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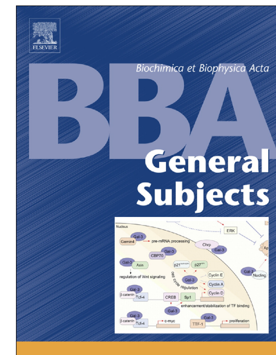

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PrfA activation in *Listeria monocytogenes* increases the sensitivity to class IIa bacteriocins despite impaired expression of the bacteriocin receptor

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ABSTRACT

Background

The scope of the present work was to characterize the activity of class IIa bacteriocins in *Listeria (L.) monocytogenes* cells that constitutively express an activated form of PrfA, the virulence master regulator, since bacteriocin sensitivity was only characterized in saprophytic cells so far. The mannose phosphotransferase system (Man-PTS) has been shown to be the class IIa bacteriocin receptor in *Listeria*; hence, special attention was paid to its expression in virulent bacteria.

Methods

L. monocytogenes FBprfA* cells were obtained by transconjugation. Bacterial growth was studied in TSB and glucose containing-minimal medium. Sensitivity to antimicrobial peptides was assessed by killing curves. Membranes of *L. monocytogenes* FBprfA* cells were characterized using proteomic and lipidomic approaches.

Results

The mannose phosphotransferase system (Man-PTS) was downregulated upon expression of PrfA*, and these cells turned out to be more sensitive to enterocin CRL35 and pediocin PA-1, while not to nisin. Proteomic and lipidomic analysis showed differences between wild type (WT) and PrfA* strains. For instance, phosphatidic acid was only detected in PrfA* cells, whereas, there was a significant decline of plasmalogen-phosphatidylglycerol in the same strain.

Conclusions

Our results support a model in which Man-PTS acts just as a docking molecule that brings class IIa bacteriocins to the plasma membrane. Furthermore, our results suggest that lipids play a crucial role in the mechanism of action of bacteriocins.

General Significance

This is the first demonstration of the link between *L. monocytogenes* virulence and the bacterial sensitivity toward pediocin-like peptides.

Keywords: bacteriocins; virulence; antimicrobial activity; *Listeria*

ABBREVIATIONS

CL Cardiolipin

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Man-PTS mannose phosphotransferase system

PBS Phosphate-buffered saline

PrfA* cells cells expressing the $PrfA_{L140F}$ protein

TE buffer Tris-EDTA buffer

TSB Tryptone Soya Broth

PA Phosphatidic Acid

PG Phosphatidyl glycerol

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1. INTRODUCTION

Bacteriocins from lactic acid bacteria are antimicrobial peptides that deserve significant attention since they have a promising future as food biopreservatives, and even as modifiers of the gut microbiota [1]. Among them, enterocin CRL35 was extensively characterized [2]. It has been proposed that all class IIa peptides interact with sensitive bacteria in a similar fashion, i.e. the positively charged N-terminal domain of these bacteriocins may bind to the N-terminal loop of the IIC subunit of the mannose phosphotransferase system (Man-PTS). It has been additionally proposed that not only IIC but also IID would then engage in helix–helix interactions with the C-terminal domain of the bacteriocins during pore formation [3,4]. Lack of expression of the Man-PTS renders cells highly resistant to these peptides [5]. Diep *et al.* nicely presented the final model for the receptor and its interaction with the bacteriocin and the immunity protein [6]. In addition to the receptor, the physicochemical properties of the cell wall and the composition of the plasma membrane seem to play a role in the interaction between bacteriocins and target cells [7,8]. Interestingly, bacteriocins can interact and even disrupt membranes lacking the bacteriocin receptor [9]. However, much higher peptide concentrations are needed for inducing membrane leakage. Importantly, anionic phospholipids seem to be essential for proper binding of peptides to membranes in the absence of receptor [9–11].

Listeria monocytogenes can live as saprophyte in the environment. However, once ingested, this bacterium can switch its metabolism and undergo remarkable adaptations in order to survive and replicate within the host [12]. The most important factor mediating this transition is PrfA, the master regulator of *Listeria* virulence. It is well established that sugar PTS complexes can shutdown PrfA when bacteria are consuming these carbohydrates, e.g. during the saprophytic life style [13–15]. Conversely, a low activity of PTS systems is expected in cells expressing activated PrfA.

Up to date, *in vitro L. monocytogenes*-related studies on class IIa bacteriocins action have been conducted with the organism in a saprophytic state, i.e. measuring bacterial growth and viability in regular culture media where no expression of virulence factors is detected whatsoever. Therefore, the main objective of the present study was to characterize the sensitivity of *L. monocytogenes* cells constitutively expressing active PrfA to pediocin-like peptides. Our working hypothesis was that virulent bacteria with activated PrfA might display a reduced sensitivity to these peptides,

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because of the attenuated expression or lower activity of the Man-PTS. However, we found the opposite result, which correlated with major changes not only in the profile of proteins associated to plasma membrane but also in the phospholipid composition of the membrane.

2. MATERIAL AND METHODS

2.1. Bacterial strains and growth conditions

E. coli DH5α was used as a host strain for recombinant plasmids, whereas *E. coli* SM10, a kind gift from Dr. García Vescovi (IBR, Argentina), was used as the donor strain for the conjugation assay. Both strains were grown in Luria Broth (LB) medium at 37°C. The *L. monocytogenes* FBUNT strain is a 4b serotype isolated from the first clinical case of neonatal meningitis due to *Listeria monocytogenes* in the province of San Luis [16]; it expresses Man-PTS and was deposited at the Culture Collection of the Bacteriology Department from the Facultad de Bioquímica, Química y Farmacia - UNT (Tucumán, Argentina). The strain FBUNT, a clinical isolate, is sensitive to both enterocin CRL35 and pediocin PA-1; it has been extensively used in our lab [2,17,18] and its genome was recently sequenced (AN: NZ_PVZU00000000.1). In addition, we used the resistant isolates *L. monocytogenes* INS7 R2 and *L. monocytogenes* INS7 R3, as well as the parental sensitive strain *L. monocytogenes* INS7, that were recently characterized [19]. Unless stated otherwise, *L. monocytogenes* strains were routinely grown in TSB medium at 37°C.

