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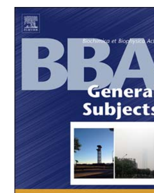

Impairment of the class IIa bacteriocin receptor function and membrane structural changes are associated to enterocin CRL35 high resistance in *Listeria monocytogenes*

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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 µg·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·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·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·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·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

beneath the lipid monolayer under constant stirring. Changes in surface pressure were followed as a function of time until steady measurements were obtained (typically, 15 min). Final results are the average of at least five independent experiments.

2.8. FTIR spectroscopy

Interaction of enterocin CRL35 with lipids purified from different *Listeria* strains was also studied in bilayer model systems by means of infrared spectroscopy. Films obtained by drying the lipid extracts with nitrogen were rehydrated in 20 mM HEPES, 145 mM NaCl prepared in D_2O (Sigma), pD 7.4. The multilamellar vesicles thus obtained were sonicated till formation of small unilamellar vesicles. The peptide was dissolved in the same deuterated buffer and added to the liposomes in a molar ratio of 10:1 (lipid:peptide).

Transmission demountable liquid cells (Harrick Sci) equipped with CaF_2 windows and 56 µm PTFE spacers were loaded with 23 µl of samples. The temperature of the cell was controlled by means of water bath circulator and a custom made thermocouple for measuring the temperature of the cell. Measurements were carried out in a Nicolet 5700 spectrometer (Thermo Nicolet, Madison, WI). Thermal scans in the range of 15 to 80 °C with a 5 min waiting time at each point were undertaken. Spectra were collected by averaging 16 scans at a final resolution of 2 cm^{-1} by means of the software provided by the manufacturer (OMNIC).

The order of the hydrophobic core and the hydration level of the interfacial region of the membranes were assessed by following the thermal evolution of the symmetric stretching of methylene (circa 2850 cm^{-1}) and stretching of carbonyl groups (circa 1740 cm^{-1}).

3. Results

3.1. Enterocin CRL35-resistant isolates displayed slower growth rates

The growth and metabolism of these strains was first monitored at 30 °C in TSB medium. Enterocin CRL35-resistant isolates not only grew slower than the parental sensitive strain but also they acidified the medium (Fig. 1A) and consumed glucose (Fig. 1B) at lower rates. The main metabolic products of all *Listeria* strains tested were lactic acid as the major product and acetic acid. However, *L. monocytogenes* INS7 produced more lactic acid as compared to the resistant isolates. On the contrary, R2 and R3 produced slightly more acetic acid as compared to the sensitive strain (Table 1). No significant amounts of formic acid were observed in any of the strains under study.

The growth in a defined minimal medium with glucose as a sole energy source was also evaluated. The main objective of this experiment was to assess the functionality of the Man-PTS complex. Fig. 2 shows that resistant isolates were unable to grow in the defined medium, in contrast to the sensitive parental strain. This result strongly suggests that both *Listeria* resistant isolates do not have a functional Man-PTS complex, hence they cannot properly grow with glucose as the sole carbon and energy source.

3.2. Enterocin CRL35 can interact with resistant cells, although to a lesser extent

Enterocin CRL35 was derivatized using fluorescamine to obtaining a fluorescent peptide with unaltered antimicrobial activity [12]. As expected, the fluorescence anisotropy of the peptide in solution was quite low (Table 2). Once cells were added, the fluorescence anisotropy values went up indicating that enterocin CRL35 was interacting with bacteria, resulting in impaired free rotational movements of the fluorescent peptide. Both resistant isolates were able to bind enterocin CRL35, although fluorescence anisotropy values were not as high as those obtained in the presence of the sensitive strain. R2 and R3 isolates proved to be different from each other since R3 protoplasts were able to

