Enhancement of antibiotic activity by sub-lethal concentrations of enterocin CRL35

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Objective: The aim of this study was to evaluate the interaction of several conventional antibiotics with sub-lethal concentrations of enterocin CRL35, a cationic peptide, on Listeria innocua 7.

Methods: Susceptibility of L. innocua 7 cells to the combination of enterocin CRL35 and non-peptide antibiotics (cefalexin, ampicillin, ciprofloxacin, nalidixic acid, erythromycin, chloramphenicol, vancomycin and tetracycline) was assayed using the broth dilution method and killing curves. Fractional inhibitory concentration (FIC) index was calculated to assess synergy. The transmembrane electrical potential and pH gradient were determined by specific fluorescent probes.

Results: We found positive interactions between the cationic peptide and three conventional antibiotics (tetracycline, erythromycin and chloramphenicol) which are excluded from the cells by efflux pumps dependent on the membrane proton gradient. Furthermore, enterocin CRL35 even at sub-lethal concentrations induced the dissipation of both components of the proton motive force (Δp), i.e. transmembrane electrical potential and pH gradient and hence the alteration of processes dependent on it.

Conclusion: We hypothesize that enterocin CRL35 increases the effectiveness of these antibiotics by impairment of the bacterial active efflux systems and the consequent accumulation of these toxic compounds in the cytoplasm.

Keywords: antibiotic, cationic peptides, bacteriocins, multidrug efflux

Introduction

The emergence of antibiotic-resistant bacteria is of worldwide concern. Antibiotics have been indiscriminately used and this has contributed to the rise in antibiotic resistance in a wide range of bacteria, using a variety of resistance mechanisms. To extend the range of therapeutic options, non-peptide antibiotics could be used in combination with cationic peptides. It is well documented that some mixtures have synergic interactions, nevertheless the mechanisms of these positive interactions appear to be complex and are not fully understood.

Bacteriocins are bioactive peptides, and most are cationic at physiological pH. Although they have potential to inhibit some foodborne pathogens, continuing attempts are being made to find applications in veterinary medicine and medicine. Typical examples are nisin and lactacin 3147, which have proved to be effective agents in the treatments of mastitis. Additionally, nisin is currently being considered for the treatment of stomach ulcers caused by Helicobacter pylori. Enterocin CRL35 is a bacteriocin produced by Enterococcus mundtii CRL35, a strain isolated from regional Argentinean (Tafi) cheese. This peptide is highly active against bacteria from the genus Listeria. It displays a dual mode of action: at high concentrations, it produces localized holes in the cell wall and cellular membrane with leakage of macromolecules such as proteins into the external medium; at lower concentrations, it modifies the ion permeability of the cells, dissipating both components of the proton motive force. Enterocin CRL35 also has antiviral activity against herpes simplex virus (HSV) types 1 and 2 in Vero and BHK-21 cells at concentrations 15-fold lower than the cytotoxic concentration.

The aim of the current study was to examine the role played by proton gradient extrusion pumps in the synergy between certain antibiotics and sub-lethal concentrations of enterocin CRL35.

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Materials and methods

**Bacterial strains and media**

*Bacillus subtilis* CRL35, the producer strain, was grown at 30°C in Laptg broth without Tween 80 as described by Raibaud *et al.*

*L. innocua* 7, the susceptible strain, was grown in Trypticase Soy Broth at 30°C. *E. mundtii* CRL35 was obtained from the CERELA (Centro de Referencia para Lactobacilos, Tucuman, Argentina) stock collection and *L. innocua* 7 from the Unité de Recherches Laitières et Génétique Appliquée, INRA, Jouy-er-Josas, France.

**Enterocin CRL35 purification, viability, MIC and FIC assays**

The bacteriocin was purified from supernatants of the producing strain as described elsewhere. The procedure yielded a preparation that appeared homogeneous on analytical RP-HPLC and SDS-PAGE systems.

For viability determinations *L. innocua* 7 cells were grown at 30°C up to mid-log phase, harvested by centrifugation (10 min at 8000 g), and resuspended to approximately 2 × 10^7 cells/mL in 50 mM MOPS-K buffer pH 7.2 in the presence of different concentrations of enterocin CRL35. Aliquots were taken at appropriate times and the number of viable cells was determined.

**Cellular extrusion of ethidium bromide**

*L. innocua* 7 depleted in energy stores and suspended in 50 mM MOPS-K buffer pH 7.2 were incubated with ethidium bromide (5 μM final concentration). The entry of this compound was monitored by an increase in the fluorescence, setting the excitation and emission wavelengths at 500 and 580 nm, respectively. When the fluorescence reached a plateau, the efflux of ethidium bromide was activated by the addition of 25 mM glucose. Enterocin CRL35 was added at inhibitory or sub-lethal concentrations and the changes in the fluorescence were monitored with time. Nigericin (1 μM) was used to achieve complete inhibition of the efflux process.

**Preparation of inside-out cell membrane vesicles**

Inside-out membrane vesicles were obtained from *L. innocua* 7 as described by Kodama *et al.* Briefly, susceptible cells were grown to early exponential phase. Then the antibiotic to be tested was added and the culture was incubated with shaking for 3 h. Cells were harvested and washed twice with 10 mM HEPES-K buffer pH 8 containing 50 mM KCl and 5 mM MgSO_4_, suspended in the same buffer and passed through a French press cell at a pressure of 2400 kg/cm². After centrifugation at 10000 g for 15 min, cell debris and unbroken cells were discarded. Inverted membranes were collected by ultracentrifugation at 40 000 g for 1 h and suspended at 8 mg protein per mL in the same buffer supplemented with 10% glycerol. The vesicles were stored in aliquots at −70°C until used.

