bac$^-$ mutant were obtained as described by Leenhouts *et al*. (1990). Plasmid DNA from *E. coli* JM 110 transformants was isolated by the alkaline lysis of Birnboim and Dolly (1979).

Table 1. Bacterial strains and plasmids
Preglednica 1. Bakterijski sevi in plazmidi

Strain or plasmid	Characteristics	Reference or source
Bacteria		
L. acidophilus LF221	bacteriocinogenic strain (bac^+), child's faeces isolate	Bogovič Matijašič *et al*., 1998
L. acidophilus LF221-1	non-bacteriocinogenic derivative (bac^-), NN-nitrosoguanidin mutant	Čanžek Majhenič and Rogelj, 1998
E. coli JM 110	plasmid-free strain	Stratagene
Vectors		
pCR-Blunt	3.5 kb, Km^r, lethal gene ccdB	Invitrogen

Molecular cloning, transformation and DNA sequencing

Probes, used in this study, were prepared as already described (Čanžek Majhenič and Rogelj, 1998). Briefly, from the N-terminal AA sequence of purified acidocin LF221 A and B, the degenerate primers (bacA5', bacA3', bacB5', bacB3') were constructed and used in PCR reactions, where chromosomal DNA of LF221 and its non-bacteriocinogenic derivative served as a template. Generated PCR fragments were ligated and transformed into *E. coli* JM 110. Plasmid DNA of grown transformants was analysed with *Bam*HI/*Xba*I (Boehringer) digestion and later on sequenced by the dideoxy chain termination method of Sanger *et al*. (1977) using a DNA sequencing with 7-deaza-dGTP and T7 Sequenase DNA polymerase kit (Amersham). [^{35}S]dATP (New England Nuclear) was used for labelling. pCR-Blunt specific primers BL1 and BL2, used in sequencing reactions, are listed in Table 2. When necessary, the recombinant DNA of the clones was used as a probe, where the AA sequence derived from the nucleotide sequence was homologous to the N-terminal AA sequence determined from the purified acidocins.

Southern hybridization

For localization of the structural genes for acidocin LF221 A and B, single and double digestion of the chromosomal DNA of bac^+ and bac^- strain was performed with the following restriction enzymes: *Hind*III, *Eco*RI, *Bam*HI, *Bgl*II, *Hae*III and *Eco*RV as recommended by the producer (Boehringer). Fragments were separated on a 1 % agarose gel for 20 h at 15 V. Southern transfer of DNA from agarose gels to MagnaGraph nylon transfer membranes (Micron Separations Inc., Westboro, Massachusetts) was done as described by Sambrook *at el*. (1989). DNA was fixed onto membrane with the UV light (Stratalinker^{TM} 1800, Stratagene). DIG-dUTP (Boehringer) labelling of the probes was realized in PCR reactions by using the Taq DNA polymerase as outlined by the manufacturer (Boehringer Mannheim Biochemicals, The Genius^{TM} System User's Guide for Membrane Hybridization). Conditions in 'labelling' PCR reaction were as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 30 sec, and polymerization at 72 °C for 45 sec through 35 cycles. Recombinant DNA of previously sequenced pCR-Blunt clones served as a template. Vector specific primers BL1 and BL2, used in PCR reactions are shown in Table 2. Qiaquick PCR Purification Kit Protocol (Qiagen) was used to purify amplified labelled probes from the PCR reactions.

Prehybridization and hybridization with DIG labelled probes was performed at 68 °C for 2 h and overnight respectively according to the protocol of DIG DNA Labelling and Detection Kit (Boehringer).

Table 2. Primers used for PCR and DNA sequencing
Preglednica 2. Oligonukleotidni začetniki, uporabljeni v reakcijah PCR ali sekvenciranju DNK

Primer	Technique	Sequence
BL1	SEQ/PCR	5'-GCA TCA AGC TTG GTA CCG AG-3'
BL2	SEQ/PCR	5'-CCC TCT AGA TGC ATG CTC G-3'
BL3	PCR	5-'CGC GTT AGA ATA CTC AAG C-3'
seqA5'	PCR	5-ATA TGG GAT TCC CGA AAT ATA-3'
seqA3'	PCR	5-ATA TTA GGG TGC GCT AACGGA GC-3'