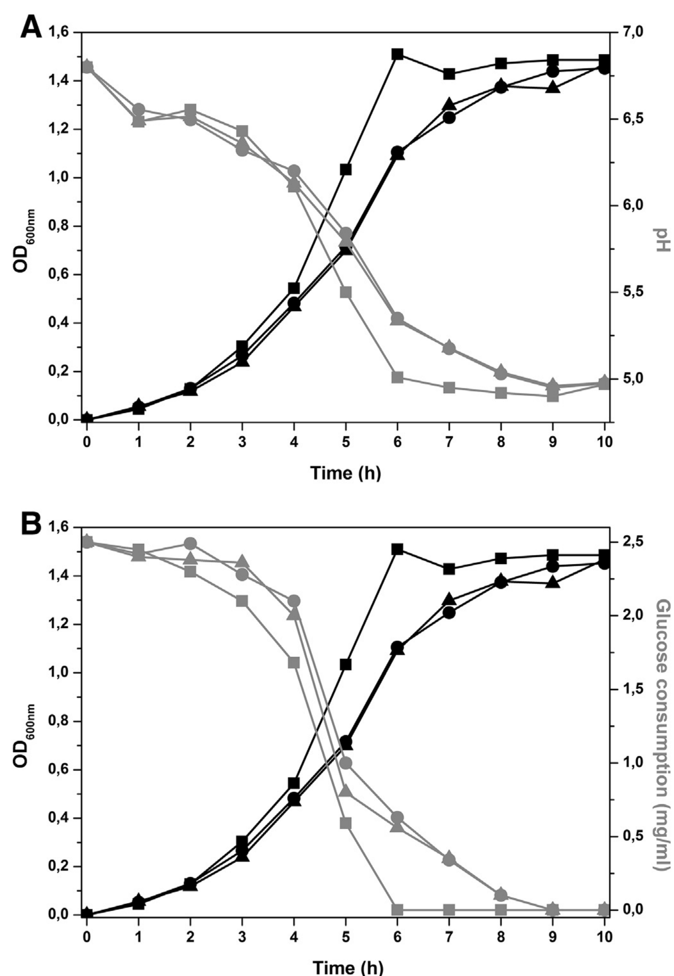

Fig. 1. Bacterial growth in TSB medium. The growth of *L. monocytogenes* INS7 (■) as well as the resistant isolates R2 (▲) and R3 (●) at 30 °C was monitored over time (black lines and symbols). The pH of the culture medium (A) and the concentration of glucose were also tested (B). These results are representative of three independent experiments.

Table 1
Organic acids production in *L. monocytogenes* INS7, *L. monocytogenes* INS R2 and *L. monocytogenes* INS R3.

	Organic acids (g/L)	
	Lactic acid	Acetic acid
INS7	1.71 ± 0.07	0.18 ± 0.009
INS7 R2	1.32 ± 0.06	0.21 ± 0.010
INS7 R3	1.38 ± 0.03	0.22 ± 0.011

bind enterocin CRL35 to a greater extent than R2 protoplasts (Table 2).

Enterocin CRL35 was unable to dissipate Δψ of R2 and R3 cells (see Supplementary information, Fig. S2), which indicates that the bacteriocin would not get inserted into the plasma membrane of the resistant isolates. The binding observed in protoplasts may be due to the interaction of enterocin CRL35 with some membrane proteins or phospholipid head groups but this interaction would not render in any insertion into the phospholipid bilayer.

3.3. INS7 R2 and R3 isolates feature differences in the cell wall

INS7 R2 and R3 isolates were more resistant to lysozyme (Fig. 3A). The killing curve assay clearly demonstrated that R2 and R3 cell counts were at least one order of magnitude higher than the parental strain after incubating them with 0.5 $mg.ml^{-1}$ lysozyme. These differences

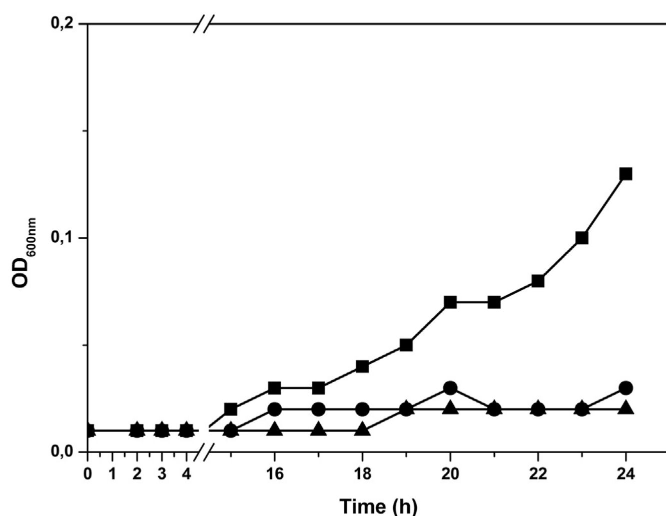

Fig. 2. *Listeria* growth in defined medium. A glucose-containing minimal medium was prepared as described by Premaratne et al. [19]. Overnight cultures of sensitive and resistant cells were pelleted, washed and inoculated. The bacterial growth was followed for up to 24 h at 30 °C. *L. monocytogenes* INS7 (■), *L. monocytogenes* INS7 R2 (▲), *L. monocytogenes* INS7 R3 (●).

