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# 28-mer Fragment Derived from Enterocin CRL35 Displays an Unexpected Bactericidal Effect on *Listeria* Cells

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**Abstract:** Two shorter peptides derived from enterocin CRL35, a 43-mer bacteriocin, were synthesized *i.e.* the N-terminal fragment spanning from residues 1 to 15, and a 28-mer fragment that represents the C-terminal of enterocin CRL35, the residues 16 to 43. The separate peptides showed no activity when combined. On one hand, the 28-mer peptide displayed an unpredicted antimicrobial activity. On the other, 15-mer peptide had no consistent anti-*Listeria* effect. The dissociation constants calculated from experimental data indicated that all peptides could bind at similar extent to the sensitive cells. However, transmembrane electrical potential was not dissipated to the same level by the different peptides; whereas the full-length and the C-terminal 28-mer fragment induced almost full dissipation, 15-mer fragment produced only a slow and incomplete effect. Furthermore, a different interaction of each peptide with membranes was demonstrated based on studies carried out with liposomes, which led us to conclude that activity was related to structure rather than to net positive charges. These results open up the possibility of designing new peptides based on the 28-mer fragment with enhanced activity, which would represent a promising approach for combating *Listeria* and other pathogens.

**Keywords:** Bacteriocins, enterocin CRL35, *Listeria,* synthetic peptides.

## 1. INTRODUCTION

Ribosomally-synthesized antimicrobial peptides from lactic acid bacteria (LAB), also known as bacteriocins, have awaken great interest in the last decades [1]. These peptides are not only highly efficient in controlling the pathogen *Listeria monocytogenes* and other bacteria but also no toxic effects have been reported so far [2]. Bacteriocins belonging to the subclass IIa, the so-called pediocin-like bacteriocins, are the most-well studied ones and several patents have been released for potential applications [3, 4]. Enterocin CRL35 is a member of this subclass mainly active against *L. monocytogenes, Staphylococcus aureus*, other close-related LAB strains and the Herpes virus simplex type 1 and 2 [5, 6].

It was demonstrated that enterocin CRL35, as other cationic peptides, interacts with plasma membrane of target cells, inducing the leakage of ions and small solutes, with the concomitant dissipation of the transmembrane electrical potential and the drop of the intracellular pH of sensitive cells [7-9]. Thus, combination of enterocin CRL35 with other membrane-active compounds like monensin, bacitracin or gramicidin could render in positive interactions [10].

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Furthermore, synergistic interactions between enterocin CRL35 with some clinical antibiotic such as erythromycin, chloramphenicol and tetracycline was demonstrated, which opens up the potential application of this bacteriocin as an adjuvant to antibiotic therapies [9]. Actually, enterocin CRL35 might be used in the treatment of multidrug-resistant bacteria that exclude antibiotics by efflux pumps dependent on the membrane proton gradient, as it was proposed by other bacteriocins such as peptide ST4SA [11].

Since the N-terminal fragment from enterocin CRL35 was already studied in some detail [12, 13], the aim of the present paper was to study the molecular interactions of the full length enterocin CRL35 as well as the C-terminal fragment of enterocin CRL35 (28-mer peptide) with both sensitive cells and liposomes. In this regard, it was previously shown by Bhugaloo-Vial *et al* that a 26-mer peptide derived from the C-terminus of divercin V41 (DV41 18-43) still conserved activity [14]. The authors hypothesized that the presence of a second disulfide bond in this part of the molecule was responsible for the activity, since treatments disrupting this bond inhibited the antimicrobial effect [14]. Therefore, we analyzed if the 28-mer C-terminal region of the enterocin CRL35 (16-43) is endowed of antimicrobial activity, since enterocin CRL35 lacks the second disulfide bond in its structure. Possible reconstitution of the activity upon mixing of 15-mer and 28-mer fragments was also analyzed. The final

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scope was to characterize shorter enterocin CRL35-derived peptides that may keep some activity against *L. monocytogenes*. The key point of analyzing the activity of shorter peptides derived from bacteriocins is that they will be easier to synthesize and less expensive, which would be important for clinical applications.

