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Identification and nucleotide sequence of genes involved in the synthesis of lactocin 705, a two-peptide bacteriocin from *Lactobacillus casei* CRL 705

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Abstract

The structural gene determinants of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705, have been amplified from a plasmid of approximately 35 kb and sequenced. Lactocin 705 is a class IIb bacteriocin, whose activity depends upon the complementation of two peptides (705α and 705β) of 33 amino acid residues each. These peptides are synthesized as precursors with signal sequences of the double-glycine type, which exhibited high identities with the leader peptides of plantaricin S and J from *Lactobacillus plantarum*, brochocin C from *Brochotrix campestris*, sakacin P from *Lactobacillus sake*, and the competence stimulating peptides from *Streptococcus gordonii* and *Streptococcus mitis*. However, the two mature bacteriocins 705α and 705β do not show significant similarity to other sequences in the databases.

Keywords: Lactocin 705; Two-component bacteriocin; *Lactobacillus casei*

1. Introduction

Lactic acid bacteria (LAB), used for centuries by man to preserve foods, produce a wide variety of antagonistic compounds, including lactic acid, hydrogen peroxide and bacteriocins. Bacteriocins are antimicrobial peptides which are bactericidal towards bacteria taxonomically close to the producer [1]. Three main classes of bacteriocins are produced by LAB [2]: (i) class I: lantibiotics, small (< 5 kDa) peptides which contain lanthionine and/or β-methyl-lanthionine residues; (ii) class II: non-lantibiotic, low-molecular mass (< 10 kDa), heat stable peptides; and (iii) non-lantibiotic, large (> 30 kDa) heat labile peptides. Most of the bacteriocins produced by LAB belong to the class II bacteriocins, which have a limited spectrum of antimicrobial activity. These bacteriocins, produced as prepeptides which are activated by a cleavage of the N-terminal leader peptide, can be subdivided into (iiA) *Listeria*-active peptides, (iiB) two-peptide bacteriocins, (iiC) Sec-dependent bacteriocins and (iiD) class II bacteriocins that do not belong to the other subgroups [2].

Lactocin 705, a bacteriocin produced by the meat starter culture *Lactobacillus casei* CRL 705, is active against several Gram-positive bacteria, including food-borne pathogens [3], and hence is a good candidate to be used for biopreservation of fermented meats. A single peptide associated with lactocin 705 antagonism has been previously purified, sequenced and characterized [4]; it is a small basic protein which contains a high ratio of glycine residues and does not show lanthionine or β-methyl-lanthionine residues [4]. In this work, the identification and DNA sequencing of the plasmid-encoded lactocin 705, and the application of lactocin 705-derived synthetic peptides, allowed us to reclassify lactocin 705 as a class IIb two-component bacteriocin.

2. Materials and methods

2.1. Microorganisms, media and growth conditions

The strains used in this study were obtained from the collection of the Centro de Referencia para Lactobacilos

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(CERELA; CRL collection). The bacteriocin producer strain *L. casei* CRL 705 and the indicator strain *Lactobacillus plantarum* CRL 691 [5], as well as strain 28B (this work), a plasmid-cured derivative of CRL 705 (phenotype Bac−), were maintained at −20°C in MRS [6] containing 10% glycerol. Cultures were grown in MRS broth at 30°C. When needed, agar was added to the MRS broth to make solid media.

2.2. Assay of bacteriocin production and synthesis of lactocin α and β peptides

Bacteriocin production and radial diffusion assays were done as described by Palacios et al. [4]. Synthesis of the 31- and 33-amino acid lactocin 705α peptides and of the 33-amino acid 705β peptide were done in Geminys Biotech (Alachua, FL, USA) and Bio-synthesis (Lewisville, TX, USA), respectively.