Table 2
Enterocin CRL35 binding to *L. monocytogenes* cells and protoplasts.

	Fluorescence anisotropy	
	Cells	Protoplasts
Control	0.0008 ± 0.0005	0.0007 ± 0.0001
INS7	0.1955 ± 0.0029	0.2121 ± 0.0049
INS7 R2	0.1538 ± 0.0077	0.1374 ± 0.0024
INS7 R3	0.1450 ± 0.0055	0.2109 ± 0.0076

were also confirmed by electron microscopy (Fig. 3B). In fact, a number of intact enterocin CRL35-resistant cells could be found in each field after lysozyme treatment, whereas rod-shaped cells were barely found among the enterocin CRL35-sensitive cells after lysozyme incubation. However, no statistical difference in the cell wall charges of sensitive and resistant strains was detected by the cytochrome *c* binding assay (see Supplementary information, Fig. S3).

3.4. Enterocin CRL35-resistant isolates also display changes in their plasma membranes

Besides cell wall analyses, *Listeria* membrane lipids were studied by means of FTIR. The stretching of carbonyls was assessed in order to know whether the peptide was able to get close to the membrane-water interface. Table 3 summarizes the results of the deconvolved carbonyl stretching band in its components in order to quantify the ratio of hydrated/dehydrated carbonyls (see Supplementary information for the complete set of data). It can be observed that the high wavenumber component (1744 cm^{-1}) had a higher contribution to the overall band when the peptide was present. The addition of enterocin CRL35 almost doubled the population of dehydrated carbonyls at the interfacial region of the three different membranes (from an average of 17% to 37% of the total carbonyls, Table 3).

Fig. 4 shows the symmetric stretching of methylenes as a function of the temperature, which is a good marker of the order degree of the membrane hydrophobic core. Membranes from sensitive and resistant cells grown at 30 °C seemed to have a rather non-cooperative phase transition below 20 °C. Membranes of both sensitive and resistant cells displayed the exact same profile with temperature, and spanned a range from wavenumber values close to gel ones (2850 cm^{-1}) at 15 °C, up to wavenumbers typically found in liquid crystalline membranes (2854 cm^{-1}) at 55 °C. Membranes derived from WT cells showed an increase of wavenumber values upon addition of the peptide, suggesting that the presence of the bacteriocin increased the amount of the trans-gauche isomers. On the contrary, no change was observed in R2 and R3 membranes (Fig. 4).

Membrane lipids were also studied by the Langmuir monolayer technique to complement the FTIR results. Enterocin CRL35 showed an important interfacial activity, lowering the surface tension of the aqueous subphase by 12 $mN{\cdot}m^{-1}$ at a concentration of 0.2 μM (data not shown). The peptide was able to induce even greater surface pressure changes (Δπ) when injected into subphases covered with

