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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

Table 1
Primers used to identify the structural genes of salivaricin CRL 1328.

Primer set	Sequence (5′ → 3′)	Product size (bp)	Bacteriocin target	Reference
ABP118-Fw	GGNAAACGNGGNCCNAAC	343	ABP-118	[16]
ABP118-Rev	CCACCAGAAATTCCACCCGCT			
OR7-Fw	ACNAAYGGNGTNCAYTGYAC	103	OR-7	[40]
OR7-Rev	TRTCYTGNAGNCGNCCCCATR			
B2-Fw	ATGAATAGCGTAAAAGAATTA	197	Carnobacteriocin B2	[33]
B2-Rev	CGGTCTCCTACCAATGGATCC			
BM1-Fw f	ATGAAAAGCGTTAAAGAACTAAAT	184	Carnobacteriocin BM1	[33]
BM1-Rev	ATGTCCCATTCCTGCTAAACT			
Cur-Fw	ATGAATAATGTAAAAGAATTAAGT	180	Curvacin A	[42]
Cur-Rev	TTACATTCCAGCTAAACCACTAGC			
Mun3-Fw	TAGCTACTGGTGGAGCAGCT	116	Enterocin CRL35	[36]
Mun2-Rev	ACCTATAAATAAGAATACTC			
Ent A-Fw	GGTACCACTCATAGTGGAAA	137	Enterocin A	[3]
Ent A-Rev	CCCTGGAATTGCTCCACCTAA			
Ent B-Fw	GAAAATGATCACAGAATGCCTA	151	Enterocin B	[8]
Ent B-Rev	AGAGTATACATTTGCTAACCC			
Ent P	GCTACGCGTTCATATGGTAAT	86	Enterocin P	[10]
Ent Pr	TCCTGCAATATTCTCTTTAGC			

deposited in the GenBank database under accession no. EF592482.

2.4. Salivaricin peptide synthesis and bacteriocin assays

Salivaricin-α (Salα) and salivaricin-β (Salβ) peptides were synthesized by Genbiotech (Buenos Aires, Argentina), with a purity level no lower than 80%. The synthesized peptides were purified by reverse-phase HPLC and their molecular masses confirmed by MALDI-TOF MS. Synthetic peptides were dissolved in water and diluted to appropriate concentrations. Single peptides and mixtures of Salα and Salβ at different molar ratios were tested for antibacterial activity as described below.

2.5. Quantification of antimicrobial activity of synthetic peptides

Synthetic bacteriocin activity was estimated by two different methods, the agar well diffusion method [20] and a microtiter plate assay [29]. In the agar well diffusion method, aliquots (25 µl) of Salα and/or Salβ peptides in a range of concentrations from 0.5 to 50 µM were poured into the 4 mm holes performed in LAPTg agar plates (LAPTg 1% agar) which contained vaginal *E. faecalis* MP97 (10^6 CFU/ml). Plates were incubated for 2 h at room temperature and then for 24 h at 37 °C. The presence and size of inhibition halos were recorded. In addition, antibacterial activities at different concentrations and various molar ratios of Salα and Salβ were quantified in a microtiter plate assay system. Briefly, each well of a microplate contained 200 µl LAPTg broth inoculated with the indicator strain *E. faecalis* MP97 at an optical density of around 0.08 at 600 nm (OD_{600}) and the peptides, individually or combined at different concentrations (from 0 to 6 µM) and also at various molar ratios. The microtiter plates were incubated for 6 h at 37 °C, growth was measured spectrophotometrically at 600 nm (Versamax Plate Reader, Molecular Devices, CA, USA) and the percentage of inhibition of *E. faecalis* MP97 growth was determined.