2.3. Molecular techniques

Standard techniques were used for *Escherichia coli* plasmid isolation, DNA modifications, endonuclease restrictions, ligations and plasmid transformations [7]. Plasmid pBlueScript SKII+ (Stratagene, CA, USA) was used for cloning. Plasmid DNA was isolated from *L. casei* using the method of Muriana and Klaenhammer [8] and resolved on 0.65% agarose at 20 V for 17 h. Alternatively, plasmid DNA was purified using a CsCl–EtBr ultracentrifugation three-step density gradient [7]. Plasmid curing was done by growing a 2.0% culture of CRL 705 at elevated temperature (39°C) in the presence of 80 μg ml^{-1} of acridine orange. The culture was diluted to 10^{-5}, pour-plated on MRS plates and incubated overnight at 30°C. Cells negative in the production of lactocin 705 were characterized by 16S rRNA sequencing, using primers PLB16 (5′-AGAGTTTGATCCTGGCTCAG-3′) and MLB16 (5′-GGCTGCTGGCACGTAGTTAG-3′), SDS–PAGE of total proteins and plasmid DNA analysis [7]. Colony hybridization and DNA labelling were carried out by standard methods [7]. Sequences were obtained from double-stranded plasmid DNA or PCR products by the dideoxy chain terminating method [9], using the universal primers T7 or T3 and specific primers to the lactocin 705 sequence at the BioResource Center Cornell University (Ithaca, NY, USA) and NCSU DNA Sequencing Facility North Carolina State University (Raleigh, NC, USA). Sequences were analyzed and aligned using the program Biology Work-bench NCSA version 3.0 (http://biology.ncsa.uiuc.edu) and homology searches were performed with the BLAST and FASTA programs [10]. The sequence of lactocin 705 was submitted to the GenBank database under accession number AF200347.

2.4. PCR conditions

Amplification of the bacteriocin genetic determinants by PCR was performed with the single specific primer-PCR (SSP-PCR) described by Shyamala and Ames [11]. Ten μg of plasmid DNA from CRL 705 was cut with *Hin*dIII and DNA fragments ranging from 1 to 3 kb were recovered in a volume of 15 μl, using Prep-A-gene kit (Bio-Rad). Ten μl was then ligated with 1 μg of *Hin*dII-digested pBlueScript SK (Stratagene) at 14°C for 16 h. The sample was recovered using Prep-A-gene kit, and resuspended in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). A 2-μl aliquot of the ligation mix was amplified in a total volume of 25 μl which contained 1×PCR buffer (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 2.5 μM (mix) deoxynucleoside triphosphate, 1 U of Taq DNA polymerase (Promega) and 2.5 pM of primer T3 or T7 plus primer lac705 (5′-GGNTAYATHCARGGNATHC-3′). DNA amplification was carried in a DNA Thermal cycler 480 (Perkin-Elmer Co., Norwalk, CT, USA) using the following program: one cycle of 5 min for 94°C; 30 cycles of: denaturation, 94°C for 3 min; primer annealing 45°C for 1 min and 30 s; and primer extension 72°C for 2 min; and one cycle of 72°C for 5 min. Five μl of the reaction mix was analyzed by electrophoresis on a 1.2% agarose gel. Amplified DNA fragments were blunt-ended, cloned into the *Sma*I site of pBlueScript SK and sequenced at the North Carolina State University Sequencing Facility. A further SSP-PCR reaction was carried out with the specific primer Bac1 (5′-ATTAGCTGCGCTTATTCC-3′).

3. Results and discussion

3.1. Localization of the lactocin 705 genes

L. casei CRL 705 contains four plasmids of 4.2, 6, 35 and >50 kb (data not shown). Screening for the occurrence of negative variants (Bac−) of lactocin 705 after cell growth at sublethal temperatures (39°C), irrespective of the presence or absence of acridine orange, yielded almost 10% Bac− cells, which suggests the lactocin 705 genetic determinants are located on plasmid DNA. Comparison of plasmid contents of strain CRL 705 and one derivative Bac− strain (strain 28B) indicated that plasmids of 6 and 35 kb were absent in the mutant strain 28B (data not shown). Hybridization experiments, using specific DNA sequences of lactocin 705 (see below), gave one strong hybridization signal with the 35-kb plasmid DNA (data not shown).

3.2. PCR amplification and sequencing analysis of the lactocin 705 genes

Based on the available amino acid sequence of the lactocin 705 protein, the degenerate primer lac705 was de-