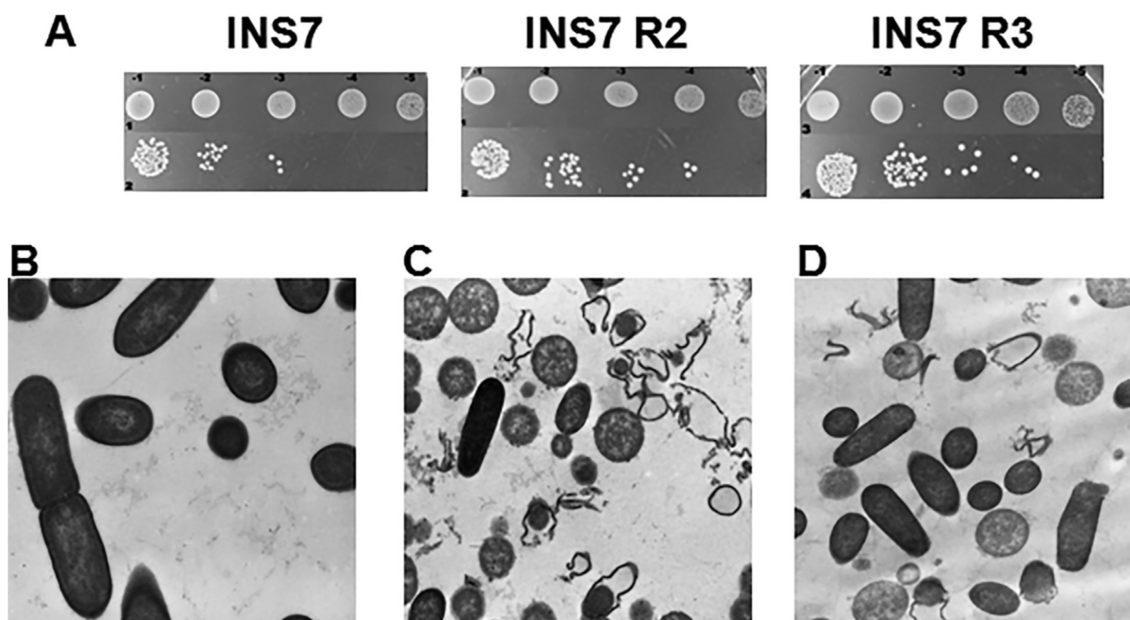

Fig. 3. Sensitivity to lysozyme. Cells were treated with 2 $mg{\cdot}ml^{-1}$ lysozyme, then viable cell counts were estimated by the spot assay as described in Materials and methods (A). In addition, bacterial suspensions were observed by transmission electron microscopy. B) Untreated *L. monocytogenes* INS7 cells, which is representative of all three *Listeria* strains under study. C) *L. monocytogenes* INS7 suspension after lysozyme treatment, where a number of protoplasts can be observed. D) *L. monocytogenes* INS7 R3 suspension treated with lysozyme is a mixed population with prevalence of intact cells.

Table 3
Hydrated/dehydrated carbonyls of *Listeria* membranes in the presence and in the absence of enterocin CRL35.

Carbonyls	INS7		INS7 R2		INS7 R3	
	Non-hydrated	Hydrated	Non-hydrated	Hydrated	Non-hydrated	Hydrated
Control	1744.0 cm^{-1} 17.50%	1728.0 cm^{-1} 82.50%	1743.9 cm^{-1} 19.50%	1727.9 cm^{-1} 80.50%	1744.1 cm^{-1} 13.40%	1729.1 cm^{-1} 86.60%
Enterocin CRL35	1743.7 cm^{-1} 37.70%	1726.9 cm^{-1} 62.20%	1743.9 cm^{-1} 37.40%	1727.4 cm^{-1} 62.60%	1744.1 cm^{-1} 36.50%	1729.5 cm^{-1} 63.50%