2.6. Mechanistic studies of salivaricin: effect on proton motive force of E. faecalis

2.6.1. Cell membrane electric potential (Δψ) of the sensitive strain

The effect of the synthetic bacteriocin peptides on membrane potential was evaluated on indicator *E. faecalis* MP97 cells with fluorescent dye 3.3-dipropylthiadicarbocyanine iodide [DiSC3(5)] (Molecular Probes, Oregon, USA) according to Breeuwer and Abee [6]. Briefly, cells in exponential growth phase ($OD_{600} = 0.6$) were harvested by centrifugation (3000 × *g*, 10 min, 4 °C), washed twice with ice-cold 50 mM potassium phosphate buffer, pH 7.0, resuspended in the same buffer and stored on ice until use. Then the cell suspension was diluted to a final OD_{600} of 0.5 in 50 mM potassium phosphate buffer, pH 7.0, containing 10 mM glucose and 5 µM DiSC3(5). Fluorescence measurements were performed with a Cary Eclipse spectrofluorometer (Varian, Victoria, Australia) at 30 °C with continuous stirring. An excitation wavelength of 643 nm and an emission wavelength of 666 nm were used. After a steady-state of Δψ was reached, Salα and Salβ peptides were added to the reaction. At the end of the assay, K^+ ionophore valinomycin (1 µM) was added to dissipate the membrane potential (ΔΨ).

2.6.2. Effect of salivaricin on intracellular pH (ΔpH) of the indicator strain

The transmembrane **Δ**pH of *E. faecalis* MP97 cells was determined by monitoring the fluorescence intensity of the pH-sensitive fluorescent probe 5- (and 6-)-carboxyfluorescein diacetate succinimidyl ester (cFDASE, Molecular probes) as described previously [6]. Cells containing the fluorescent probe were diluted to a concentration of approximately 10^7 cells per ml in a 3-ml glass cuvette and placed in the

thermostated cuvette holder of the spectrofluorometer. Fluorescence intensities were detected in a Cary Eclipse spectrofluorometer at 30 °C with continuous stirring at excitation wavelengths of 500 (pH sensitive) and 440 nm (pH insensitive) by rapidly alternating the monochromator between both wavelengths. The emission wavelength was detected at 530 nm for both excitation wavelengths. The excitation and emission slit widths were 5 and 10 nm, respectively. The 500-to-440-nm ratios were corrected for background signal due to the buffer. At the end of each assay, the extracellular fluorescence signal (background) was determined in the filtrate obtained from filtration of the cell suspension through a 0.22-μm-pore-size membrane filter. The calibration curve of pH was performed with buffers with pH values ranging from 4 to 8. Buffers were prepared with glycine (50 mM), citric acid (50 mM), $Na_2HPO_4 \cdot 2H_2O$ (50 mM), and KCl (50 mM); the pH was adjusted with either NaOH or HCl. The pHin and pHout were equilibrated by addition of nigericin (1 μm).

2.7. Ultrastructural modifications produced by synthetic peptides on E. faecalis: transmission electronic microscopy (TEM)

In order to evaluate ultrastructural modifications produced by salivaricin on *E. faecalis* MP97, samples were taken from the exponentially grown cells of *E. faecalis* MP97 before and 30 min after addition of the synthetic peptides Salα and Salβ (5 μM). Cells were harvested by centrifugation and resuspended in 1 volume of 0.1 M phosphate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde and 1% (w/v) $CaCl_2$. The mixture was centrifuged (3000 ×*g*, 5 min) and maintained at 4 °C for 16 h. The samples were included in agar and fixed in phosphate buffer containing 1% (w/v) osmium tetraoxide at 20 °C for 2 h. Dehydration was performed using an increased serial dilution of ethanol concentration (30%, 50%, 70%, 85%, 90%, 100%) and finally dried with 100% acetone. Samples were included in Spurr medium [39] and stained according to Reynolds [35]. The cells were examined in an electron microscope (EM 109 Zeiss, Germany).