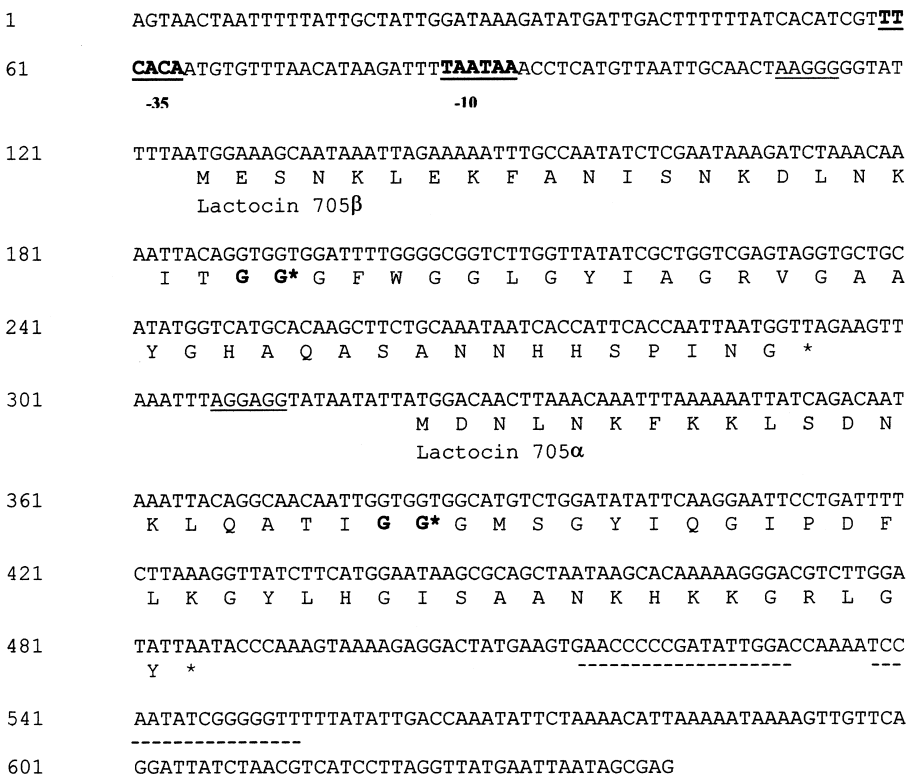

Fig. 1. Nucleotide sequence and derived amino acid sequence of lactocins 705α and 705β. The putative RBSs (AAGGG and AGGAGG) are underlined. The −35 and −10 positions of a putative promoter are indicated. Amino acid residues (1–22 of lactocin 705α and 1–23 of 705β) represent typical signal sequences. Processing sites of the prebacteriocins are marked by asterisks. The characteristic rho-independent terminator region is indicated by dotted lines. The GenBank accession number is AF200347.

fined and used to amplify, in a SSP-PCR reaction, the gene involved in lactocin 705 production. Plasmid DNA from *L. casei* CRL 705 was digested with *Hin*dIII, ligated to pBlueScript SKII+ and amplified using primer lac705 and the universal primer T3 or T7 (see Section 2). A unique 1.5-kb lac705-T7 DNA fragment (the ends of this fragment are defined by primers lac705 and universal T7) was amplified, isolated and cloned into the *Sma*I site of pBlueScript SKII+, and its nucleotide sequence determined in both directions. At the lac705 end of the lac705-T7 DNA fragment, an open reading frame (ORF) was identified which corresponded with the internal region of the lactocin 705 gene (now called *lac*705α; see below) (Fig. 1). DNA sequences upstream of the *lac*705 gene were further amplified and sequenced through SSP-PCR reactions with specific primer Bac1.

The 642-bp DNA sequence shown in Fig. 1 revealed the presence of a putative promoter sequence and two complete ORFs, designated α and β. A putative ribosome-binding site (RBS) preceded the start codon of ORFβ, named *lac*705β, by 9 bp. The *lac*705β gene was 168 bp in length and encodes a protein of 56 amino acid residues. Downstream of *lac*705β, separated by 27 bp, the 162-bp lactocin 705α gene was found, with a potential RBS 9 bp

Table 1
Alignment and identity of the α and β lactocin 705 subunits with other signal sequences

Signal sequences	Microorganism	Identities
MD-NLNK-FKKLSDNKLQATIGG	Lactocin705α- *Lactobacillus paracasei* CRL 705	100 %
MESNKLEKFANISNKDLNKITGG	Lactocin705β- *Lactobacillus paracasei* CRL 705	30 %
MHKVK-----KLNNQELQQIVGG	Brochocin C –*Brochothrix campestris*	44 %
----KLEQFVALKEKDLQKIKGG	Competence stimulating peptide- *Streptococcus mitis*	57 %
LQ–QFEIL---TDNKLQTVIGG	Competence stimulating peptide- *Streptococcus gordonii*	61 %
LD--VVDAFAPISNNKLNGVVGG	Plantaricin J – *Lactobacillus plantarum*	38 %
MD–KIIKFQGISDDQLNAVIGG	Plantaricin S, β protein- *Lactobacillus plantarum*	45 %
ME-----KFIELSLKEVTAITGG	Sakacin P- *Lactobacillus sake*	50 %
* *◊ ◊◊ * *	Consensus[a]	-

◊ and * indicate hydrophilic and hydrophobic amino acid residues, respectively.
[a]Consensus according to Håvarstein et al. [16].