2.2. Generation of *L. monocytogenes* FBUNT with activated *prfA* allele

The integration vector pNF1002 (pPL2-*prfA*L140F) was used for inducing *L. monocytogenes* FBUNT to constitutively express virulence factors [20]. For that purpose, 250 µl of pNF1002-bearing *E. coli* SM10 culture were mixed with 150 µl of *L. monocytogenes* FBUNT. The bacterial mixture was put onto a 0.45-µm-pore-size HA-type filter (Millipore) on a Triptone Soy Broth (TSB) agar plate without antibiotics, and incubated at 30°C for 2 h. Afterward, cells were recovered in 2 ml of TSB and 200 µl of bacterial suspension were mixed with 3 ml of LB top agar and overlaid onto TSB plates containing 7.5 $\mu g.ml^{-1}$ of chloramphenicol and 25 $\mu g.ml^{-1}$ of nalidixic acid. Plates were incubated overnight at 30°C and then shifted to 37°C for 2 days. Colonies were plated on *Listeria* selective culture medium (Oxoid, Bioartis S.R.L, Argentina). Finally, colony PCR was

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performed to confirm the integration of the constitutively activated *prfA* allele, as described elsewhere [20]. The primers used were the following: 5′-GTCAAAACATACGCTCTTATC-3′ and 5′-ACATAATCAGTCCAAAGTAGATGC-3′. The selected clone was named *L. monocytogenes* FBprfA*. The activated PrfA (L140F mutation) will be denoted as PrfA* in the manuscript. In parallel assays, *L. monocytogenes* INS7 and its enterocin CRL35-resistant clones were also conjugated with pNF1002-bearing *E. coli* SM10.

2.3. Characterization of *L. monocytogenes* FBprfA*

The growth of both *L. monocytogenes* FBUNT and *L. monocytogenes* FBprfA* was tested in minimal medium supplemented with 50 mM glucose [21]. For this purpose, starting cultures were grown overnight at 37ºC in TSB, then cells were harvested, washed twice and suspended in PBS. Minimal medium was then inoculated (1:100) and cell growth was monitored at 37°C for 24 h. This medium enhances the differences in growth rates between PrfA*-expressing *Listeria* cells and the saprophytic parental strains, since glucose is the only source of energy, and *L. monocytogenes* cells that express PrfA* heavily rely on sugar phosphates instead [22, 23]. In addition, growth in TSB medium was analyzed. *L. monocytogenes* expressing the L140F allele settles at the bottom of the culture tubes when growing in a rich culture medium [20]. Therefore, we analyzed this phenotype to confirm the correct generation of the PrfA* variant derived from *L. monocytogenes* FBUNT. The hemolytic activity of *L. monocytogenes* FBprfA* was also tested. Bacterial cultures were grown at 37°C till OD_{600nm}=0.9. The culture supernatants were assayed for hemolytic activity as previously described [20]. The 100% control was achieved upon addition of 0.1% Triton X-100.

2.4. RNA isolation and quantitative real-time PCR measurements

L. monocytogenes FBUNT and *L. monocytogenes* FBprfA* cells were harvested at mid-exponential phase by centrifugation and washed with cold TE buffer, [24]. Cells were suspended in 500 µl of the same buffer and then the following reagents were sequentially added: 0.6 g of glass beads, 170 µl of 2% macaloid slurry, 500 µl of TE saturated phenol with chloroform:isoamyl alcohol (1:1) and 50 µl of 10% SDS. Bacterial disruption was achieved in a Mini-Beadbeater Cell Disrupter (Model 607EUR, Biospec Products). After 15 min centrifugation at 12,000 x *g*, the aqueous supernatant containing the RNA was extracted with 1 volume of phenol-chloroform-isoamyl alcohol

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and precipitated with 3 M sodium acetate and 3 volumes of absolute ethanol. Then, RNA was recovered by centrifugation, washed with 70% ethanol and dissolved in RNAse free water. Isolated RNA was treated with recombinant Turbo^{TM} DNAse (Ambion-Thermo Fischer Scientific) prior to cDNA synthesis according to the manufacturer's instructions.

DNA-free RNA (1 µg) was used to synthesize cDNA using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Thermo Fisher Scientific). qRT-PCR reactions were carried out in duplicate using 50 ng of cDNA, 0.3 µM of each primer and 10 µl of iQ™ SYBR® Green Supermix (Bio-Rad) for a final reaction volume of 20 µl. Reactions were performed in an iQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) under the following conditions: 3 min at 95°C, 40 cycles of 20 seg at 95°C, 20 seg at 52°C and 20 seg at 72 ºC, followed by melting curve cycles. For each reaction, non-template controls (NTC) were included. qPCR primers were designed with PrimerQuest® Tool from IDT (http://www.idtdna.com/primerquest/Home/Index), and are shown in Supplementary Table S1. The expression of the following genes was studied: *mptC* and *mptD* which encode the IIC and IID subunits of Man-PTS, respectively, *prfA* and *hly* (listeriolysin O gene). The set of primers used in these experiments were designed to be highly specific for *mpt* (see alignment in Supplementary information). The 16S rRNA gene was chosen as the housekeeping gene because its expression was invariable under the tested conditions. The changes in the expression of these genes in *L. monocytogenes* FBprfA* were expressed by means of the double $\Delta\Delta C_T$ method [25].

2.5. Membrane proteomics

L. monocytogenes FBUNT and *L. monocytogenes* FBprfA* were grown till mid-exponential phase in TSB medium at 37ºC and then they were harvested by centrifugation, washed twice with PBS and sonicated with 15 s pulses under nitrogen stream and keeping the samples at 0-4ºC. Afterward, unbroken cells and debris were discarded by centrifugation (20,000 x *g* for 30 minutes). The resulting supernatants were centrifuged at 150,000 x *g* in order to collect plasma membranes and the associated cell wall. Note that bacteria were not treated with lysozyme or pancreatic enzymes prior to plasma membrane isolation in order to avoid any artifact derived from a plausible differential sensitivity of the strains under study toward these enzymes. Pellets obtained after

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ultracentrifugation were resuspended in double distilled water and protein content was measured by the Lowry method [26]. Then, membrane suspensions were dissolved with 2X Laemmli sample buffer (Sigma) and they were electrophoresed till the dye front entered 1 cm into the separation gel, which was prepared at 10%. Gels were stained with colloidal Coomassie G-250, the protein spots were excised and the proteomic study was carried out at the mass spectrometry facility of Cequibiem (UBA, Argentina) with a nanoHPLC coupled to a mass spectrometer with Orbitrap technology. Quantification of each protein was obtained using the proteome Discover software (Thermo Scientific) and the statistical analysis was done with Perseus free software [27].

