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Characterization of salivaricin CRL 1328, a two-peptide bacteriocin produced by *Lactobacillus salivarius* CRL 1328 isolated from the human vagina

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Abstract

Salivaricin CRL 1328 is a heat-stable bacteriocin produced by *Lactobacillus salivarius* CRL 1328, a strain isolated from healthy human vagina, with potential applications for preventing urogenital infections. The objective of this study was to characterize the locus responsible for salivaricin CRL 1328 production and its mechanism of action against *Enterococcus faecalis* MP97 as the sensitive strain. Oligonucleotides were designed based on sequences of antimicrobial peptides previously described in the literature. The salivaricin CRL 1328 cluster was identified, sequenced and analyzed. This cluster was similar to the previously described ABP118 which codified for a two-peptide bacteriocin. The putative mature peptides of salivaricin CRL 1328, Salα and Salβ were chemically synthesized. These peptides did not show bacteriocin activity when assayed individually. Both peptides exhibited optimal antimicrobial activity at an equimolar ratio. Spectroscopic fluorescence assays were carried out using the synthetic peptides to study the effect of salivaricin on proton motive force. This bacteriocin was shown to dissipate membrane potential and the transmembrane proton gradient, both components of proton motive force. *E. faecalis* MP97 cells treated with salivaricin CRL 1328 peptides were observed in transmission electron microscopy which revealed ultrastructural modifications of the cell wall.

Keywords: Bacteriocin; Salivaricin CRL1328; Bacteriocin cluster; Synthetic peptides; Pathogen inhibition; Vagina; Pharmaceutical applications

1. Introduction

Bacteriocins are a structurally diverse group of ribosomally synthesized antimicrobial peptides which display antimicrobial activity against different bacteria [12]. Their activities can be either narrow- or broad-spectrum, capable of targeting bacteria within the same species or across genera, respectively [12]. Bacteriocins produced by lactic acid bacteria (LAB), a group of Generally Regarded As Safe (GRAS) microorganisms, have an interesting potential for use as food biopreservatives and pharmaceutical products in order to prevent growth of undesirable microorganisms in food and to prevent infections. Furthermore, because of the emergence and dissemination of antibiotic resistance and their association with continuous use of antibiotics, therapies based on antimicrobial peptides are attractive candidates as valid alternatives to antibiotic treatments. These antimicrobial therapies offer additional advantages over drug therapies currently used, because bacteriocins are considered as natural bioactive compounds; they are present in foods consumed since ancient times, and they represent a natural means of reducing or avoiding pathogenic growth via their antimicrobial activity. Moreover, the targets of these peptides often involve general but fundamental structures of sensitive microorganisms; therefore, the probability of emergence of resistance would be reduced compared with that observed in most current antibiotics or antimicrobials, which have more specific molecular targets [21].

In the last few years, major advances in the bacteriocin field have been observed in the LAB group. Research on this

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subject has identified several novel bacteriocins, many of which are produced by intestinal and food-borne bacteria [4,38]. Bacteriocinogenic probiotic LAB strains may play a role during in vivo interactions in the gastrointestinal tract, thus contributing to gut health [11]. *Lactobacillus salivarius* subsp. *salivarius* UCC118, a probiotic human strain, produces in vivo a bacteriocin that can protect mice against *Listeria monocytogenes* infection [11]. As concerns the urogenital tract, Lactocin 160, an antimicrobial peptide produced by *Lactobacillus rhamnosus*, did not show adverse effects in a rabbit vaginal irritation model used for in vivo safety evaluation of this type of product [14].

Salivaricin CRL 1328 is a heat-stable bacteriocin produced by *L. salivarius* CRL 1328, a strain isolated from human vagina [28]. This bacteriocin is active against potentially urogenital pathogenic bacteria such as *Enterococcus faecalis*, *Enterococcus faecium* and *Neisseria gonorrhoeae* [28]. A combination of tricine-SDS-PAGE, lumitein protein gel staining and a bioassay for antibacterial activity indicated that the molecular mass of salivaricin CRL 1328 was approximately 4.5 kDa [43].