## 2. MATERIALS AND METHODS

### 2.1. Synthesis and Purification of Enterocin CRL35 and its Derivatives

Three peptides were synthesized: 43-mer (KYYGNGV SCNKKGCSVDWGKAIGIIGNNSAANLATGGAAG-WKS), which is the full-length enterocin CRL35, 15-mer (KYYGNGVSCNKKGCS) that represents the 1-15 enterocin CRL35 variant and 28-mer (VDWGKAIGIIGNN-SAANLATGGAAGWKS), the 16-43 bacteriocin. These peptides were manually synthesized on Rink amide 4-methylbenzhydrylamine resin according to the standard Nα-Fmoc protocol [15]. Cleavage of the peptides from the resin and removal of the side chain protecting groups were performed with a mixture of 90% trifluoracetic acid (TFA), 5% triisopropylsylane and 5% Milli-Q water. Afterward, crude peptides were precipitated with ethyl ether in order to separate them from soluble non-proteinaceous material and then extracted into a 30% acetonitrile/$H_2O$ solution (v/v) prior to lyophilization. Except for the 28-mer peptide, the oxidized peptide was obtained by air oxidation of a solution of reduced peptides (0.1 mM) in aqueous solution, pH 8.0, at room temperature with stirring. Purification was achieved by using a semi-preparative reverse phase $C_{18}$ HPLC column (μBondapack, Waters), applying a linear gradient between 20 mM ammonium acetate, pH 5.0 and 90% acetonitrile in ammonium acetate buffer or using a linear gradient 40–80% between 0.045% (v/v) TFA/$H_2O$ and with a 0.036% (v/v) TFA/acetonitrile for 120 min. The flow rate was set at 5 ml/min. Purity of the samples was checked in an analytical HPLC column (Kromasil 300 Å, C18, 4.6 x 150 mm, 5 μm particle size), using bidistilled water with 0.045% TFA as solvent A and acetonitrile, 0.036% TFA as solvent B. The separation was performed with a linear gradient of 5–95% of solvent B in 30 min, at a flow rate of 1 ml/min, following the elution at 220 nm. Peptide purity and identity was confirmed by Electrospray Mass Spectrometry on Bruker model apparatus (Germany) (not shown).

### 2.2. Antimicrobial Assays

The sensitive strain *L. monocytogenes* INS7 was grown in Trypticase Soy Broth (TSB) medium supplemented with yeast extract and 25 μg/ml of nalidixic acid [16]. Anti-*Listeria* activity was assayed by a modified spot-on-lawn assay. Briefly, dilutions of each sample (10 μl) were absorbed directly onto sterile TSB plates, then 5 ml of soft agar (0.6 % (w/v)) inoculated with $10^6$ cells of the sensitive strain *L. monocytogenes* INS7 were added and plates incubated 16 h at 30°C. The use of culture medium for preparing the soft agar as indicated in the original method was avoided because cleaner and more reproducible halos can be obtained in this way. Antimicrobial activity was detected as clear halos of growth inhibition.

In addition, a microplate-based assay was used to determine the MICs of synthetic peptides, using a VersaMax Microplate Reader (Molecular Devices, CA). The final concentrations ranged from 10 nM to 500 nM for the full-length peptide, and from 0.2 μM to 100 μM for both shorter peptides. The MIC was defined as the lowest concentration of peptides that inhibited the growth more than 50% as compared to the untreated control.

The possible synergistic effect of peptides with clinical antibiotics was assessed by determining the fractional inhibitory concentration (FIC) [17].

For viability assays (time-kill curves), mid-log phase *L. monocytogenes* INS7 cells were suspended to approximately $10^8$ cells/ml in 50 mM HEPES-Na buffer pH 7.4 in the presence of different concentrations of peptides. Aliquots were taken at appropriate times and serially diluted. 5 μl of each dilution were plated onto TSB plates and incubated 16 h at 30°C for colony visualization.

