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 (705K and 705L) 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 705K and 705L do not show significant similarity to other sequences in the databases. © 2000 Published by Elsevier Science B.V. All rights reserved.

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...
(CERELA; CRL collection). The bacteriocin producer strain \textit{L. casei} CRL 705 and the indicator strain \textit{Lactobacillus plantarum} CRL 691 [5], as well as strain 28B (this work), a plasmid-cured derivative of \textit{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 Geninmys Biotech (Alachua, FL, USA) and Bio-synthesis (Lewisville, TX, USA), respectively.

2.3. Molecular techniques

Standard techniques were used for \textit{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 \textit{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 10⁻², poured 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 HindIII 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 HindIII-digested pBlue-Script 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 MgCl₂, 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 Smal 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'-ATTAGCTGCGCTTATCC-3').

3. Results and discussion

3.1. Localization of the lactocin 705 genes

\textit{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-
and used to amplify, in a SSP-PCR reaction, the gene involved in lactocin 705 production. Plasmid DNA from \textit{L. casei} CRL 705 was digested with \textit{HindIII}, ligated to \textit{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 SmaI site of \textit{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 \textit{lac705K}; see below) (Fig. 1). DNA sequences upstream of the \textit{lac705} 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 \textit{K} and \textit{L}. A putative ribosome-binding site (RBS) preceded the start codon of ORF \textit{L}, named \textit{lac705L}, by 9 bp. The \textit{lac705L} gene was 168 bp in length and encodes a protein of 56 amino acid residues. Downstream of \textit{lac705L}, separated by 27 bp, the 162-bp lactocin 705 \textit{K} gene was found, with a potential RBS 9 bp upstream of the start codon of ORF \textit{K}, named \textit{lac705K}, by 9 bp.

<table>
<thead>
<tr>
<th>Signal sequences</th>
<th>Microorganism</th>
<th>Identities</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD-NLNK-FKLSDNKLQATIGG</td>
<td>Lactocin705α - \textit{Lactobacillus paracasei} CRL 705</td>
<td>100 %</td>
</tr>
<tr>
<td>MESNLKEFANISNKDLKINIGG</td>
<td>Lactocin705β - \textit{Lactobacillus paracasei} CRL 705</td>
<td>30 %</td>
</tr>
<tr>
<td>MHKV-KLNNQEQQQIVGG</td>
<td>\textit{Brochothrix campesiris}</td>
<td>44 %</td>
</tr>
<tr>
<td>----KLNNQEQQQIVGG</td>
<td>Competence stimulating peptide - \textit{Streptococcus mitis}</td>
<td>57 %</td>
</tr>
<tr>
<td>----KLNNQEQQQIVGG</td>
<td>Competence stimulating peptide - \textit{Streptococcus gordonii}</td>
<td>61 %</td>
</tr>
<tr>
<td>LD-VVDAFAPISNKLNGVGG</td>
<td>\textit{Lactobacillus plantarum}</td>
<td>38 %</td>
</tr>
<tr>
<td>MD-KIKFQGSDQMQVGG</td>
<td>\textit{Lactobacillus plantarum}</td>
<td>45 %</td>
</tr>
<tr>
<td>ME-KIKFQGSDQMQVGG</td>
<td>\textit{Sakacina} P - \textit{Lactobacillus sake}</td>
<td>50 %</td>
</tr>
</tbody>
</table>

and * indicate hydrophilic and hydrophobic amino acid residues, respectively.

*Consensus according to Håvarstein et al. [16].
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 P60959). 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 deduced sequences. As shown 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 II 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 lac705β indicate the presence of putative immunity determinants and genes encoding proteins with similarities to maturation and transport proteins in other class II bacteriocin systems.

Acknowledgements

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References