Colony hybridization and analysis of the recombinant DNA of the hybrids

To clone the structural gene for acidocin LF221 A, the region containing specific *Hind*III/*Bam*HI for acidocin LF221 A was purified from 1 % agarose gel, using Qiaex kit (Qiagen). Similarly, *Hind*III/*Eco*RI specific region for acidocin LF221 B was extracted. Furthermore, purified fragments were ligated into pCR-Blunt vector as recommended by manufacturer (Invitrogen) at 16 °C overnight with T4 DNA ligase (Boehringer) and the recombinant DNA was transformed into *E. coli* JM 110 by heat shock (90 s, 42 °C). After transformation, cells were incubated for 1 h at 37 °C in nonselective media prior to plating onto selective media. Putative pCR-Blunt clones were detected with the help of the lethal gene. After the growth, transformants were transferred onto Whatman 541 filter paper (Whatman Ltd. Maidstone), 9 cm in diameter. Lysis of the cells, denaturation and immobilization of the DNA were carried out as described by Gergen *et al*. (1979). Probe was labelled at the 5' with [γ-^{32}P]dATP (Amersham) with T4 polynucleotide kinase (Boehringer) and then purified through a Nuctrap push column (Stratagene). Hybridization was accomplished at 68 °C overnight (Sambrook *et al*., 1989). To detect positive hybrids, the membrane was exposed to an autoradiography film.

When necessary, plasmid DNA was isolated from the positive hybrids and analysed with restriction and PCR respectively. To examine the acidocin LF221 A gene in positive clones, endonucleases *Hind*III/*Xba*I were used (Boehringer). Moreover, same recombinant DNA was also subjected to PCR reactions, where different combinations of the acidocin LF221 A gene specific primers seqA5' (Ile^{20}-Tyr-Phe-Gly-Asn-Pro-Ile-Leu^{27}) and seqA3' (Ile^{26}-Leu-Gly-Cys-Ala-Asn-Gly-Ala^{33}) and vector primers BL2 and BL3 were used. Conditions of PCR reaction were the same as decribed above, except that annealing and polymerization times were extended to 1 min and 3 min respectively. Primer sequences are listed in Table 2. Amplified PCR products were electrophoresed on a 2 % agarose gel.

RESULTS AND DISCUSSION

Detection of the structural genes for acidocin LF221 A and B

The results of Southern hybridization with acidocin LF221 A probe are shown in Figure 1. As it can be revealed, the same hybridization pattern was obtained, either when the chromosomal DNA of bac$^+$ strain or its non-bacteriocinogenic derivative was used as a template. Same results were determined with acidocin LF221 B probe (data not shown). These results strongly suggest that the non-bacteriocinogenic strain might still contain the structural genes for acidocin LF221 A and B. Similar observations that bac$^-$ derivative contains structural genes for both acidocins

were already reported by Čanžek Majhenič and Rogelj (1998), as PCR reactions with acidocin LF221 A and B degenerate primers amplified same size and identical fragments when either DNA of LF221 strain or its bac- mutant was used as a template. Another interesting fact is that the probe for acidocin LF221 B, used in Southern hybridization, was constructed in PCR reaction, where plasmid DNA with cloned part of acidocin LF221 B structural gene from bac$^-$ strain was used as a template.

*Hind*III/*Bam*HI digestion of LF221 chromosomal DNA gave a single 0.9 kb fragment that hybridized with acidocin LF221 A probe whereas 1.6 kb *Hind*III/*Eco*RI fragment of LF221 DNA hybridized with acidocin LF221 B probe. The 0.9 kb *Hind*III/*Bam*HI fragment was ligated into pCR-Blunt and introduced to *E. coli* JM 110.