2.6. Membrane lipidomics

All *Listeria* strains were grown at 37ºC till the end of exponential phase in 500 ml TSB culture medium supplemented with nalidixic acid. Cells were harvested by centrifugation at 8,000 x *g* during 20 min and washed twice with double distilled water. The bacterial pellets were resuspended in water, lyophilized overnight, then suspended in methanol-chloroform-water (2:1:0.8) mixture was sonicated for 2 minutes in a cold bath. Afterward, 1 ml of chloroform and 1 ml of 0.63% LiCl were added, and, the samples were vigorously vortexed for 30 minutes. The final solution was centrifuged at 2,000 x *g* for 10 minutes to form two layers. The bottom layer was carefully transferred to a new vial, dried under nitrogen and the resulting lipid film was stored at -20ºC until use. Phospholipid content was estimated by measuring phosphate, according to the Ames method [28]. The polar lipids of *Listeria* membranes were characterized by linear ion-trap (LIT) multiple-stage high resolution mass spectrometry (MS^n), as described previously [29]. To determine the structures of the fatty acid substituents in the lipid, the lipid extraction was hydrolyzed with tetrabutylammonium hydroxide (40 wt% solution in water) in a centrifuge tube. After heating at 100 ºC for 60 min,1:1 0.63% LiCl/ hexane was added, vortexed, and centrifuged at 1,200 x *g* for 2 min to form two layers. The upper layer, containing the free fatty acids, was transferred to a clean tube, dried under nitrogen and N-(4-aminomethylphenyl) pyridinium (AMPP) derivative was made with the AMP+ Mass Spectrometry Kit, according to the user's instruction [29]. High resolution LIT MS^n experiments were conducted on a Thermo Scientific LTQ Orbitrap Velos mass spectrometer with Xcalibur operating system (San Jose, CA). Samples were loop injected

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into the electrospray ion source where the needle was set at 4.0 kV and the temperature of the capillary was 300 ºC. Helium was used as the collision and the buffer gas. The mass spectra were accumulated in the profile mode, typically for 3-10 min for MS^n spectra (n=2,3,4).

2.7. Antimicrobial activity of enterocin CRL35

Enterocin CRL35 and pediocin PA-1 were purified from the supernatants of *Enterococcus mundtii* CRL35 grown in LAPTg broth and *Pediococcus acidilactici* PAC1 grown in MRS broth, respectively. Both strains were cultured overnight at 30°C and bacteriocins were obtained by a two step protocol consisting in a precipitation with ammonium sulfate followed by reverse phase HPLC using a C_{18} column with a non-linear gradient of acetonitrile [30]. Nisin was purchased from Sigma (Research AG, Argentina) and also purified by reverse phase HPLC C_{18}.

For viability assays, *L. monocytogenes* cultures were harvested at mid-exponential phase, cells were washed and suspended in 50 mM HEPES-K buffer, pH 7 to approximately 10^7 cells ml^{-1}. Class IIa bacteriocins were added at a final concentration of 5 nM and cell suspensions were incubated for 30 min at 37°C. This peptide concentration was chosen because it allows a clear differentiation in the sensitivity of *L. monocytogenes* FBUNT and *L. monocytogenes* FBprfA*. Since FBUNT strain is already hypersensitive to class IIa, concentration of bacteriocin of 10 nM and higher results in complete elimination of these bacteria. In the experiments assessing sensitivity to nisin, this lantibiotic was used at a final concentration of 30 nM.

Samples from culture supernatants were taken, serially diluted and plated onto TSB plates. Colonies were counted after 16–h incubation at 37°C according to Masias *et al.* [31]. In addition, sensitivity of *L. monocytogenes* INS7 wild type, *L. monocytogenes* INS7 R2 and *L. monocytogenes* INS7 R3 to enterocin CRL35 was also studied in order to analyze whether the expression of PrfA* leads to a change in the phenotype of these strains. Particularly, we focused on the sensitivity of the enterocin CRL35-resistant clones R2 and R3 [19].

2.8. Binding of enterocin CRL35 to *Listeria* cells

90 µl of 100 µM enterocin CRL35 solution was mixed with 10 µl of fluorescamine prepared in acetone (1 $mg.ml^{-1}$) in an eppendorf tube wrapped in tin foil. After vigorous vortexing, the tube was kept at 4°C until use. It is important to note that this derivatized peptide keeps the antimicrobial

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activity displayed by the native peptide [31]. Cells of *L. monocytogenes* FBUNT and *L. monocytogenes* FBprfA* that have grown in TSB at 37°C were harvested by centrifugation, washed twice with HEPES-K buffer, pH 7.4, and kept on ice. Then, cell suspensions (10^9 cells.ml^{-1}) were prepared in the same buffer and 200 nM fluorescent-labeled enterocin CRL35 was added. The peptide was allowed to be in contact with *Listeria* cells for 5 minutes and then fluorescence was measured in the supernatants as an indirect estimation of peptide binding. Control of enterocin CRL35 in a cell-free medium was used for calculating the percentage of unbound enterocin CRL35 that remained in the supernatants. The λ_{exc} was set at 395 nm, whereas the λ_{em} was set at 475 nm. Fluorescence was monitored in an ISS PC1 spectrofluorimeter. These results were confirmed by measuring total fluorescence upon excitation at 395 nm by using a SLM 4048c fluorometer equipped with a 3-71 Corning filter placed right before the emission photomultiplier.

2.9. Insertion of enterocin CRL35 into *Listeria*-derived lipid bilayers

0.125 µmol of lipids extracted from both *Listeria* strains were dried under nitrogen, resuspended in 250 µl of 20 mM Tris-HCl buffer containing 1 mM EDTA and vigorously vortexed till complete formation of multilamellar vesicles. Then, liposomes were sonicated 20 min and the small unilamellar vesicles (SUV) thus obtained were used within 3 h.

Enterocin CRL35 was diluted in the same buffer till a final concentration of 1.5 µM and tryptophan spectra were taken upon addition of increasing concentrations of SUV, from 0 to 16 µM. Samples were excited at 280 nm and the measurements were carried out at 37°C in a ISS PC1 spectrofluorometer. Raw spectra were corrected by substracting the scattering signals derived from liposomes.