Although production of bacteriocins by *L. salivarius* has been previously reported [4,7,16,31,40,41], the mode of action of these antimicrobial peptides has not been elucidated at the present time. An understanding of the mode of action of salivaricin CRL1328 would help in finding an effective application of this bacteriocin. Thus, the aim of the present study was to characterize the locus responsible for salivaricin CRL 1328 production and its mechanism of action against uropathogenic *E. faecalis* MP97 using chemically synthesized peptides.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacteriocin-producing *L. salivarius* CRL 1328 isolated from healthy human vagina and sensitive strain *E. faecalis* MP97 isolated from urogenital infection were obtained from the CERELA Culture Collection (Tucumán, Argentina) and the Instituto de Microbiología of the Universidad Nacional de Tucumán (Argentina), respectively. Both strains, originally isolated from human vagina [28] by our research group, were grown in LAPTg broth (15 g/l peptone, 10 g/l tryptone, 10 g/l yeast extract, 10 g/l glucose, and 1 ml/l tween-80, final pH 6.5 [17]) at 37 °C without aeration. *Listeria innocua* 7 (Unité de Recherches Laitiéres et Génetique Appliqueé, INRA France), used as indicator strain, was grown in Brain Heart Infusion (BHI, Difco) broth at 37 °C. All strains were stored in milk-yeast extract at −20 °C.

Escherichia coli DH10B (Stratagene, California, USA), used for transformations, was grown in Luria–Bertani (LB) broth at 37 °C, with vigorous agitation [37]. For selection of transformants harboring recombinant pCR2.1-TOPO or pBlueScript plasmids, LB agar was supplemented with 100 μg/ml ampicillin, 100 mM isopropyl thiogalactoside (IPTG) and 40 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal). Agar media were prepared by adding 1.5% (w/v) granulated agar to broth media.

2.2. DNA manipulations for genetic characterization of the salivaricin gene cluster

Genomic DNA of *L. salivarius* was isolated as described previously by Pospiech and Neumann [32].

Plasmid DNA was isolated from *E. coli* by an alkaline lysis procedure [5]. Agarose gel electrophoresis was carried out through standard methods. DNA ligation was performed with T4 DNA ligase (Invitrogen, Karlsruhe, Germany) in 20 μl reactions at 4 °C overnight and then stopped by ethanol precipitation. *E. coli* $CaCl_2$-competent cells were used, and heat shock transformation and recombinant DNA techniques were performed according to the method of Sambrook and Russell [37]. For routine PCR amplification, *Taq* polymerase (Invitrogen) was used. For cloning and sequencing purposes, high-fidelity *Vent* polymerase (New England Biolabs, Hertfordshire, UK) was used.

2.3. DNA amplification, sequencing and analysis

To identify salivaricin genes in *L. salivarius* CRL 1328 degenerate primers were designed based on sequence homologies of antimicrobial peptide genes from LAB (Table 1). Primers were synthesized by Invitrogen. PCRs were carried out in a MyCycler thermocycler (BioRad, California, USA). PCR mixtures (50 μl) contained 15 ng of DNA, 2.5 mM $MgCl_2$, the four deoxynucleoside triphosphates at 100 μM, each primer at 1 μM in *Taq* buffer and 2.5 U of *Taq* polymerase. PCR conditions included 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 or 50 °C (depending on the average melt temperature of the primer set) for 30 s, and extension at 70 °C for 3 min. The reactions were terminated with 5 min of incubation at 70 °C, and then chilled to 4 °C. PCR products were purified by the use of agarose gel electrophoresis and by the commercial GFX™ PCR DNA gel band purification kit (Amersham Biosciences, New York, USA). The fragments were cloned in *E. coli* DH10B into pCR2.1-TOPO vector by using the Invitrogen TOPO TA cloning kit. *E. coli* transformants were selected by their growth in LB broth supplemented with ampicillin, X-Gal and IPTG. Since the ABP118 primer set was the only that allowed amplification of the expected size, a series of primers based on the ABP-118 cluster sequence [16] were used to amplify fragments of about 3.5 kb. These amplicons were cloned into pCR2.1-TOPO vector and the sequence of these cloned fragments was determined by a primer walking strategy. The cloned PCR products were sequenced by Ruralex SRL (Buenos Aires, Argentina). Database searches were performed using the BLAST program of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST). Sequence comparisons and alignments were performed with BioEdit software using the ClustalW algorithm. The presence of putative promoter elements was predicted by Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Transcriptional terminators were predicted with the FindTerm algorithm (http://www.softberry.ru/berry.phtml). The DNA sequence described in this article has been