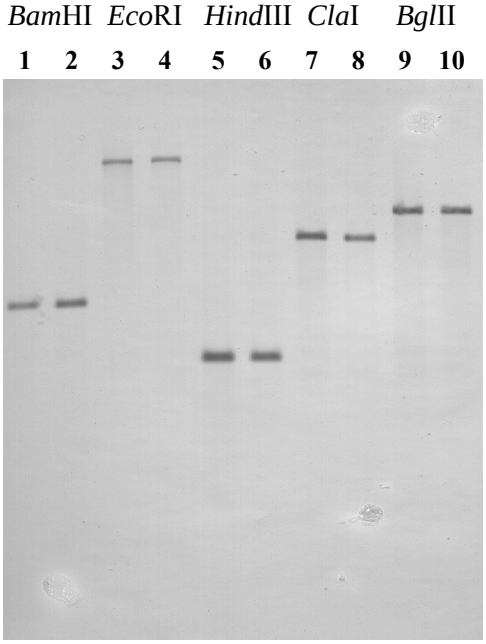

Figure 1. Results of Southern hybridization with acidocin LF221 A probe and digested chromosomal DNA of bac$^+$ and its bac$^-$ mutant as template. Lane 1,3,5,7,9: digested chromosomal DNA of bac$^+$; lanes 2,4,6,8,10: digested chromosomal DNA of bac$^-$ mutant.

Slika 1. Rezultati Southern hibridizacije, v kateri smo uporabili sondo za acidocin LF221 ter razrezani kromosomski DNK seva bac$^+$ in njegovega mutanta bac$^-$. Stolpec 1,3,5,7,9: razrezana kromosomska DNK seva bac$^+$; stolpec 2,4,6,8,10: razrezana kromosomska DNK mutanta bac$^-$.

Colony hybridization and analysis of the recombinant DNA of positive clones

Radioactive labelled probe for acidocin LF221 A was used to detect a positive clone(s), carrying 0.9 kb *Hind*III/*Bam*HI insert with the acidocin LF221 A structural gene. Ten colonies out of 700 screened gave positive signal and their recombinant DNA was further analysed. Results of *Hind*III/*Xba*I restriction are shown in Figure 2.

Restriction analysis showed that only colonies 1, 5, 6 and 10 might carry the expected fragment with the acidocin LF221 A structural gene. But to confirm this speculation, recombinant DNA of these four clones was analyzed with PCR reactions. Only with clone 1 the 0.9 kb *Hind*III/*Bam*HI insert with acidocin LF221 A gene was confirmed (Figure 3). Examinations of the recombinant DNA of putative positive clones assured only one as a really positive one (Figures 2 and 3). It is possible that with the other 9 colonies the hybridization signal was unspecific.

The comparison of the primary structure of acidocins LF221 A and B with already known bacteriocins showed that acidocin LF221 A has 50 % identity over a small region of the ThmB peptide of thermophilin 13, whereas acidocin LF221 B has 50 % identity with a small region of the LafX peptide of lactacin F. According to the results obtained so far, we assume that both LF221 bacteriocins are novel representatives of the II class of bacteriocins.

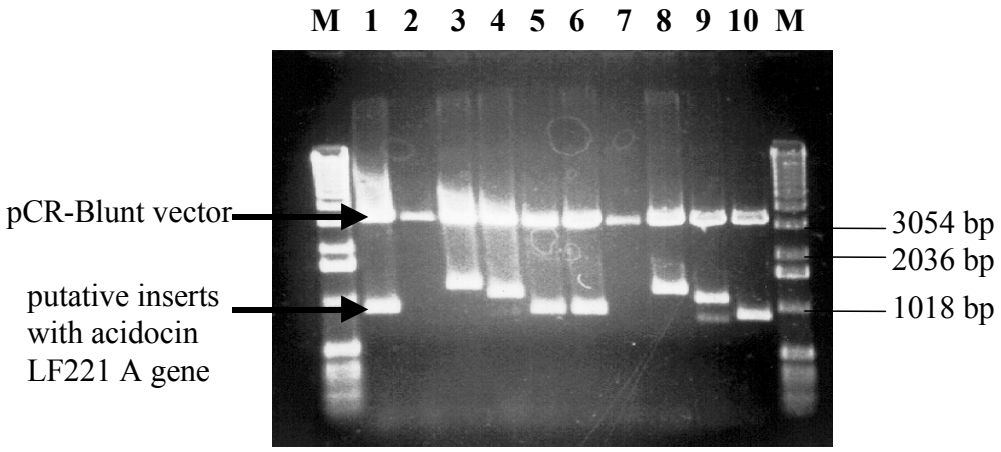

Figure 2. Analysis of the digested recombinant DNA of ten putative positive clones. Lane M: marker 1 kb; lane 1,2,3,4,5,6,7,8,9 and 10: results of *Hind*III/*Xba*I digested recombinant DNA of ten positive clones. 3.5 kb fragment: pCR-Blunt vector; 0.9 kb fragment: putative *Hind*III/*Bam*HI fragment of chromosomal DNA containing LF221 A structural gene