### 2.3. Isolation of *L. monocytogenes* INS7 Mutants Resistant to Enterocin CRL35 (43-mer Peptide)

200 μM enterocin CRL35 solution (43-mer) were spotted onto TSB agar plates and covered with 5 ml of soft agar containing $10^8$ CFU/ml *L. monocytogenes* INS7 cells. Spontaneous resistant colonies were picked from the inhibition halos and re-isolated in PALCAM medium. Taxonomic identification of each isolate was performed by PCR and DNA sequencing of 16S rRNA gene (Sequencing Service of CCT-CONICET-Tucumán) (Masias, unpublished results). Afterward, 15-mer and 28-mer peptides were tested against these cells.

### 2.4. Peptides-Sensitive Cells Interactions

In order to study the interaction of complete molecule of enterocin CRL35 with living cells, the peptide was labeled with the fluorescent probe fluorescamine (Molecular Probes-Life Technologies). 90 μL of 100 μM solution of each peptide prepared in HEPES buffer pH 8 was mixed with 10 μl of a freshly acetone dissolved fluorescamine solution (1 mg/ml). Samples were incubated 10 min in the dark at room temperature and kept at 4°C under N2. *L. monocytogenes* INS7 cells were harvested at mid-log phase, washed and suspended in HEPES-Na buffer, pH 7.4 and kept up to two hours on ice. An aliquot of each fluorescamine-derivatized peptide was diluted in 2 ml of HEPES buffer containing 12.5 mM glucose and steady-state fluorescence polarization (*p*) was measured upon addition of increasing concentrations of sensitive cells under constant stirring. The steady-state fluorescence polarization was determined in an ISS PC1 Photon Counting Spectrofluorimeter thermostatized at 30°C by means of an external circulating bath (Cole Parmer), adjusting the excitation and emission wavelengths at 390 nm and 475 nm, respectively. Fluorescence polarization according to:

$$p=\frac{I_{vv}-I_{vh}}{I_{vv}+I_{vh}}$$

where Ivv is the fluorescence intensity recorded with both the analyzing and the excitation beam polarizers vertically oriented, whereas Ivh is the fluorescence intensity recorded in crossed polarizers condition, in which excitation beam polarizer is vertically oriented whereas the emission channel is horizontally oriented. Fluorescamine-labeled peptides were not further purified as the excess of fluorescent probe is readily hydrolyzed to non-fluorescent compounds [18]. However, controls were run by mixing fluorescamine solutions with peptide-free buffer before performing the measurements, in order to ensure no significant signal could be detected after adding cells.

### 2.5. Dissipation of Transmembrane Electrical Potential

The transmembrane electrical potential ($\Delta\psi$) was measured with the fluorescent probe 3,3′-dipropylthiadicarbocyanine iodide (DisC3[5]) (Life Technologies, CA). Changes in fluorescence was measured with an ISS PC1 Photon Counting Spectrofluorimeter thermostatized at 30°C with constant stirring, adjusting the excitation and emission wavelengths at 622 nm and 684 nm respectively.

### 2.6. Membrane-Peptides Interactions

Single lamellar liposomes composed of dimyristoyl phosphatidylcholine/dimyristoyl phosphatidylglycerol (9:1) were prepared by extrusion or sonication as described elsewhere [19]. Light scattering of 50 μM liposome suspensions in the presence and in the absence of peptides was measured in an ISS PC1 Photon Counting Spectrofluorimeter by setting excitation and emission wavelengths at 370 nm [19]. Additionally, tryptophan fluorescence of the unlabeled peptides was measured in order to study membrane partitioning of the peptides. Spectra were taken using 2 μM peptide solutions in the absence and in the presence of 100 μM liposomes. Excitation wavelength was set at 280 nm while emission was recorded from 300 to 400 nm [20]. Measurements were carried out at 15°C, which guarantees a gel phase of lipids that is optimal for enterocin CRL35 interaction with membranes [20]. All the studies were carried out by triplicate at least five independent times.