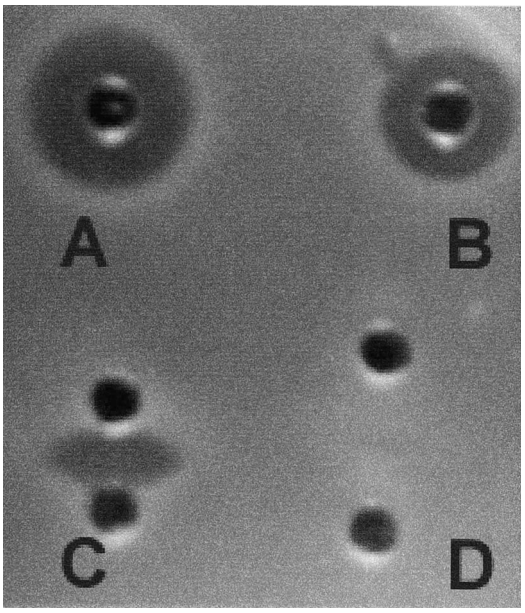

Fig. 2. Complementary activity of the two components of lactocin 705 as shown by radial diffusion assay. (A) Mixture of synthetic lactocins 705α and 705β (15 μg of each peptide); (B) supernatant from a CRL 705 culture. In (C and D), the synthetic peptides 705α and 705β were seeded in separated wells. No antagonistic activity was observed around the wells, except in the zone of confluency. The dark areas represent clearing (lysis) of bacteria *L. plantarum* CRL 691; the white area represents bacterial growth.

upstream. The *lac*705α gene encodes a protein of 54 amino acid residues; the C-terminal 33-amino acid part of the protein corresponded with the 31-amino acid protein purified and sequenced by Palacios et al. [4]; two extra amino acids (G and Y) at the C-terminal end were revealed from the deduced amino acid sequence of the *lac*705α gene. The short intergenic region between the *lac*705β and *lac*705α genes suggested that these two genes might be cotranscribed. A perfect inverted repeat structure, which represents a putative rho-independent transcription terminator, was detected 31 nucleotides downstream of the stop codon of *lac*705α.

Amino acid sequence analysis of the lactocin 705β and 705α proteins revealed that they are translated as bacteriocin precursors which include an N-terminal leader sequence of the double-glycine type ([12]; Table 1). When the N-terminal sequences of lactocin 705α and lactocin 705β, 21 and 23 amino acids, respectively, were compared with other amino acid sequences in the databases, the highest similarity scores were obtained with the leader peptides of plantaricin S and J from *L. plantarum*, brochocin C from *Brochotrix campestris*, sakacin P from *Lactobacillus sake*, and the competence stimulating peptides from *Streptococcus gordonii* and *Streptococcus mitis* (Table 1). However, no significant amino acid sequence homologies with other amino acid sequences were found for the mature lactocin 705α and lactocin 705β proteins.

3.3. Lactocin 705 is a two-component bacteriocin

Purification and amino acid sequencing of lactocin 705α have been reported earlier [4]. Edman degradation analysis of the purified lactocin 705α showed that the primary structure of the bacteriocin contained 31 amino acid residues ([4]; Swiss-Prot accession number P80959). DNA sequence analysis of the lactocin 705α gene revealed two extra amino acids (glycine and tyrosine) at the C-terminal.

In order to determine which peptide was responsible for the antimicrobial activity, synthetic peptides were prepared from the deducted sequences. As showed in Fig. 2, the synthetic lactocin 705α peptides, of 31 (data not shown) and 33 amino acid residues, as well as the synthetic lactocin 705β peptide, did not show bacteriocin activity when tested against the indicator strain *L. plantarum* CRL 691. Bacteriocin activity was only detected upon the complementation of peptides 705α plus 705β (Fig. 2). The response shown by peptides 705α and 705β is typical of class IIb bacteriocins [13,14].

The data of this work showed that the genes of lactocin 705 have been identified and sequenced. The structural genes of this non-lantibiotic bacteriocin, associated with a plasmid of approximately 35 kb, encode precursors of two proteins, lactocin 705α and lactocin 705β, with its corresponding signal sequences. These types of leader peptides are cleaved following two highly conserved glycine residues by the proteolytic activity on an ABC transporter protein, resulting in the mature peptides [15]. Preliminary DNA sequencing data from the region upstream of *lac*705β indicate the presence of putative immunity determinants and genes encoding proteins with similarities to maturation and transport proteins in other class II bacteriocin systems.

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