3. Results

3.1. Salivaricin cluster identification

In PCR experiments with primers listed in Table 1 and genomic DNA from *L. salivarius* CRL 1328 as template, only oligonucleotides designed for bacteriocin ABP-118 enabled amplification of a DNA fragment of the expected size (~350 bp). The PCR fragment was sequenced, and the deduced amino acid sequence was found to be similar to ABP-118, a bacteriocin produced by *L. salivarius* subsp. *salivarius* UCC118. The complete nucleotide sequence of the salivaricin cluster was determined by a primer-walking strategy. Using this technique, 7759 bp of DNA surrounding the initial 343 bp fragment were sequenced (GenBank accession number EF592482). Sequence analysis revealed the presence of 11 complete putative open reading frames (ORFs) which are transcribed in the same direction (Fig. 1). These ORFs were designated *sal* when functions could be assigned to the encoded products (according to the nomenclature used by Flynn et al. [16]). The database analysis of this salivaricin CRL 1328 locus showed 99% identity (DNA-DNA) with the ABP-118 cluster reported by Flynn et al. [16]. Analysis of this cluster indicated that salivaricin CRL 1328 was a two-peptide-bacteriocin. The structural genes encoding salivaricin CRL 1328 activity were designated *salα* and *salβ,* with the latter located directly downstream of *salα* (Fig. 1). The ORF located immediately downstream of structural gene *salβ* probably encodes the putative immunity protein. This organization is a common feature of LAB bacteriocin operons [25].

3.2. Antimicrobial activity analysis of synthetic peptides

In order to investigate the optimal molar relationship of peptides encoded by *salα* and *salβ* genes, mature amino acid sequences of Salα and Salβ were deduced from the nucleotide sequence and these peptides were chemically synthesized. The sequences of Salα and Salβ were KRGPNCVGNFLGGLFA GAAAGVPLGPAGIVGGANLGMVGGALTCL and KNGYG GSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFT SCR, respectively. Salα and Salβ were tested individually or combined at different concentration ratios for their bacteriocin activity against the indicator strain *E. faecalis* MP97. The antibacterial activity at different concentrations and various molar ratios is shown in Fig. 2. The peptides did not show bacteriocin activity even at concentrations of 50 μM when assayed individually (Figs. 2 and 3). Salα consistently required complementary peptide Salβ to exhibit antibacterial activity, and vice-versa. However, when one of these peptides was in excess, the intensity of antibacterial activity was dependent on the peptide present at a lower concentration. In other words, higher inhibitory activities were observed at equimolar concentrations of Salα and Salβ indicated in the lineal tendency in the plot shown in Fig. 2. These data indicate that Salα and Salβ are functional only in 1:1 complexes. The minimal inhibitory concentration (MIC) value for the combination of Salα and Salβ at a molar ratio of 1:1 was 1 μM.

The complementary activity of Salα and Salβ was confirmed by spotting both peptides individually or combined,

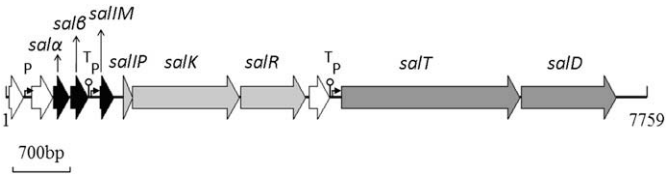

Fig. 1. Schematic representation of the genetic organization of the salivaricin CRL 1328 locus. Putative ORFs are indicated by arrows in the proposed directions of transcription. ORFs with deduced functions for production and secretion of active salivaricin CRL 1328 are indicated by *sal*. ORFs assumed to be dedicated to production and immunity, regulation, and transport are indicated with black, light gray and gray arrows, respectively. ORFs encoding unknown proteins are represented by white arrows. Putative promoters (P) and sequences resembling rho-independent terminators (T) are depicted.

