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Impairment of the class IIa bacteriocin receptor function and membrane structural changes are associated to enterocin CRL35 high resistance in *Listeria monocytogenes*

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ABSTRACT

Background: Enterocin CRL35 is a class IIa bacteriocin with anti-*Listeria* activity. Resistance to these peptides has been associated with either the downregulation of the receptor expression or changes in the membrane and cell walls. The scope of the present work was to characterize enterocin CRL35 resistant *Listeria* strains with MICs more than 10,000 times higher than the MIC of the WT sensitive strain.

Methods: Listeria monocytogenes INS7 resistant isolates R2 and R3 were characterized by 16S RNA gene sequencing and rep-PCR. Bacterial growth kinetic was studied in different culture media. Plasma membranes of sensitive and resistant bacteria were characterized by FTIR and Langmuir monolayer techniques.

Results: The growth kinetic of the resistant isolates was slower as compared to the parental strain in TSB medium. Moreover, the resistant isolates barely grew in a glucose-based synthetic medium, suggesting that these cells had a major alteration in glucose transport. Resistant bacteria also had alterations in their cell wall and, most importantly, membrane lipids. In fact, even though enterocin CRL35 was able to bind to the membrane-water interface of both resistant and parental sensitive strains, this peptide was only able to get inserted into the latter membranes.

Conclusions: These results indicate that bacteriocin receptor is altered in combination with membrane structural modifications in enterocin CRL35-resistant *L. monocytogenes* strains.

General significance: Highly enterocin CRL35-resistant isolates derived from *Listeria monocytogenes* INS7 have not only an impaired glucose transport but also display structural changes in the hydrophobic core of their plasma membranes.

1. Introduction

Bacteriocins are antimicrobial peptides produced by most bacteria genera. Among them, bacteriocins from Lactic Acid Bacteria (LAB) are thought to have great potential as food preservatives [1,2]. So far, only nisin has been approved as food additive [3]. In addition, ferments produced by *Pediococcus acidilactici* PA1, the pediocin PA-1 producer strain, are also used in biopreservation (ALTA 2431, Quest International) [4].

There are several classes of LAB bacteriocins, whose classification was recently revised [4,5]. The class IIa peptides, also known as pediocin-like bacteriocins, are potent anti-*Listeria* agents that are active in nanomolar concentrations [6]. The class IIa peptide enterocin CRL35, produced by *Enterococcus mundtii* CRL35, was tested as food preservative in the manufacture of artisan cheeses and it was effective in controlling the growth of *L. monocytogenes* [7]. As other IIa peptides, enterocin CRL35 acts by disturbing the cell membrane, leading to dissipation of the proton motive force [8].

Man-PTS complex is the receptor of the IIa peptides in *L. monocytogenes* and its down-regulation is associated to high level of resistance [9]. Changes in phospholipid head groups as well as fatty acid composition can also be involved in the rise of resistance to class IIa bacteriocins [9]. We have previously demonstrated that enterocin CRL35-resistant *L. innocua* 7 cells own more disordered membranes as

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judged by the DPH fluorescence polarization values [10]. A similar feature, i.e. less ordered membranes in resistant cells, was also reported by others [11].

While characterizing shorter enterocin CRL35 peptides in our lab, we isolated two *L. monocytogenes* clones resistant to this bacteriocin [12]. Therefore, the main scope of the present work was to analyze the resistant *L. monocytogenes* isolates derived from *L. monocytogenes* INS7. We observed an impairment in glucose uptake and a severe alteration of the bacterial growth in glucose-containing synthetic medium. Differences in the cell wall and membrane composition were also found, as reported by other groups [11,13,14]. Moreover, the bacteriocin interacts with bacterial membrane surfaces of sensitive and resistant cells at the same extent but the peptide could not get inserted into the hydrophobic core of resistant bacteria. This result indicates that regardless the initial binding to the membrane surface, the phospholipid fatty acid composition might be crucial in determining the resistance to class IIa bacteriocins alongside the presence of the receptor.

2. Materials and methods

2.1. Synthetic peptide

Enterocin CRL35 was manually synthesized on Rink amide 4-methylbenzhydrylamine resin according to the standard Nα-Fmoc protocol [15]. Peptide was cleaved from the resin and purified as described by Masias et al. [12]. The purity of enterocin CRL35 was checked by Electrospray Mass Spectrometry in positive ion mode (Bruker). Purified peptides were aliquoted and stored at − 20 °C.

2.2. Characterization of L. monocytogenes INS7 mutants by PCR analyses

L. monocytogenes INS7-derived cells that were resistant to enterocin CRL35 were previously isolated from Trypticase Soy Broth (TSB) agar plates containing 200 μM enterocin CRL35 [12]. Colonies were re-isolated in a selective differential medium (BD PALCAM Listeria Agar) and then they were routinely grown in TSB medium supplemented with 25 $\mu g \cdot ml^{-1}$ of nalidixic acid. Finally, two resistant clones were isolated: *L. monocytogenes* INS7 R2 and *L. monocytogenes* INS7 R3. Total DNA was purified from sensitive bacteria as well as from the R2 and R3 isolates by the method of Pospiech and Neumann [16]. Taxonomic identification of each isolate was performed by PCR and DNA sequencing of 16S rRNA gene (Sequencing Service of CERELA, CCT-CONICET/Tucumán). Repetitive element palindromic PCR analysis (rep-PCR) was carried out in order to confirm that resistant isolates derived from the same sensitive parental strain. The $(GTG)_5$ primer was used [17] and the fragments were visualized using ChemiDoc MP Imaging System (Biorad, Tecnolab, Argentina) after GelRed staining (Biotium, Genbiotech).