## 3. RESULTS

### 3.1. Anti-*Listeria* Activity of Enterocin CRL35 Derived N-and C-Terminal Peptide Fragments

Peptide fragments derived from enterocin CRL 35 synthesized as described in M&M section, were tested for their anti-*Listeria* activity using a spot-on-lawn assay. Fig. **1** clearly shows that 28-mer peptide (16-43) was able to inhibit the growth of the sensitive strain *L. monocytogenes* INS7 although to a lesser extend when compared with the full-length parental bacteriocin enterocin CRL35. Nevertheless, the fact that inhibition halos were produced by this peptide was highly unexpected. Importantly, 15-mer fragment only produced faint and turbid halos. Microplate-based assay indicated that the MIC of the full-length bacteriocin is 10 nM, while the 28-mer peptide presented a MIC of 20 μM.

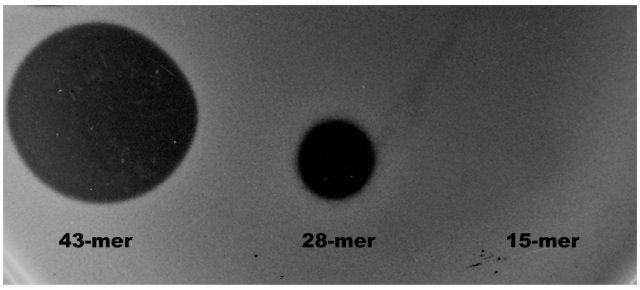


**Figure 1. 28-mer fragment induces inhibition halos in *L. monocytogenes* INS7 cultures.** 10 μl of 20 μM stock solution of either 28-mer peptide or 15-mer peptide were placed onto TSB agar plates as described in Materials and Methods. Besides, 10 μM solution of enterocin CRL35 was used. Inhibition halos were developed after an overnight incubation at 30°C. The result is representative of four independent experiments.

The activity of 15-mer and 28-mer mixtures at different ratios were always in the micromolar range, thus no synergistic effect was achieved upon combination of the two parts of enterocin CRL35. In the same trend, no consistent synergistic effect was found when these peptides were tested in the presence of the clinical antibiotics tetracycline and erythromycin. In fact, FIC values were always close to 1 (data not shown).

Then, time-kill curves were carried out in buffer supplemented with glucose as described in Materials and Methods. The 28-mer fragment turned out to be a bactericidal peptide, in a similar way full-length enterocin CRL35 is, although at higher concentrations, as discussed above. On the contrary, 15-mer peptide was unable to reduce *L. monocytogenes* INS7 (Fig. **2**). Importantly, *L. monocytogenes* INS7 derived-cells resistant to the full-length bacteriocin were also resistant to the 28-mer peptide as well even at concentrations as high as 200 μM (data not shown).

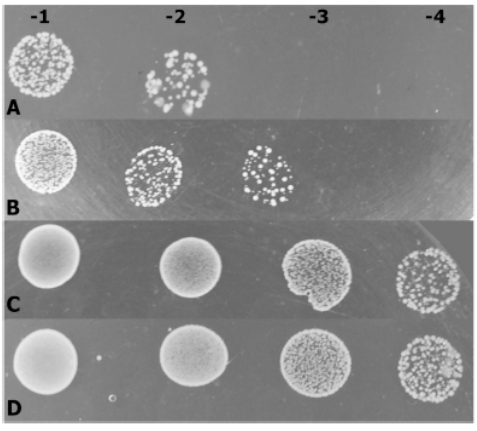


**Figure 2. 28-mer fragment acts as a bactericidal peptide.** 40 nM enterocin CRL35 (**A**), 40 μM of 28-mer peptide (**B**) and 100 μM of 15-mer peptide (**C**) were added to *L. monocytogenes* INS7 suspensions in glucose-containing buffer. Control cells with no peptide were also platted (**D**). Cells were incubated at 30°C and aliquots were taken at different times, serially diluted and spotted onto TSB plates. The result is representative of three independent experiments.