For assessing the affinity constants, we plotted the blue shift ($\Delta\lambda_{max}$) against lipid concentration and data was fitted to a hyperbole using the following equation:

$$\beta = \frac{\beta_{max} . [lipid]}{Kd + [lipid]}$$

where β is the tryptophan blue shift measured after each addition of lipids, β_{max} is the maximal blue shift (asymptote of the hyperbola), Kd is the affinity constant, which represents the lipid concentration ([lipid]) needed for reaching a 50% of the β_{max}.

3. RESULTS

3.1. *L. monocytogenes* grows poorly in glucose-based minimal medium upon introduction of PrfA*

The constitutively activated *prfA* L140F allele was introduced into *L. monocytogenes* FBUNT, an enterocin CRL35 – sensitive indicator strain [18], by conjugation, as described by Wong and Freitag [20]. The resulting isolate, *L. monocytogenes* FBprfA*, bear pPL2-*prfA*L140F plasmid integrated within the $tRNA^{Arg}$ gene in its chromosome (Fig. S1A and B). As expected, the resultant mutant strain displayed increased hemolytic activity (Fig. S1C) and autoaggregation phenotype when growing overnight in TSB medium (Fig. S1D).

L. monocytogenes FBprfA* showed a fitness defect in TSB culture medium, as previously observed for other *Listeria prfA** strains [32] (Fig. 1A). Most notably, it displayed a marked growth impairment in glucose-containing minimal medium, as reported for *L. monocytogenes* EGD-derived strains [32]. The FBprfA* mutant exhibited poor growth in the presence of glucose (Fig. 1B), suggesting that glucose transport is impaired by the presence of the activated *prfA** allele.

3.2. Expression of IIC and IID subunits of Man-PTS complex is reduced in *L. monocytogenes* FBprfA*

Man-PTS is the physiological transporter of glucose in *L. monocytogenes* [33]. Therefore, the Man-PTS expression could be downregulated after the introduction of *prfA** in the FBUNT strain. Thus, we assessed the relative expression of membrane proteins of *L. monocytogenes* FBUNT and *L. monocytogenes* FBprfA* by means of a proteomic approach. At first, we analyzed all proteins corresponding to the Man-PTS systems present in *Listeria* (MptACD and MpoABCD) (Tables 1 and S2). In the case of MptA, MptC and MpoB proteins, no significant differences were detected between the two strains (p-value> 0.05). In contrast, MptD and MpoD levels were about 44 and 55% lower in FBprfA* than FBUNT (Table 1). In addition, we did find the known regulators of *mpt*

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operon, ManR and ResD, associated to the membrane fraction of *L. monocytogenes* FBprfA* samples (Table S3). Moreover, ManR was not detected in the membrane fraction of *L. monocytogenes* FBUNT, whereas ResD was expressed 3.66 fold higher in FBprfA* than FBUNT samples (Table S3). These results showed that Man-PTS proteins were down regulated in *L. monocytogenes* FBprfA*. It is important to note that a number of proteins increased their association to membranes upon expression of PrfA*, not only the virulence factors that are already known to be regulated by PrfA (Table S3). Among these proteins, several enzymes related to fatty acid synthesis were found in the membrane fraction, which is a puzzling finding based on the above results.

We next analyzed the expression of *mptC* and *mptD,* encoding the IIC and IID subunits of the Man-PTS, respectively, in *L. monocytogenes* FBprfA* using qRT-PCR. Expression of two PrfA-regulated genes, *prfA* itself and *hly,* was also measured. The level of both *mptC* and *mptD* transcripts was reduced by 65% in *L. monocytogenes* FBprfA* as compared to those observed in the parental strain (*mptC* 0.35 ± 0.07; *mptD* 0.38 ± 0.08). Consistent with the introduction of the constitutively activated *prfA** allele, *prfA* and *hly* were overexpressed in *L. monocytogenes* FBprfA*. Indeed, the 16-fold increase in *hly* gene expression (encoding the pore forming hemolysin listeriolysin O or LLO) explains the enhanced hemolytic activity of the virulent mutant strain (*prfA* 3.97 ± 0.66, *hlyA* 16.59 ± 5.58). The *prfA**-dependent reduction in the expression of *mptC* and *mptD* differs somewhat from that reported by Marr *et al.* [32]; however, their data were obtained from microarrays studies, which, in contrast to qPCR, usually cannot accurately distinguish relatively minor changes in gene expression levels [34].

3.3. *L. monocytogenes* containing constitutively activated PrfA are more sensitive to pediocin-like bacteriocins

Since MptC/MptD (IIC/IID proteins of the Man-PTS) have been reported to constitute the class II bacteriocin receptor, we tested the sensitivity of our PrfA*-expressing strain to pediocin-like bacteriocins by performing killing curves analyses, according to the protocol reported by Masias *et al.* [31]. We observed less than 20% decrease in *L. monocytogenes* FBUNT colony forming units (CFU) after 30 min incubation with 5 nM enterocin CRL35. In contrast, more than 90% of the *L. monocytogenes* FBprfA* bacteria were killed under the same conditions (Fig. 2). Similar results

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were obtained for pediocin PA-1 (Fig. S2). To confirm the increased sensitivity to class IIa bacteriocins, we next constructed another PrfA* mutant strain on a different *Listeria* background, but still sensitive to enterocin CRL35, *L. monocytogenes* INS7, and two bacteriocin – resistant derivatives which are Man-PTS-deficient [19]. *L. monocytogenes* INS7 became more sensitive to enterocin CRL35 following the introduction of *prfA** (Fig. 3). However, INS7-derived resistant cells, with impaired expression of the bacteriocin receptor, did not become more sensitive upon expression of PrfA* (Fig. 3). Overall, these results support the hypothesis that the so-called receptor for class IIa bacteriocins is not the only factor that dictates how sensitive a certain strain is, but it is still important for the activity. Importantly, *L. monocytogenes* FBprfA* strain did not display any change in its sensitivity toward nisin (Fig. S3), strongly suggesting that the expression of activated PrfA did not influence the mechanism of action of lantibiotics.