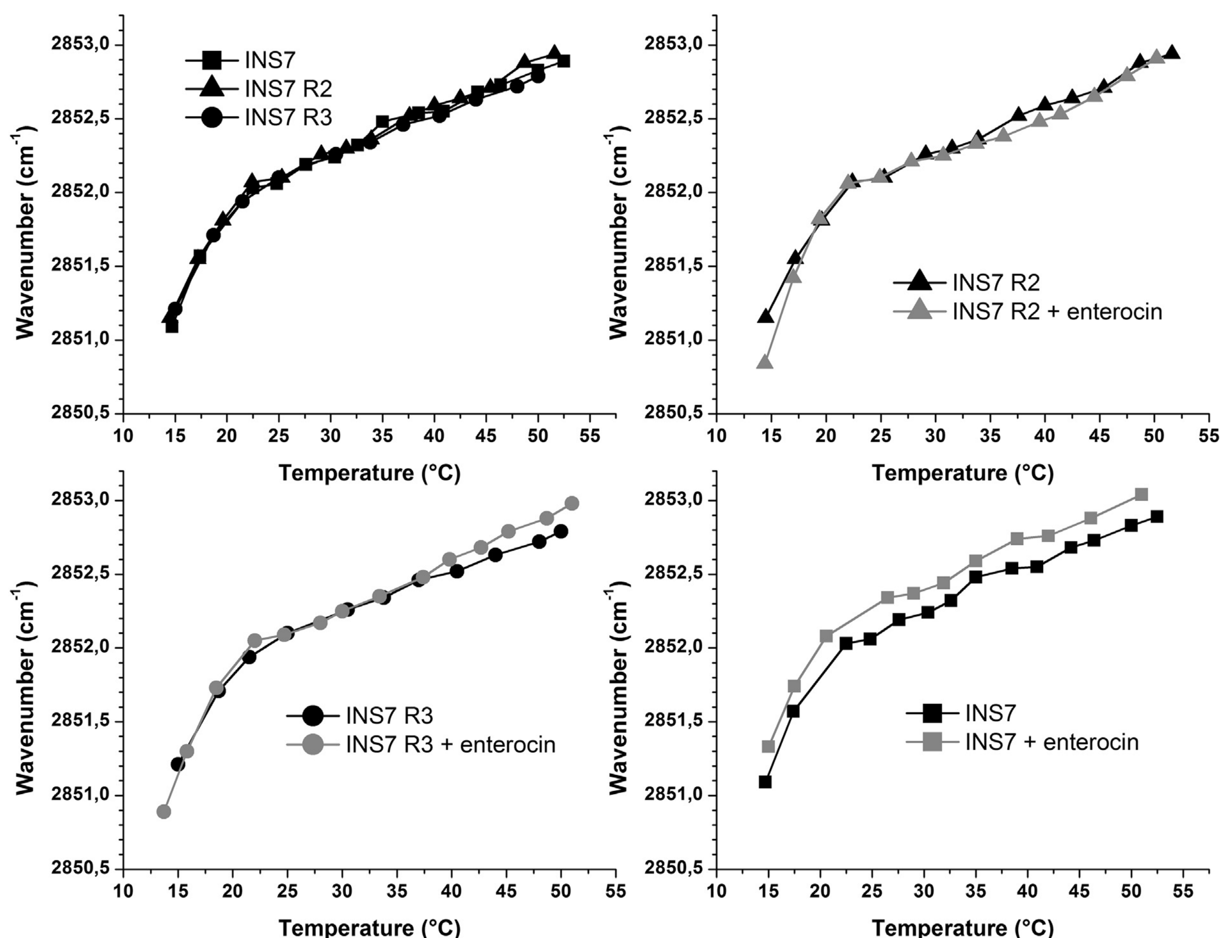

Fig. 4. Peak position of the symmetric stretching vibration of the methylene groups versus temperature. Total lipids from *L. monocytogenes* INS7 (■), R2 (▲) and R3 (●) were purified as described in Materials and methods. Liposomes were prepared and the symmetric stretching of methylenes was monitored as temperature was increased from 15 °C to 55 °C.

phospholipid monolayers. As it can be seen in the Fig. 5, enterocin CRL35 increased the surface pressure inversely proportional to the initial surface pressure of the monolayer (π_i). The exclusion surface pressures were obtained by extrapolating the values of $\Delta\pi$ to the abscissa axis intersection (π_{excl}). This analysis showed that enterocin CRL35 interacted with higher affinity with the lipids purified from the *L. monocytogenes* INS7 as compared to the lipid monolayers from R2 and R3 resistant cells (Fig. 5 and Supplementary information). This finding also points out that lipids from WT and resistant cells are different, which may explain why enterocin CRL35 can insert into WT membranes but it cannot insert into resistant ones.

4. Discussion

The main mechanism underlying resistance to class IIa peptides is associated to a reduced or lack of expression of the mannose PTS complex, the physiological transporter of glucose in *Listeria* cells [9,22]. This type of bacteriocin-resistant cells displays a marked alteration in the growth rates when glucose is used as the main carbon source [14,23,24]. Many different mutations could be involved in this phenotype, from mutations in Man-PTS subunits to any regulator involved in the expression of Man-PTS genes [9,25]. Slower growth rates of class IIa bacteriocin resistant cells have been previously reported using TSB or BHI. We refined the system by using a synthetic medium with glucose as a sole carbon source. In this particular medium, sharp differences between the WT sensitive strain and the resistant isolates were found. R2 and R3 isolates did not have a functional Man-PTS system, hence glucose could not be efficiently transported.