### 3.2. Interaction of Enterocin CRL35 as Well as the N-and C-Terminal Peptide Fragments with *L. monocytogenes* INS7 Cells

Since fluorescence polarization gives a good estimation of the mobility or the size of the fluorescent probe [21], it represents an excellent tool for studying peptide-cell interaction, because peptides in cell-free buffer must have a low capability of emitting polarized light in the same plane that they were irradiated, owing their small size and higher mobility. However, when they are immobilized or attached to bigger objects, like when they interact with cells; the rotational and translational freedom degrees would be markedly reduced. Therefore, a concomitant increase in fluorescence polarization should be expected. As it can be observed in Fig. **3**, all three peptides were cell-associated, since fluorescence polarization of fluorescamine-labeled peptides increased upon addition of cells. Experimental data were fitted to a hyperbole using Origin 8.0 software (OriginLab, MA), and dissociation constants (Kd) were calculated. Surprisingly, Kd values were very close to each other *i.e.* 10.05, 6.11 and 12.06 for 43-mer, 28-mer and 15-mer respectively. This result indicates that all three peptides were able to bind to the cells to a similar extent, as opposed to the predicted reduced interaction of 15-mer peptide based on the results described above.

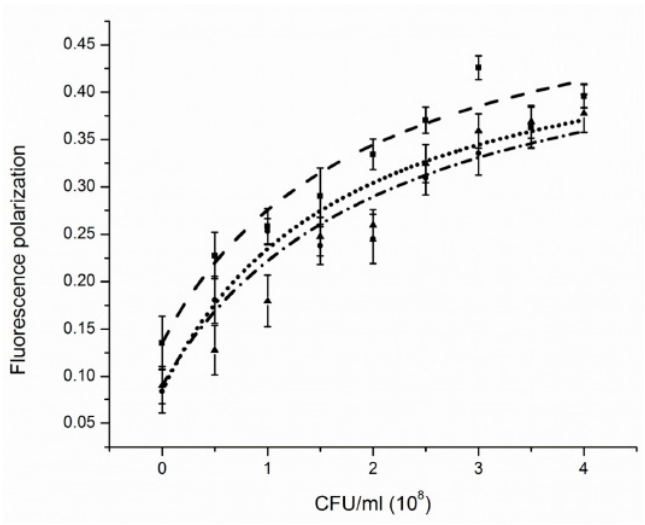


**Figure 3. All peptides interact with sensitive cells to a similar extent.** Increasing concentrations of *L. monocytogenes* INS7 cells were added to buffer containing either 15-mer peptide ( − · − ), 28-mer peptide ( − − ) or 43-mer peptide ( · · ). All peptides were previously derivatized with fluorescamine as described in Materials and Methods. Fluorescence polarization was calculated and plotted against cell number. The result is representative of five independent experiments.

### 3.3. Transmembrane Potential Dissipation by Enterocin CRL35 and its Derivatives

Since differences among the bactericidal activity of all three peptides were observed, while similar association could be assumed based on the fluorescence polarization results, the ability of dissipating the membrane electrical potential was assayed on living *Listeria* cells. Fig. **4** shows that 43-mer peptide completely dissipated the $\Delta\psi$ as it was previously described [9, 12], whereas the 15-mer peptide only induced a 40% $\Delta\psi$ dissipation when compared to the dissipation induced by the full-length bacteriocin. Interestingly, the 28-mer peptide showed an intermediate effect, inducing a 60% dissipation of the transmembrane electrical potential of *L. monocytogenes* INS7.