3.4. Membrane phospholipid composition changes upon expression of PrfA*

A correlation between protein levels of Man-PTS components and sensitivity toward enterocin CRL35 and pediocin PA-1 was not observed. However, we did detect changes in the association to membranes of a number of enzymes related to lipid metabolism. Based on this result, we hypothesized that the two strains might have different lipid abundances. Therefore, we pursued a detailed analysis of the membrane lipids present in FBprfA* and FBUNT strains. For this purpose, we purified lipids from both strains and subsequently carried out a comprehensive lipidomic analysis using the approaches previously developed in our laboratory [29]. As shown in Table 2, some differences were found when PrfA* was expressed. For instance, phosphatidic acid family (PA), that is absent in wild type cells, was detected as a minor species in *L. monocytogenes* FBprfA* strain. Conversely, plasmalogen phosphatidylglycerol species that is prominent in *L. monocytogenes* FBUNT, is significantly lowered in the FBprfA* strain (Fig. 4). All the lipids present e.g., phosphatidylglycerol (PG), phosphatidic acid (PA), cardiolipin (CL) and digalactosyldiacylglycerol (DGDG) are negatively charged and no zwitterionic lipids such as lysyl-cardiolipins previously described in *L. monocytogenes* was found [29]. Hence, difference in the total charge is not expected.

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The phospholipids present in both strains used in this study, including PG, CL, PA, and DGDG, contain 15:0- and 17:0-FA substituents at sn-1 or sn-2 of the glycerol backbone, respectively, (Table 2) as the dominant species, whose structures were identified as anteiso 17:0 (a-17:0) and anteiso 15:0 (a-15:0) fatty acids [29]. For example, ions at m/z 721, 707, and 693 represent a17:0/a15:0-, a17:0/16:0-, and a15:0/a15:0-PG, respectively. The major CL species observed at m/z 1323 and 1351 represent (a15:0/a15:0) (a17:0/a15:0)-CL and (a15:0/a17:0) (a17:0/a15:0)-CL, consistent with the observation of the ions of m/z 661.46 and 675.48, representing the doubly charged counterpart, respectively. We could not find marked differences in PG and CL between the two strains. However, *L. monocytogenes* FBprfA* consistently contained a 2-fold higher fraction of a17:0- and a15:0-FA as compared to FBUNT (data not shown in our repeated experiments). Based on the proteomic analysis, it is evident that several enzymes related to fatty acid synthesis such as acetyl-CoA carboxylase (AccC), the dehydratase FabZ, FabH that participates in the fatty acid elongation, the trans-2-enoyl-ACP reductase (FabI), also the reductase FabK1, the fatty acid kinases FakA and FakB2, were associated to the membrane fraction in *L. monocytogenes* FBprfA* samples. The observed changes in the FA profile is in accordance with the association of these enzymes of fatty acid synthesis. However, it remains unclear whether the increase of a17:0- and a15:0-FA in FBprfA* is related to the unknown activity of PrfA, the virulence master regulator in *L. monocytogenes*.

3.5 Binding and insertion of enterocin CRL35 into *Listeria* plasma membranes

In addition to differences in membrane composition, we had also observed unexpected changes in the membrane association of proteins related to bacterial cell wall synthesis upon expression of PrfA*, e.g. UDP-N-acetylmuramate--L-alanine ligase (MurC) and UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1 (MurA1) (Table S3, Supplementary information). The bacteriocin bound both cell types, i.e. FBprfA*and FBUNT at the same degree, based on the estimations obtained with the fluorescent derivative of enterocin CRL35 (Fig. 5A). In fact, *Listeria* cells bound one third of the total peptides regardless the expression of PrfA* with the concomitant reduction in the cell wall net negative charge and the reduced expression of the bacteriocin receptor.

Since the antimicrobial activity of bacteriocins is related to the insertion of these peptides into plasma membranes of target microorganisms rather than the mere association to the cell wall, we

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decided to analyze whether enterocin CRL35 would bind and insert the lipid bilayer of both *Listeria* strains (FBUNT and FBprfA*) besides its association to the bacterial outer surface structures. To this end, liposomes were prepared from *Listeria* lipids and the penetration of enterocin CRL35 into the membrane was assessed by taking advantage of a very well-known property of tryptophan (Fig. 5B); its fluorescence spectrum undergoes a blue shift when enters a more hydrophobic/apolar environment [11]. The affinity for lipids estimated was obtained from the binding curve of experimental data. Our results conclusively show that the affinity of enterocin CRL35 for FBprfA* lipids is 4 times higher than for FBUNT lipids (Kd FBUNT: 18.83 μM Vs. Kd FBprfA*: 4.46 μM). This result can explain the higher sensitivity of *L. monocytogenes* FBprfA* to enterocin CRL35 as compared to *L. monocytogenes* FBUNT.

4. Discussion

We showed that bacteria expressing *prfA** have a marked reduction in the expression of Man-PTS genes and yet they are even more susceptible to pediocin-like peptides. One of the first mechanisms proposed to explain pore formation was based on the premise that Man-PTS acts as an anchor for pediocin-like bacteriocins, helping these peptides to reach the plasma membrane and eventually alter membrane permeability [35]. Later, this model was modified [4,6], and the concept that pediocin-like bacteriocins may induce a conformational change in the Man-PTS that leads to pore opening was proposed [36,37].

In the present work, we demonstrated that the overexpression of *prfA** resulted in a partial downregulation of Man-PTS complex based on the qPCR results. Besides, two transcription factors involved in the expression of the *mptACD* operon, i.e., ResD and ManR, were associated to the plasma membrane in *L. monocytogenes* FBprfA*. Since it has been reported that some transcription factors are associated to membranes when inactive [38], this finding agrees with Man-PTS mRNA expression results. Furthermore, we were able to detect by mass spectrometry a significant downregulation of the IID subunit, which further supports the conclusion that Man-PTS complex expression is compromised in *L. monocytogenes* FBprfA* cells. Interestingly, we found a reduction in the expression of the IID subunit encoded by the *mpt* operon and also in the homolog protein encoded by the *mpo* operon.