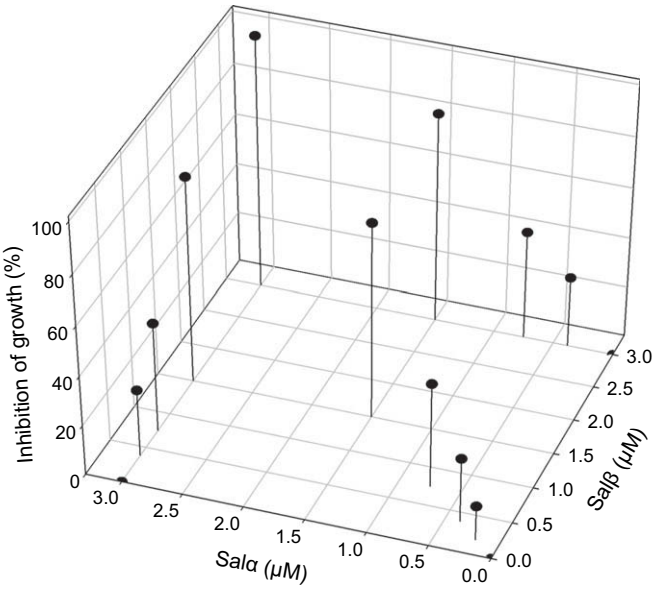

Fig. 2. Complementary antimicrobial activities of Salα and Salβ. The percentage of inhibition of growth of *E. faecalis* MP97 strain is indicated with bars and dots.

added directly onto a lawn of the indicator strain. No inhibitory activity was observed for the individual peptides even at a concentration of 50 μM (Fig. 3). However, in the zone of confluence of the two peptides, antagonistic activity could be observed, as shown in Fig. 3.

Partially purified salivaricin CRL 1328 [43] and chemically synthesized Salα and Salβ peptides (1.5 μM of each peptide) showed the same antimicrobial activity against *Listeria innocua* 7 and other sensitive strains (data not shown). However, inhibitory activity was only observed when both peptides were added together (data not shown).

3.3. Effect of salivaricin CRL1328 on Δψ and ΔpH of sensitive cells

The capacity of Salα and Salβ to dissipate Δψ in the *E. faecalis* MP97-sensitive strain was determined using the fluorescence intensity of the cyanine dye DiSC3(5). In cells energized with glucose, rapid quenching of fluorescence was detected upon addition of the dye (data not shown), showing the generation of Δψ. After the addition of Salα and Salβ, an increase in the fluorescence intensity of DiSC3(5) was observed, indicating that the Δψ of *E. faecalis* MP97 was dissipated (Fig. 4). Salα and Salβ dissipated the Δψ of the sensitive strain more gradually than that obtained with the K^+ ionophore valinomycin at 1 μM (Fig. 4). The individual peptides (Salα or Salβ) showed no detectable activity toward the Δψ of *E. faecalis* MP97 (Fig. 4).

The generation of a ΔpH in *E. faecalis* MP97 cells was analyzed by determining changes in intracellular pH with the pH-sensitive fluorescent probe cFDASE. Modifications in the fluorescence intensity of this reagent by extrusion or loss of intracellular cFDASE indicated depletion of membrane ΔpH. The fluorescence intensity of the probe was increased upon addition of Salα and Salβ, indicating depletion of membrane ΔpH of the sensitive strain (Fig. 5). The ΔpH of *E. faecalis* MP97 was dissipated almost completely by salivaricin CRL 1328 treatment, as shown by a slight increase in the fluorescence probe due to the addition of nigericin.

The results described above indicate that salivaricin CRL 1328 was able to disrupt both components of the proton motive force (Δψ and ΔpH) of sensitive cells.

3.4. Ultrastructural modifications of sensitive strains by transmission electronic microscopy (TEM) studies

Ultrastructural cell modifications of indicator *E. faecalis* MP97 strains incubated with salivaricin CRL 1328 peptides were evaluated through TEM. The formation of an electron-transparent layer between the plasma membrane and the cell wall layer in *E. faecalis* MP97 cells treated with Salα and Salβ was observed (Fig. 6). Other cell modifications were detected at the level of the cell wall, showing disorganization of the structural component, while the cell wall of the control group was shown to be intact (Fig. 6).