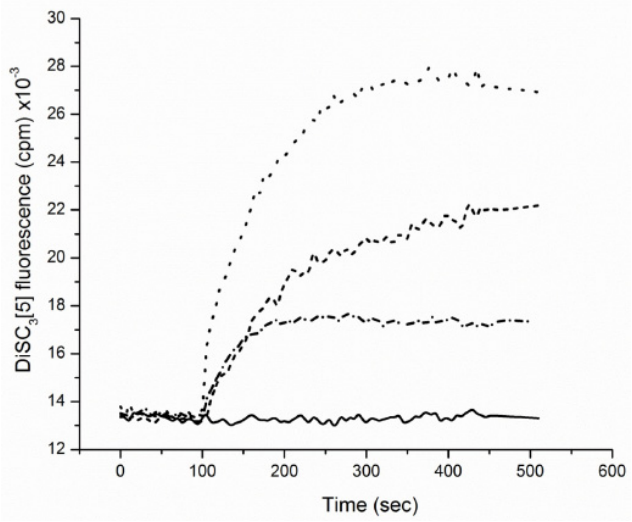


**Figure 4. Enterocin CRL35 and its shorter derivatives dissipate *L. monocytogenes* INS7 membrane potential.** *L. monocytogenes* INS7 were suspended in buffer at a final concentration of $10^8$ cells/ml. Then the fluorescent probe $DisC_3$ [5] and glucose were added and cells were maintained at 30°C with constant stirring. Changes in fluorescence were monitored upon addition of either 15-mer peptide ( − · − ), 28-mer peptide ( − − ) or 43-mer peptide ( · · ). $\lambda_{exc}$: 622 nm, $\lambda_{em}$: 684 nm. Control cells with no peptide added is shown in solid line. The result shown in this figure is representative of at least five independent experiments.

### 3.4. Interaction of Enterocin CRL35-Derived Peptides with Model Membranes

As it was previously demonstrated, enterocin CRL35 interacts with anionic membranes with gel-like domains [20]. Therefore, a comparative study of the interaction of enterocin CRL35-derived peptides with liposomes was carried out. As a first approach, peptides were added to liposome suspensions and light scattering was measured as described above. The full-length enterocin CRL35 induced vesicle aggregation as estimated from the light scattering values whereas 15-mer peptide induced a less pronounced increase of light scattering. Surprisingly, 28-mer peptide induced just slight changes in light scattering values (Fig. **5**). Overall, this result indicates that peptides interact with membranes but it does clearly not correlate with the antimicrobial activity findings.

As an alternative strategy for searching some clues about the differences observed in the anti-*Listeria* activity, the possible insertion of the peptides in the phospholipid bilayers was analyzed by following the blue shift in tryptophan fluorescence of peptides [20]. Since 15-mer peptide does not

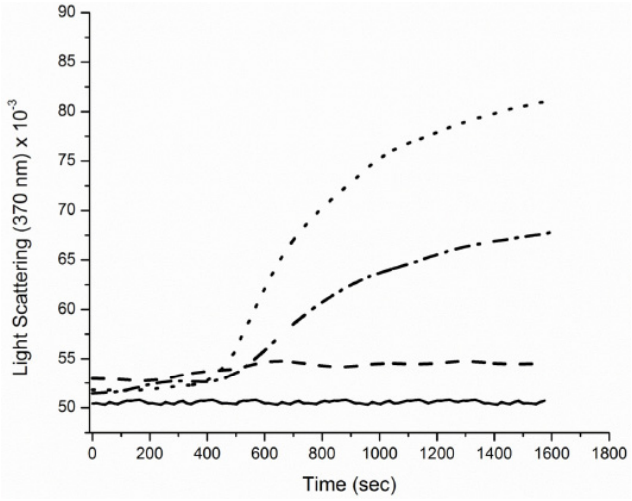


**Figure 5. Liposome aggregation induced by peptides.** Liposomes of dimyristoyl phosphatidylcholine/ dimyristoyl phosphatidylglycerol were prepared either by extrusion or by sonication and diluted to a final concentration of 50 μM. Then, peptides were added under constant stirring and light scattering was followed during 20 min. All the measurements were carried out at 15°C.. 15-mer peptide ( − · − ), 28-mer peptide ( − − ) or 43-mer peptide ( · · ). $\lambda_{exc}$ and $\lambda_{em}$ were set at 370 nm. Control with no peptide added is shown in solid line. The result shown in this figure is representative of five independent experiments made by triplicate.