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The partial reduction in the expression of Man-PTS should impair pore formation, if this complex were the only proteins involved in this structure. Our results suggest a different mechanism, and support the model in which Man-PTS acts just as a docking molecule that brings bacteriocins to the plasma membrane for the subsequent aggregation into a pore. In this regard, Barraza *et al.* [39] have demonstrated that Man-PTS can be dispensable in the bacteriocin mechanism of action, and their results also favored a role as a mere docking molecule for Man-PT*S* [39]. These conclusions were based on experiments carried out in *Escherichia coli* cells that heterologously expressed the *munA* gene fused to another docking protein. We propose that Man-PTS would be part of the mechanism of action, since no bactericidal activity is displayed by enterocin CRL35 when no receptor is expressed in *L. monocytogenes*. In fact, the results obtained with *L. monocytogenes* INS7-derived resistant cells support the contribution of Man-PTS to enterocin CRL35 sensitivity, presumably to ensure binding and insertion of antimicrobial peptides into the plasma membrane of target cells. However, the increased sensitivity of PrfA* strains suggests that some other PrfA-regulated component enhance sensitivity to bacteriocins when PrfA is activated. The lipidomic analysis shed some light on the differences in the plasma membranes of the two *Listeria* strains, which may help understand the sensitivities observed to class IIa bacteriocins. For instance, phosphatidic acid was present only in PrfA, being absent in FBUNT and it is tempting to speculate that PA might have an important role. In addition, the decrease in plasmalogen PG in FBPrfA* may also be crucial. Plasmalogen phospholipids have been thought to confer certain protection against extreme pH, high temperatures, organic solvents and antibiotics [40,41]. Therefore, the abrupt reduction of plasmalogens in *L. monocytogenes* FBprfA* may predispose this strain to be more sensitive to class IIa peptides. So far, phosphatidylglycerol and cardiolipin are the only two phospholipid families taken into account for analyzing sensitivity of bacteriocins to *Listeria* [42]. Conversely, the lysinylation of these phospholipids is associated to increased resistance because of the positive charge that is introduced [8].

PA contains a large negative curvature that can form HII phase or cubic phase [43]. On the contrary, PG, the main phospholipid in *Listeria* is a lipid with a small curvature that can form a lamellar phase, which is a typical lipid bilayer. It has been reported that a hybrid bacteriocin composed by pediocin PA-1 and plantaricin 149 can induce a negative curvature of the membrane

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with a concomitant disruption of the bilayer [44]. Thus, if enterocin CRL35 interacts with membranes in the same manner, even a slight increase in PA could enhance the membrane disruption triggered by the cationic peptides.

In the same context, the increase of the major a 17:0- and a-15:0-FA in *L. monocytogenes* FBprfA* can increase the total negative charge of its membrane; more importantly, these FFA can also change the properties of the bilayer. It has been suggested that the addition of fatty acids to cylindrical phospholipids, such as phosphatidylcholine (and probably this can also be applied to PG), induces type II lyotropic liquid crystalline phases, such as the inverse bicontinuous cubic phases and the inverse hexagonal phase [45,46]. Therefore, the presence of fatty acids in the plasma membrane of *Listeria* can play a significant role in the interaction of class IIa bacteriocins with these cells and in their mechanism of action. In this regard, Marr *et al.* [32] reported that the expression of several enzymes associated with fatty acid synthesis and metabolism was increased in cells expressing PrfA*. Even though they did not analyze the membrane composition, their microarray assays led to speculate that some changes in the plasma membrane should take place. For instance, they found that *fabH* gene was upregulated. This gene is known to encode the enzyme that carries out the condensation reaction in the initiation of type II fatty acid biosynthesis [47]. Therefore, any change in its expression can have important consequences in the architecture of *Listeria* membrane. We found an increased fraction of FabH associated with FBprfA* membranes. It is important to note that FabH in *Listeria* is related to the synthesis of anteiso branched-chain fatty acids [48]. The increase of anteiso FFA (e.g., a17:0 and a15:0FA) and PA in the plasma membrane of FBprfA*, as observed in this study, is therefore in accordance with the fact that the FabH enzymes involved in FFA synthesis are intriguingly bound to plasma membranes, suggesting that an increase in turnover of these lipids induced by PrfA* may have occurred. The binding and insertion measurements of enterocin CRL35 to liposomes prepared from both *L. monocytogenes* FBUNT and *L. monocytogenes* FBprfA* lipids may reflect different composition of both membranes. We hypothesize that lipid composition might dictate the sensitivity of each strain. Moreover, we show that enterocin CRL35 has higher affinity to FBprfA* lipids.

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In agreement with our findings, López-Solanilla *et al.* [49] also reported an enhanced susceptibility of a *L. monocytogenes* PrfA* strain only to potato defensin. However, no changes were reported by the same authors with other antimicrobial peptides of animal and vegetal origin, such as thionins, magainin and human defensins. In addition, there are no reports about sensitivity of *L. monocytogenes* PrfA* strain to bacterial-derived antimicrobial peptides such as pediocin-like bacteriocins. Nonetheless, that virulent *L. monocytogenes* are more sensitive to these compounds is an issue that deserves more attention. This focus may lead to design ways to use these peptides instead of antibiotics for clinical interventions, thus avoiding drastic alterations in the patient's natural microbiota. We and others have tested bacteriocins in mice infected with *L. monocytogenes* with promising results [18,50,51]. By oral administration, Dabour *et al.* [52] have successfully treated infected mice with pediocin PA-1. Our success in the treatment of *L. monocytogenes* infections in mice with bacteriocins may be explained by the enhanced sensitivity of the virulent *L. monocytogenes* following activation of PrfA within host cells.

In summary, we have proved that *L. monocytogenes* cells expressing active PrfA are more sensitive to class IIa peptides than *Listeria* cells in saprophytic state. This unexpected result raises questions about current models regarding receptor(s) for class IIa peptides and how these bacteriocins interact with bacterial membranes. For the first time, a study provides a link between the virulence of *L. monocytogenes* based on PrfA activation and bacterial sensitivity toward pediocin-like peptides.

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Ethical approval: Not required

AUTHOR CONTRIBUTIONS

(i) Conceptualization and design of the study: CM, LS, (ii) Acquisition of data: JF, EM, FFH, NF (iii) analysis and interpretation of data: JF, FFH, LS, CM, RS, EMH (iv) writing of the manuscript: CM, LS, FFH, RS, EMH.