4. Discussion

Salivaricin CRL 1328 is a heat-stable bacteriocin produced by *L. salivarius* CRL 1328, a strain isolated from a healthy human vagina. In this work, identification of the genetic locus responsible for production of the two-component bacteriocin salivaricin CRL 1328 is reported. Nucleotide sequence analysis of the salivaricin CRL1328 gene cluster revealed an

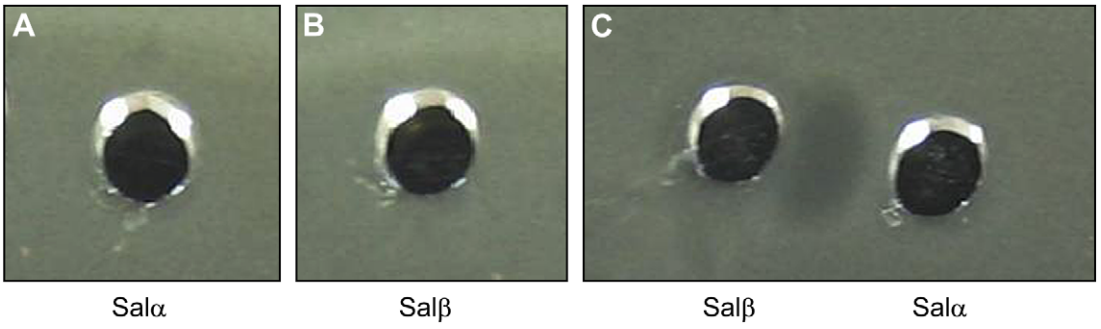

Fig. 3. Radial diffusion assay showing the complementary activity of Salα and Salβ. (A) 50 μM Salα, (B) 50 μM Salβ (C) Inhibitory activity observed in the zone of confluence of Salα and Salβ peptides. The dark halo shows the area where indicator strain *E. faecalis* MP97 did not grow.

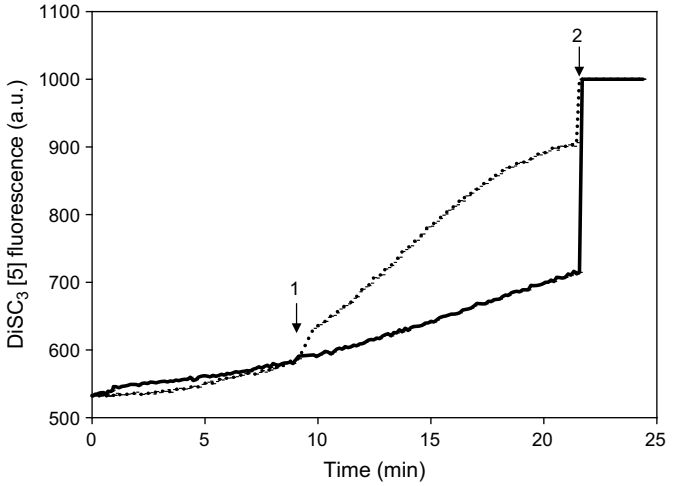

Fig. 4. Dissipation of cell membrane electric potential (Δψ) of *E. faecalis* MP97 cells induced by salivaricin CRL 1328 (continuous line). Control cells untreated with salivaricin CRL 1328 are indicated by dashed line. Cells loaded with 3,3-dipropylthiadicarbocyanine iodide [DISC3(5)] were suspended in 50 mM K-phosphate buffer (pH 7.0) and energized with 10 mM glucose at time 0. Subsequently, salivaricin CRL 1328 (arrow 1, continuous line) or water (arrow 1, dashed line) and valinomycin (arrow 2) were added. Data represent results from three independent experiments.