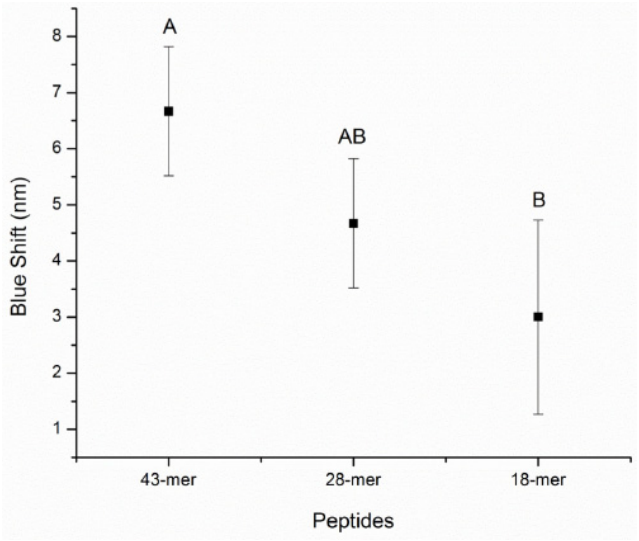


**Figure 6. All three peptides get inserted into lipid bilayer although to a different extent.** Tryptophan fluorescence of 18-mer, 28-mer and 43-mer peptides was analyzed in the absence and the presence of 100 μM dimyristoyl phosphatidylcholine/ dimyristoyl phosphatidylglycerol liposomes. Then blue shift of the tryptophan emission maximum was calculated.

contain tryptophans in its structure, a 3-amino acid longer peptide derived from enterocin CRL35 was used since tryptophan is present at position 18 and 41 in the primary sequence of the bacteriocin. As it can be observed in Fig. **6**, the maximum emission of tryptophan fluorescence was shifted 6.5 nm in enterocin CRL35 upon interaction with membranes, confirming previous results [20]. 28-mer peptide tryptophan fluorescence also showed a blue shift in the presence of liposomes, although this change was only 2 nm in average. 15-mer peptide did not show any change in the tryptophan fluorescence at the concentration of lipid used in the present work. As the 15-mer fragment, the 18-mer peptide displayed no antimicrobial activity under regular conditions, although it was able to bind to cells and induce liposome aggregation (data not shown).

## 4. DISCUSSION

From experimental data, it is well-established that class IIa bacteriocins can be structurally divided in two domains *i.e.* a three stranded β-sheet structure at the N-terminus and an amphiphilic α-helical domain, that is induced at the C-terminus upon binding to membrane-like structures [22]. In fact, bacteriocins were proposed to have a modular design where each domain has a specific function [23]. Based on this hypothesis, it would be expected that a combination of two derived peptides from a given bacteriocin would restore the antimicrobial activity present in the full-lenght peptide. To test this concept, two enterocin CRL35-based synthetic peptides were synthesized: an N-terminal fragment, consisting of the first 15 amino acids and a peptide representing the C-terminal domain spanning from amino acid 16 -43.

We have previously studied the effect of the N-terminal peptide in some detail. It was first described as a non-active peptide [12]. However, it was clear that some activity could be observed in defined media [13]. It is important to note that Bhugaloo-Vial *et al.* also reported that N-terminal peptide from divercin V41 was inactive under regular conditions [14]. Interestingly, these authors also analyzed a C-terminal fragment obtained by digestion of divercin V41 with the protease Asp-N [14]. They found that the 26-mer C-terminal peptide was active, arguably because it had a disulfide bond. In fact, they conclusively demonstrated that activity disappeared upon reduction of that S-S bond [14]. Based on this finding, we hypothesized that enterocin CRL35-derived C-terminal peptide would be inactive because disulfide bond is missing in this domain.

Even though we predicted a positive combination with the N-terminal half owing the modular nature of bacteriocins, we were unable to find positive combinations between 15-mer and 28-mer peptides. In this regard, Ovchinnikov *et al*. also reported no reconstitution of antimicrobial activity upon combination of different truncated peptides derived from the leaderless bacteriocin LsbB [24]. However, it was found that the 28-mer peptide itself was active against *L. monocytogenes* cells. Indeed, it turned out to be a bactericidal agent at concentrations equal to the MIC or higher.