Supplementary data

Supplementary material

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Figure Legends

Figure 1. Differential growth kinetics of *Listeria* cells upon expression of PrfA*. *L. monocytogenes* FBUNT (■) and *L. monocytogenes* FBprfA* (●) were grown overnight and then collected, washed in PBS and inoculated in TSB medium **(A)**, and in a glucose-containing minimal medium **(B)**. Bacterial growth at 37 °C was followed for up to 24 h by measuring the OD at 600 nm. The results presented here are representative of three independent experiments.

Figure 2. *L. monocytogenes* FBprfA* displays higher sensitivity to bactericidal activity of enterocin CRL35 compared to *L. monocytogenes* FBUNT. Cells were grown in TSB at 37 °C till mid-exponential phase. Then, cells were collected by centrifugation, washed and suspended in HEPES-K at 10^7 cells ml^{-1}. Enterocin CRL35 was added at a final concentration of 5 nM, and aliquots of cell suspensions were serially diluted and plated onto TSB agar plates after 30 minutes of incubation at 37 °C. Values shown are mean ± standard deviation of three independent assays carried out in triplicate. Significantly different values compared to the corresponding control are indicated by * ($\alpha = 0.05$).

Figure 3. Sensitivity toward enterocin CRL35 of *L. monocytogenes* INS7 and its enterocin CRL35-resistant derivatives. *L. monocytogenes* INS7 (R2 and R3) were conjugated with *E. coli* SM10 bearing pNF1002 as described in Materials and methods for *L. monocytogenes* FBUNT. Bacteria grown till exponential phase were washed and suspended in HEPES-K at 10^7 cells ml^{-1}. Enterocin CRL35 was added at a final concentration of 5 nM in the case of *L. monocytogenes* INS7 and at a concentration as high as 100 μM for the resistant isolates. Samples of each suspension were serially diluted and plated onto TSB agar plates after 30 minutes of incubation at 37 °C. Values shown are mean ± standard deviation of three independent assays carried out in duplicate. Significantly different values compared to the corresponding control are indicated by * ($\alpha = 0.05$).

Figure 4. Plasmalogen PG content in plasma membranes. The relative abundance of pPG species was estimated by mass spectrometry as described in Materials and methods. Results were normalized with the internal standards and phosphorous content. *L. monocytogenes* FBUNT

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is represented in black bars and FBprfA* in gray bars. This figure is representative of three independent experiments.

Figure 5. Binding of enterocin CRL35. *Listeria* cells were grown in TSB medium, harvested by centrifugation and suspended in HEPES-K buffer. These cells were incubated with 2 µM fluorescamine-labeled enterocin CRL35 and supernatants were collected by centrifugation and fluorescence was measured as indicated in Materials and methods. Unbound enterocin CRL35 was calculated as the percentage of initial enterocin CRL35, prior to the addition of bacteria **(A)**. Influence of lipid composition on the binding of enterocin CRL35 to lipid vesicles. Tryptophan fluorescence blue shifts ($\Delta\lambda_{max}$) were recorded in the presence of increasing concentrations of lipid vesicles derived from *L. monocytogenes* FBUNT (■) and FBprfA* (●). The means and standard errors were obtained from three independent experiments **(B)**.

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Table 1. Relative expression of ManPTS proteins in *Listeria* PrfA* compare to FBUNT.

Accession number	Description	Gene	p-value	FBprfA*/FBUNT Fold change expression	FBUNT/FbprfA* Fold change expression
Q8YAM2	lmo0096 protein	*mptA*	0.1539	0.69	1.43
Q8YAM1	lmo0097 protein	*mptC*	0.9484	0.96	1.04
Q8YAM0	lmo0098 protein	*mptD*	0.0189	0.64	1.55
Q8Y8W1	lmo0781 protein	*mpoD*	0.0089	0.69	1.44
Q8Y8V9	lmo0783 protein	*mpoB*	0.8442	0.94	1.07

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Table 2. Lipidomic analysis of FBUNT and FBprfA* plasma membranes.

FBUNT		FBprfA*				
Measured *m/z* Da	Rel. Intensity %	Measured *m/z* Da	Rel. Intensity %	Theo. Mass Da	Deviation mDa	Elementa
640.4	0.13	640.4	0.11	640.4	0.26	C68 H130
647.4	0.37	647.4	0.51	647.4	0.38	C69 H132
654.4	0.32	654.4	0.43	654.4	0.4	C70 H134
661.4	1.92	661.4	2.07	661.4	0.42	C71 H136
668.4	0.78	668.4	0.85	668.4	0.4	C72 H138
675.4	3.6	675.4	3.26	675.4	0.36	C73 H140
682.4	0.21	682.4	0.15	682.4	0.5	C74 H142
689.4	0.57	689.4	0.29	689.4	0.51	C75 H144
		1281.8	0.1	1281.8	1.37	C68 H131
1295.9	0.13	1295.9	0.28	1295.9	1.5	C69 H133
1309.9	0.09	1309.9	0.22	1309.9	1.55	C70 H135
1323.9	0.5	1323.9	1.11	1323.9	1.41	C71 H137
1337.9	0.23	1337.9	0.41	1337.9	1.76	C72 H139
1351.9	0.91	1351.9	1.3	1351.9	1.33	C73 H141
1365.9	0.05			1365.9	2.3	C74 H143
1379.9	0.15	1379.9	0.19	1379.9	1.34	C75 H145
535.3	0.26	535.3	0.61	535.3	0.14	C54 H104
542.3	0.06	542.3	0.23	542.3	0.09	C55 H106
549.3	0.56	549.3	1.37	549.3	0.17	C56 H108
		556.3	0.08	556.3	0.05	C57 H110
563.3	0.08			563.3	0.18	C57 H108
1071.6	0.09	1071.6	0.6	1071.6	0.67	C54 H105
1085.7	0.05	1085.7	0.24	1085.7	0.73	C55 H107
1099.7	0.25	1099.7	1.3	1099.7	0.63	C56 H109
		1127.7	0.12	1127.7	1.06	C58 H113

Table 2 (continued)