Slika 2. Analiza razrezane rekombinantne DNK 10-ih potencialno pozitivnih klonov. Stolpec M: marker 1 kb; stolpec 1,2,3,4,5,6,7,8,9 in 10: rezultati restrikcije rekombinantne DNK potencialno pozitivnih klonov z encimoma *Hind*III/*Xba*I. Fragment 3,5 kb: vektor pCR-Blunt; fragment 0,9 kb: verjetni *Hind*III/*Bam*HI fragment kromosomske DNK s strukturnim genom za acidocin LF221 A.

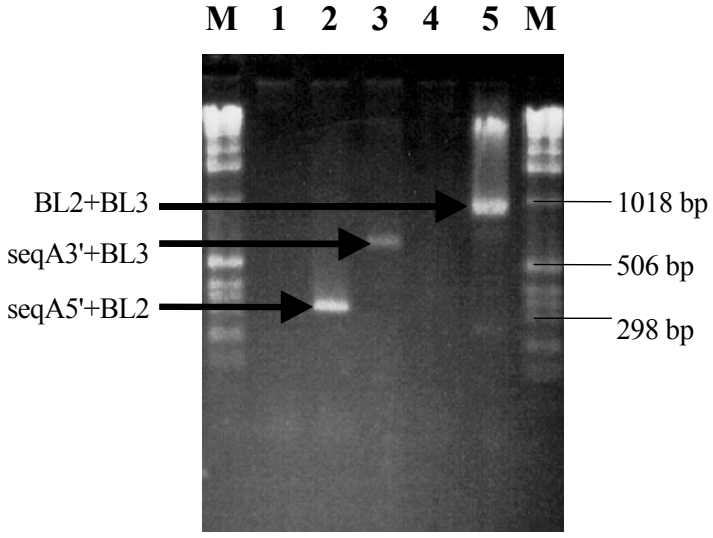

Figure 3. Amplified PCR products where recombinant DNA of the only positive clone 1 was used as a template and 5 different combinations of specific primers. Lane M: marker 1 kb, lane 1: seqA3'+BL2; lane 2: seqA5'+BL2; lane 3: seqA3'+BL3; lane 4: seqA5'+BL3; lane 5: BL2+BL3.

Slika 3. Produkti reakcije PCR, kjer smo kot tarčno DNK uporabili rekombinantno DNK pozitivnega klona 1 ter 5 različnih kombinacij oligonukleotidnih začetnikov. Stolpec M: marker 1 kb; stolpec 1: seqA3'+BL2; stolpec 2: seqA5'+BL2; stolpec 3: seqA3'+BL3; stolpec 4: seqA5'+BL3; stolpec 5: BL2+BL3.

SUMMARY

To localize the structural genes for LF221 acidocins identical hybridization signals were obtained when chromosomal DNA of either bac^+ or bac^- was used as a template in Southen hybridization. It is assumed that bac^- derivative still carries genes for acidocins. Acidocin LF221 A or B showed certain homology with thermophilin 13 or lactacin F that are already confirmed representatives of the class II bacteriocins. This finding and the fact that the biochemical properties of LF221 acidocins resemble most the class II bacteriocins, strongly support the affirmation that acidocin LF221 A and B are novel representatives of this class of bacteriocins.

POVZETEK

Pri lokalizaciji strukturnih genov za acidocina seva LF221 smo ugotovili, da smo s Southern hibridizacijo dobili identične hibridizacijske signale, ne glede ali smo kot tarčno DNK uporabili kromosomsko DNA seva bac^+ oz. njegovega mutanta bac^-. Sklepamo, da nebakteriocinogeni mutant še vedno nosi strukturne gene za oba acidocina. Poleg tega je primerjava primarnih struktur acidocinov LF221 A in B z znanimi bakteriocini pokazala, da sta delno homologna termofilinu 13 oz. laktacinu F, ki sta potrjena predstavnika II. skupine bakteriocinov. Ta ugotovitev in dejstvo, da sta se acidocina seva LF221 tudi po biokemičnih lastnostih najbolj približala tej skupini, potrjujeta, da sta acidocin LF221 A in B nova predstavnika te skupine.

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