Resistant isolates also displayed important differences in their cell walls. However, Jacquet et al. have shown that there is no clear correlation between resistance and changes in the cell wall [26]. Thus, it is not clear at this point whether the changes in the cell wall documented in the present work are related to the resistance observed. R2 and R3 cell walls seem to be different from each other, fact that could be taken as another proof that there is no direct link between cell

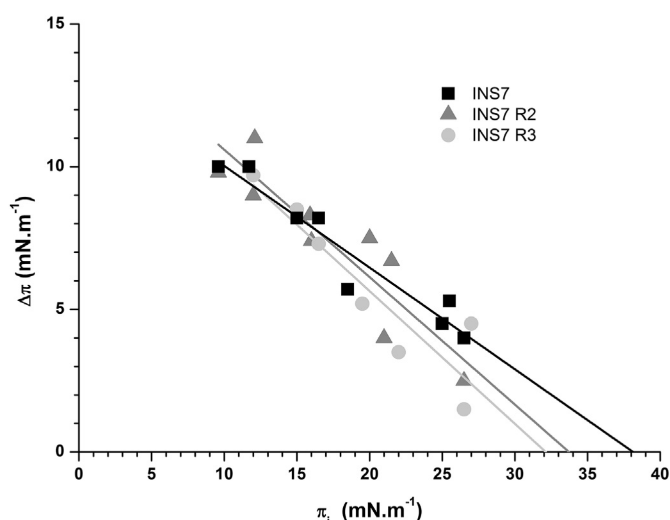

Fig. 5. Interaction of enterocin CRL35 with *Listeria* lipids. Monolayers were prepared with lipids purified from *L. monocytogenes* INS7 (■), R2 (▲) and R3 (●) at different initial surface pressures. Peptide was added beneath the monolayers at a final concentration of 0.2 μM. The different increments in surface pressure were used for determining the exclusion surface pressure for each lipid by means of lineal regression and extrapolation to the abscissa axis.

walls and resistance to bacteriocins.

We also found changes in the plasma membranes that may be associated to the resistance to enterocin CRL35. We used two biophysical tools such as FTIR and Langmuir monolayer measurements. The former technique was already used by other authors for characterizing the interaction of bacteriocins with lipid membranes [27,28]. In turn, the Langmuir monolayer is a powerful tool that can give precious information about membrane phospholipids and also about the affinity of peptides for certain membranes [29,30].

Enterocin CRL35 was able to interact with sensitive- and resistant-derived membranes at the interfacial region, but it could only be inserted into membranes derived from sensitive bacteria. Adsorption of enterocin CRL35 to Langmuir monolayers confirmed this finding since the cut-off value found in WT lipid monolayers was higher than those obtained with resistant cells. In the same trend, Verheul et al. also found differences in lipid monolayer experiments from nisin-sensitive and resistant cells [31].

It has been previously shown that enterocin CRL35 partially dissipates membrane potential even at sub-lethal concentrations [8]. The dissipation occurs because enterocin CRL35 binds and inserts into plasma membranes of target cells. The membrane potential of R2 and R3 isolates was not dissipated at any degree by enterocin CRL35, suggesting the lack of insertion of the peptide into the membranes of resistant cells. These results are in agreement with findings described above.

In general, low-level resistance to class IIa bacteriocins is associated to changes in membrane phospholipid composition [11,32], whereas high level of resistance is related to loss of the Man-PTS complex [22]. R2 and R3 cells present MICs values 10,000 times higher than the MIC of the WT sensitive strain. The mere absence of the physiological glucose transporter could not explain such an impressive degree of resistance since this type of peptides can interact and disrupt protein-free membranes in the absence of any protein receptor [33]. Moreover, it has been shown that plantaricin A induced a marked leakage from zwitterionic liposomes even in the nanomolar range [34]. Besides the Man-PTS lack of function, *L. monocytogenes* INS7 resistant isolates displayed major changes in the plasma membrane that complement the deficiency of a receptor. It is important to note that Sakayori et al., who characterized *Enterococcus faecium* mutants resistant to mundticin KS, also found significant changes in the membrane lipid composition [35].

It was believed that bacteria resistant to class IIa peptide that displayed membrane or cell wall alterations had normal or even higher levels of Man-PTS [36,37]. Tessema et al. carried out a comprehensive analysis of resistance mechanisms encompassing 30 resistant isolates where they found that only those displaying high resistance had downregulation of the receptor and at the same time had better growth fitness [37]. Indeed, this seminal study confirmed that there are several mechanisms of resistance.

Our study is the first report where both resistance mechanisms i.e. loss of Man-PTS function associated with poor growth and membrane changes seem to play together in order to confer high resistance to enterocin CRL35.

Competing interests

None declared.

Ethical approval

Not required.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2017.03.014.

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