FBUNT		FBprfA*				
Measured *m/z* Da	Rel. Intensity %	Measured *m/z* Da	Rel. Intensity %	Theo. Mass Da	Deviation mDa	Elementa
423.2	0.07	423.2	0.63	423.2	-0.12	C39 H76
847.4	0.05	847.4	0.69	847.4	0.51	C39 H77
		875.5	0.08	875.5	0.79	C41 H81
		619.4	0.22	619.4	0.58	C33 H64
		647.4	0.4	647.4	0.85	C35 H68
679.4	1.68	679.4	1.96	679.4	0.4	C35 H68
693.4	27.02	693.4	32.69	693.4	0.28	C36 H70
707.4	13.35	707.4	15.47	707.4	0.4	C37 H72
719.4	0.52	719.4	0.64	719.4	0.44	C38 H72
721.5	100	721.5	100	721.5	-0.01	C38 H74
733.5	1.05	733.5	1.13	733.5	0.2	C39 H74
735.5	3.38	735.5	2.77	735.5	0.24	C39 H76

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749.5	10.52	749.5	7.39	749.5	0.39	C40 H78
663.4	0.57	663.4	0.11	663.4	0.44	C35 H68
677.4	14.11	677.4	1.63	677.4	0.39	C36 H70
691.4	8.41	691.4	1.01	691.4	0.51	C37 H72
705.5	61.3	705.5	7.55	705.5	0.35	C38 H74
719.5	0.81	719.5	0.09	719.5	0.5	C39 H76
733.5	2.54	733.5	0.25	733.5	0.2	C40 H78
		917.6	0.06	917.6	0.66	C51 H98
		931.7	0.06	931.7	0.43	C52 H100
945.7	0.13	945.7	0.17	945.7	0.71	C53 H102
959.7	0.1	959.7	0.11	959.7	0.68	C54 H104
737.4	0.3	737.4	0.38	737.4	0.1	C39 H74
891.6	0.09	891.6	0.24	891.6	0.45	C47 H87
899.5	0.14	899.5	0.56	899.5	0.39	C45 H84
927.5	0.49	927.5	1.27	927.5	0.46	C47 H88
955.6	0.14	955.6	0.3	955.6	0.52	C49 H92

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Highlights

- Up to date class IIa bacteriocins sensitivity was evaluated in saprophytic *Listeria*.
- *Listeria overexpressing prfA shows partially reduced expression of Man-PTS complex.*
- *Sensitivity to pediocin-like bacteriocins is increased in pathogenic Listeria cells.*
- Phosphatidic acid and plasmalogen are key players in the bacteriocin mode of action.
- A link between virulence and sensitivity toward antilisterial peptides is proposed.

A

FBUNT
FBprfA*

OD_{600nm}

0
1

0 1 2 3 4 5 6 7 8 9 10 11 12 13

Time (h)

B

FBUNT
FBprfA*

OD_{600nm}

0
1

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Time (h)

Figure 1

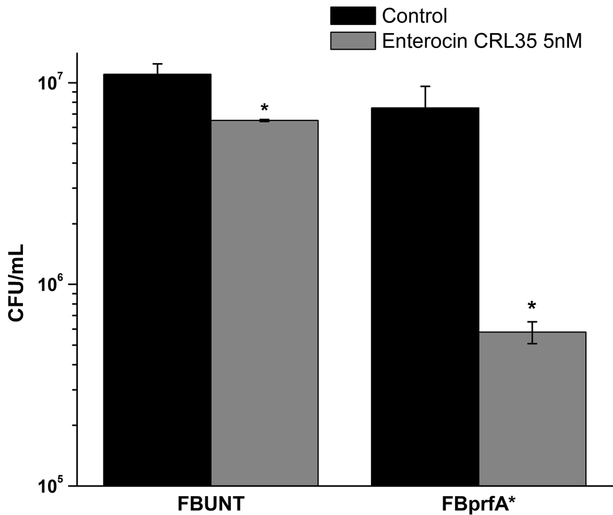

Figure 2

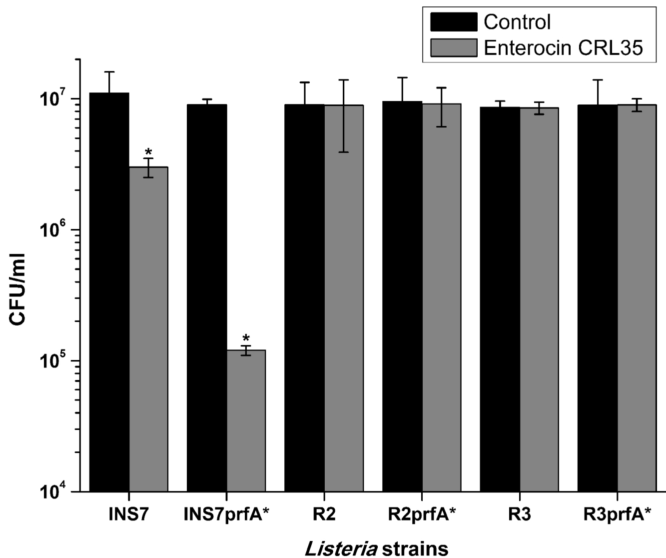

Figure 3

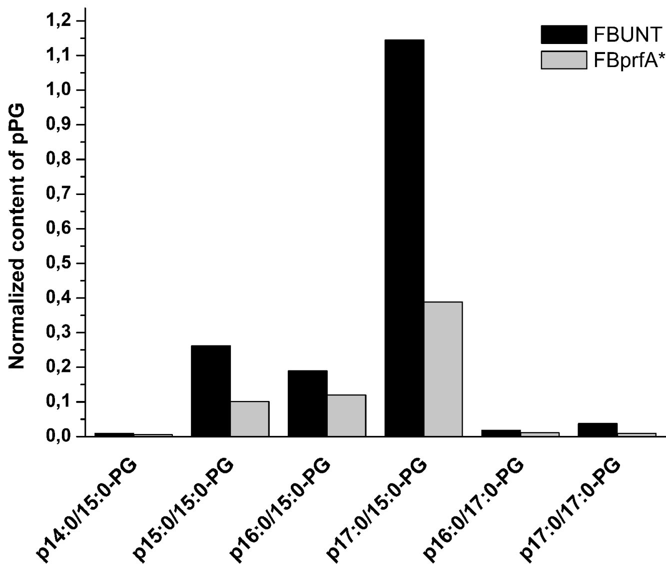

Figure 4

A

Enterocin CRL35 binding (%)

45
40
35
30
25
20
15
10
5
0

FBUNT

FBprfA*

B

FBUNT
FBprfA*

$\Delta\lambda_{max}$ (nm)

5
4
3
2
1
0

0 2 4 6 8 10 12 14 16

Lipid concentration (μM)

Figure 5