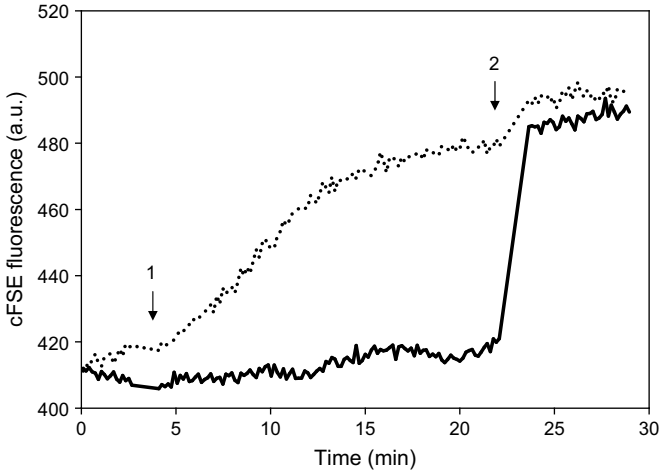

Fig. 5. Effect of salivaricin CRL 1328 (continuous line) on the ΔpH of *E. faecalis* MP97. cFSE-loaded and glucose-energized cells were diluted in 50 mM K-phosphate buffer (pH 7.0). Salivaricin CRL 1328 (continuous line) or water (dashed line) was added at the time indicated by arrow 1. Nigericin was added to completely dissipate ΔpH at the time indicated by arrow 2. Data represent results from three independent experiments.

identical gene organization (Fig. 1) and high sequence identity (DNA:DNA) with the ABP-118 cluster previously published by other authors [16]. The analysis of this region revealed that salivaricin CRL 1328 is a class IIb two-peptide bacteriocin composed of Salα and Salβ peptides. The organization of genes involved in salivaricin CRL1328 production, immunity, secretion and regulation is similar to the other class II bacteriocin gene clusters [30]. The cluster is constituted by two structural genes (*salα* and *salβ*) encoding the pre-bacteriocin peptides, an immunity gene (*salIM*) encoding a protein that protects the producer against its own bacteriocin, a putative three-component regulatory system (*salIP*, *salK*, *salR*), a gene (*salT*) encoding a membrane-associated ABC transporter that transfers the bacteriocin across the membrane and a *salD* gene (*salD*) encoding an accessory protein also needed for secretion of bacteriocin [30]. It is known that the secretion of bacteriocin peptides possessing a double glycine-type leader is mediated by a dedicated transmembrane translocator belonging to the HlyB ABC transporter superfamily and an accessory protein [19,25].

The production of bacteriocins by *L. salivarius* strains isolated from different origins has been reported previously [4,7,16,31,40]. The genetic locus responsible for production of ABP-118, a bacteriocin produced by the human intestinal probiotic strain *L. salivarius* subsp. *salivarius* UCC118, has been characterized previously [16]. Furthermore, the structural genes involved in production of the two-component class II bacteriocins, FK22 and salivaricin P (Sln1 and Sln2), were identified [4,31]. The analysis of the sequence of genetic determinants responsible for production of the companion peptides showed that salivaricin CRL 1328 studied in this work, salivaricin P, ABP-118 and FK22 shared more than 95% identity.

Although molecular characterization of different antimicrobial peptides produced by *L. salivarius* was previously reported by other authors, their mode of action was not elucidated. Therefore, on the basis of the deduced amino acid sequence, the companion peptides (Salα and Salβ) were chemically synthesized in order to further study their mechanistic behaviors. The molecular masses estimated from the deduced amino acid sequence of mature Salα and Salβ peptides were 4096.14 and 4333.12 kDa, respectively. These molecular masses were similar to that experimentally estimated previously using tricine-SDS-PAGE analysis [43].

An antimicrobial activity assay using synthetic Salα and Salβ peptides demonstrated that the two peptides exhibited full activity at an equimolar ratio, whereas each peptide individually did not show antimicrobial activity. Similar results were obtained using *E. faecalis* MP97 or *L. innocua* 7 as indicator strain. These characteristics agree with those of class IIb bacteriocins produced by LAB [27]. For certain two-peptide bacteriocins, such as lactacin F [1], thermophilin 13 [22], plantaricin E/F and plantaricin J/K [2], one or both peptides individually may display a low antimicrobial activity, whereas the two peptides lactococcin G [23], lactococcin Q [44], or lactocin 705 [13] have no activity when tested individually. In general, optimal activity of the two-peptide (class IIb) bacteriocins, like salivaricin CRL 1328, requires the presence of both peptides in almost equal amounts [27]. However, lactocin 705 exerts an antimicrobial effect with an optimal peptide ratio of 1 to 4 [13].