Naturally resistant-occurring *Listeria* cells towards 43-mer peptide were picked and the sensitivity was tested against 28-mer peptide. Surprisingly, they turned out to be completely resistant as well (data not shown). This result strongly suggests that these two peptides would share a similar mechanism of action, at least to some extent. As a matter of fact, 28-mer fragment was unable to dissipate the transmembrane potential of *Listeria* cells resistant to the full-length peptide. Another important conclusion that can be

learned from the membrane dissipation experiments is that dissipation of $\Delta\psi$ is a necessary but not sufficient condition for the antibacterial activity. In fact, as it was stated above, 15-mer peptide did induce some dissipation and yet, it was unable to act as an antibiotic peptide. In this regard, N-terminal-derived peptide was able to get bound to the membranes, although insertion was limited based on the blue-shift tryptophan fluorescence experiments. On the contrary, 43-mer peptide had the most pronounced blue shift, strongly suggesting a better interaction of this peptide with apolar regions of the membranes. 28-mer peptide had an intermediate effect, which correlated well with its antimicrobial activity. It might be speculated that antimicrobial activity would only be displayed upon interaction with membranes in a very specific way.

It is important to stress that fluorescamine derivatization did not influence the activity of peptides. In fact, they displayed the same anti-*Listeria* activity and were able to dissipate the electrical potential in the same way non-derivatized peptide did. These findings are in agreement with a previous work with divercin V41 where acetylation of $\varepsilon$-$NH_2$ of lysine residues and the N- terminus did not abolished the antimicrobial activity [14]. In this regard, the isoelectric point of full-length enterocin CRL35 is 9.45 with a net positive charge of +4 at neutral pH. The pI for the 28-mer peptide is 8.56 (+1 net charge) while the 15-mer peptide pI is calculated as 9.26 with +3 net charge at the working conditions (ExPASy server). Therefore, there is no correlation between bactericidal activity and positive charges and hence they would not be essential for enterocin CRL35 activity either. Nevertheless, charges did correlate with the ability of inducing scattering of anionic liposome suspensions, indicating that the basic residues of the peptides are important in the mechanism of membrane aggregation.

A recent paper by Bodapati *et al.* confirmed that N-terminal part of class IIa bacteriocins is crucial for activity. Indeed, they replaced the β-sheet residues of the N-terminus with shorter β-turn motifs that led to a dramatic reduction of activity [25]. This result highlighted the importance of the whole bacteriocin for the antimicrobial activity. The N-terminus that is absent in the 28-mer peptide is likely to be the responsible for the lower activity seen in this peptide as compared to native enterocin. Nonetheless, MIC of 20 μM still represents an interesting activity, similar to the reported MICs for eukaryotic peptides [26, 27]. Ongoing work at our lab is focused on designing 28-mer peptide derivatives with enhanced antimicrobial potency.

The use of peptides as anti-infective drugs was proposed as a novel approach since they can be readily synthesized and the costs of solid-phase approach would be rather inexpensive [28]. Moreover, enterocin CRL35 was already tested in a murine model, demonstrating a great potential for clinical purposes [29]. The 28-mer peptide or its derivatives would be even easier to manipulate and use as adjuvant to antibiotic therapy.

## CONCLUSION

In conclusion, a shorter peptide derived from enterocin CRL35 was found to be a bactericidal agent. This 28-mer fragment may serve as a template for designing highly active antibiotic peptides.

## ABBREVIATIONS

| | | |
|---|---|---|
| LAB | = | Lactic Acid Bacteria |
| TFA | = | Trifluoracetic acid |
| TSB | = | Trypticase Soy Broth |
| MIC | = | Minimum Inhibitory Concentration |
| FIC | = | Fractional Inhibitory Concentration |
| HEPES | = | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| DisC3[5] | = | 3,3′-dipropylthiadicarbocyanine iodide |
| Kd | = | Dissociation constant |
| $\Delta\psi$ | = | Transmembrane electrical potential |

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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