2.3. Metabolic characterization of L. monocytogenes INS7 R2 and R3 isolates

TSB was inoculated with either the WT sensitive strain or the R2 and R3 resistant isolates. The cultures were incubated at 30 °C without shaking and the growth was evaluated by following the OD at 600 nm. Absorbance readings were recorded and aliquots of each culture were taken. Cells were discarded by centrifugation, the pH of the supernatants was measured and then samples were kept at − 20 °C. Once all the culture supernatants were collected, samples were thawed and glucose levels were measured by the glucose oxidase assay, following the manufacturer's instructions (Wiener, Argentina). In addition, each sample was deproteinized as described by Ortiz et al. [18] prior to the quantification of organic acids by using an Aminex HPX-87H column associated to a HPLC. 5 mM sulfuric acid was used as the mobile phase and elution was followed by a refractive index detector.

Listeria growth was evaluated in a minimal medium supplemented with 50 mM glucose [19]. Starting cultures were grown overnight at 30 °C in TSB, then cells were centrifuged and washed once with PBS. Afterward, cells were resuspended in PBS in a concentration of 10^9 $cells \cdot ml^{-1}$ and used for inoculation of minimal medium.

2.4. Enterocin CRL35-cells interactions

Enterocin CRL35 was labeled with the fluorescent probe fluorescamine (Molecular Probes-Life Technologies) in HEPES buffer pH 8. At the same time, *L. monocytogenes* INS7 and the R2 and R3 resistant cells were harvested at mid-log phase, washed and suspended in HEPES-Na buffer, pH 7.4. Fluorescamine fluorescence anisotropy was determined in an ISS PC1 Photon Counting Spectrofluorimeter thermostatized at 30 °C in the presence of increasing concentrations of bacteria. The excitation wavelength was set at 390 nm whereas the emission wavelength was set at 475 nm. Fluorescence anisotropy was calculated as $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2\ I_{\perp})$, where $I_{\parallel}$ is the fluorescence intensity obtained with the analyzing and the excitation polarizers oriented in parallel, whereas $I_{\perp}$ is the fluorescence intensity got with polarizers perpendicularly adjusted. In a different set of experiments, fluorescence anisotropy measurements were performed with protoplasts instead of whole cells. Protoplasts were generated by sequential incubation of cells with pancreatic enzymes (0.3 $mg \cdot ml^{-1}$ lipase) followed by lysozyme as suggested by Ghosh and Murray [20]. Each incubation was carried out at 37 °C. Lysozyme incubation was done in phosphate buffer containing 2 $mg \cdot ml^{-1}$ lysozyme, 10 mM $MgCl_2$ and 0.4 M sucrose, pH 7. Protoplast formation was monitored under the microscope and checked by measuring $OD_{600\ nm}$ after diluting protoplast suspensions in distilled water (1:1000).

2.5. Assessment of the bacterial cell wall

The three different strains under study (INS7 WT, R2 and R3) were treated with 0.5 $mg \cdot ml^{-1}$ lysozyme at 37 °C. Samples were taken at determined times, diluted in distilled water and optical density was recorded at 600 nm. At the end of the incubation, aliquots were fixed with osmium tetroxide and glutaraldehyde and analyzed by transmission electron microscopy (CIME, CCT-CONICET/Tucumán). In addition, samples were serially diluted in sterile distilled water and 10 μl of each dilution was plated onto TSB agar plates in order to check the number of remaining cells that survived the treatment with lysozyme.

2.6. Listeria membrane lipids purification

Cells were grown till mid-log phase (250 ml TSB cell culture). They were harvested and protoplasts were prepared as described above and resuspended in a final volume of 1 ml in Teflon screw cap tubes. Then, 3.75 ml of chloroform: methanol (1:2) mixture was added and samples were left to stand for 30 min with vigorous vortexing every 10 min. Chloroformic layer was carefully saved and passed through Na_2SO_4 columns in order to remove traces of water. The lipid extraction protocol was repeated twice. Phospholipid concentrations were estimated on the basis of phosphorus content according to the method of Ames [21] and kept at − 20 °C under a nitrogen atmosphere.

2.7. Langmuir monolayer experiments

The interaction of enterocin CRL35 with the three *Listeria* lipid samples was studied by determining the surface pressure at which each lipid monolayer excludes the peptide from the interface. Experiments were carried out at a constant area in a custom-made PTFE trough filled with 7 ml of 145 mM NaCl. Lipid monolayers were formed by spreading chloroformic solutions of each purified lipid extract by drop-wise deposition with Hamilton syringes till different values of initial surface pressure were achieved. Monolayers were let to stand 5 min to ensure complete solvent evaporation. Then, enterocin CRL35 was injected