Some class I (or lantibiotic) bacteriocins such as nisin were shown to have a dual mode of action [12]. They bind to lipid II, preventing adequate cell wall synthesis and leading to cell death. Furthermore, nisin can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. A two-peptide lantibiotic, such as lacticin 3147, has these dual activities

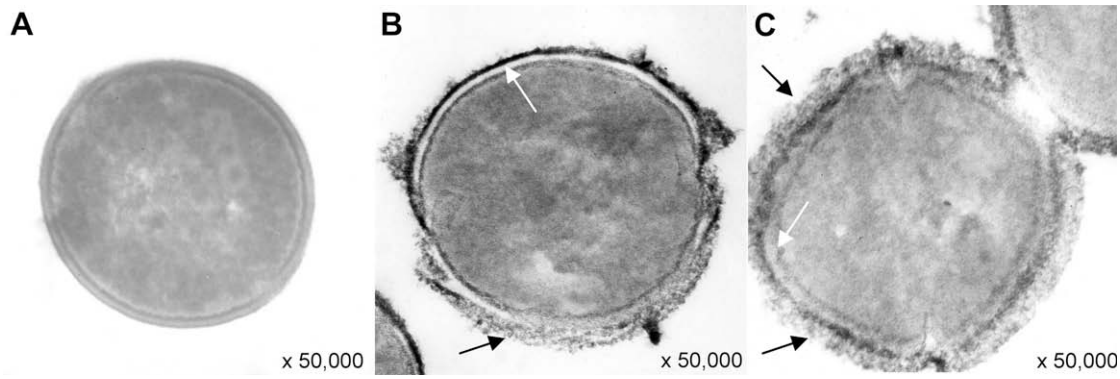

Fig. 6. TEMs of exponentially growing *E. faecalis* MP97 cells in the absence (A) and presence (B and C) of Salα and Salβ synthetic peptides at equimolar ratio (5 μM). Dark arrows indicate the retraction of the cytoplasm and white arrows indicate alterations in the cell wall.

distributed across two peptides, whereas mersacidin has only the lipid-II binding activity, but does not form pores [12]. On the other hand, pediocin-like bacteriocins (class IIa) utilize the mannose transport subunit IIc as receptor or docking site of the peptide [15]. To our knowledge, no information about a docking molecule in class IIb bacteriocins has been described [30]. These two peptide LAB bacteriocins, like salivaricin CRL 1328, appear to act by altering the permeability barrier of the cell membrane, and one of the common mechanisms of inhibition on their target cells is dissipation of the membrane proton motive force [27]. The partially purified salivaricin CRL 1328 [43] as well as the synthetic peptides Salα and Salβ showed to act on the indicator-sensitive cells by dissipating both components of the PMF, transmembrane electrical potential and transmembrane proton gradient (Figs. 4 and 5), probably originating from pores in the membrane, which results in leakage of cellular materials. Similar results were found for two peptide bacteriocins such as lactocin 705 [9], plantaricins EF and JK [24] and thermophilin 13 [22]. On the other hand, lactococcin G was found to selectively dissipate the membrane potential and hydrolyze internal ATP, leading to eventual collapse of the pH gradient [23].

TEMs of *E. faecalis* MP97 exposed to the bacteriocin showed the presence of an electrontransparent layer between the cell membrane and the cell wall layer which indicates retraction of the cytoplasm. In addition, ultrastructural modifications of the cell wall were observed. These results suggest that some type of effect on the peptidoglycan layer of *E. faecalis* MP97 could be a secondary effect of salivaricin on the indicator cells, as proposed González et al. [18].

Bacteriocinogenic LAB could prevent establishment of other bacteria, mainly pathogens from overgrowth that can thereby create altered vaginal microbiota, e.g. in the case of bacterial vaginosis [34]. Although the availability of antibiotics to treat these infections has significantly improved the health and well-being of women, their overuse has contributed to the emergence of antibiotic-resistant bacteria. This situation prompted a search for new therapeutic agents, such as bacteriocins which are distinguished from antibiotics on the basis of their synthesis, mode of action, toxicity and resistance mechanisms [26]. In this sense, the two-component bacteriocin, salivaricin CRL 1328 or the bacteriogenic strain offer a potential alternative for replacement of antibiotics in the prophylaxis of urogenital tract infections.

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