**Antipporter ATB/H⁺ activity**

The transport of antibiotics depending on proton motive force was measured by the method of quenching of quinacrine. Briefly, membrane vesicles (0.2 mg of protein) were suspended in 10 mM HEPES-K buffer pH 8, 50 mM KCl, 1 μM quinacrine hydrochloride. The pH gradient was generated by addition of 0.5 mM NADH. Antipporter activities were studied upon the addition of different antibiotics. NH_4Cl and nigericin were used as controls for complete dissipation of the proton gradient. Excitation and emission wavelengths were 424 and 500 nm, respectively.

**Other determinations**

Proteins were quantified by the method of Lowry *et al.* All the fluorescence measurements were carried out in an SLM (SLM Instruments)
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**Results**

**Effect of enterocin CRL35 concentrations on the cell viability and proton motive force**

As shown in Figure 1, enterocin CRL35 is strongly bactericidal. A concentration of 8 ng/mL induced a rapid decrease in the cellular viability of *L. innocua* 7. In fact, more than 99% of *Listeria* cells were killed after 10 min of incubation. Concentrations of enterocin CRL35 up to 4 ng/mL were designated as sub-lethal concentrations, although the viability of susceptible bacteria began to decrease slightly only after 30 min of incubation with the highest sub-lethal concentration tested.

Figure 2(a) shows the dissipation of the cell membrane electrical potential (ΔΨ) upon the addition of lethal and sub-lethal concentrations of enterocin CRL35. The inhibitory peptide concentration (8 ng/mL) induced an almost complete dissipation of ΔΨ. On the other hand, sub-inhibitory concentrations of enterocin CRL35 (4 ng/mL), despite their lack of bactericidal activity (at least until 30 min), induced an important electrical potential dissipation at a slower rate.

Figure 2(b) shows the modification of intracellular pH after the addition of enterocin CRL35. Once again, 8 ng/mL of the peptide induced a marked alteration of the cytoplasmic pH. Higher concentrations of the bacteriocin produced a more abrupt change in this parameter (not shown). Sub-lethal concentrations were effective in modifying the pH gradient as well, but at a slower rate.

**Synergic effect of antibiotics and sub-lethal concentration of enterocin CRL35**

The activity of different clinical antibiotics was studied in the presence of enterocin CRL35 at sub-lethal concentrations. The MICs of conventional antibiotics were determined alone and in the presence of 4 ng/mL enterocin CRL35 (Table 1). Combination studies showed positive interactions between the cationic peptide and chloramphenicol, erythromycin and tetracycline but not with ciprofloxacin, cefalexin, nalidixic acid, ampicillin or vancomycin. Control experiments with enterocin CRL35 alone (4 ng/mL) showed no cell growth inhibition indicating that this peptide concentration is sub-lethal in both systems: culture medium and buffer. An observed FIC index less than 0.5 for chloramphenicol, tetracycline and erythromycin confirmed the synergic effect of sub-lethal concentration of enterocin CRL35 with these antibiotics (Table 1). Killing curves with combinations of enterocin CRL35 at sub-lethal concentration and the clinical antibiotics that had showed synergic action were carried out, but no bactericidal activity was seen even at concentrations as high as four-fold the MIC of each antibiotic (data not shown).

**Antibiotic/H⁺ antiporter activity**

It is well documented that erythromycin, chloramphenicol and tetracycline are excluded by efflux pumps dependent on the membrane proton gradient. Further, a multidrug efflux pump has been described in *Listeria monocytogenes*, although its relation with antibiotic extrusion is unknown. Thus, antibiotic/H⁺ antiporter activities were screened in everted membrane vesicles obtained from *L. innocua* 7 cells. As shown in Figure 3, the addition of NADH induced a rapid decrease in quinacrine fluorescence as a consequence of the activation of proton pumping into the proteoliposome lumen and the pH gradient imposition. Once steady state was reached, the addition of different concentrations of erythromycin modified the fluorescence, confirming that the erythromycin/H⁺ activity was present in these bacterial membranes. Similar results were obtained with chloramphenicol and tetracycline (not shown). No significant effects could be demonstrated when cefalexin, ampicillin, ciprofloxacin or vancomycin were added. The controls used were nigericin and NH₄Cl; and both were equally effective in eliminating the proton gradient.

**Uptake of amino acids**

It is known that *Listeria* cells require leucine for growth. To investigate the uptake of this compound, a fluorescent method comprising the reaction of the non-incorporated amino acid with fluorescamine was carried out. As Figure 4 shows, the uptake of leucine was completely inhibited by 1 µM of either valinomycin or nigericin indicating that this transport is a proton motive force driven process. Enterocin CRL35 at inhibitory concentrations caused a total abolition of the leucine incorporation and sub-lethal concentrations of the bacteriocin partially inhibited the uptake: 4 ng/mL decreased by approximately 70% the incorporation of leucine, whereas the inhibition induced by 2 ng/mL was 47% (data not shown).

**Extrusion of ethidium bromide**

Ethidium efflux was measured indirectly by monitoring the fluorescence of the intracellular ethidium-poly nucleotide complex. A fluorescence increase represents the entry of this compound into the cell and a decrease represents an efflux of ethidium bromide from the cell. As shown in Figure 5, energization of the ethidium-incubated cells gave a decrease in the fluorescence. The addition of glucose triggered the active extrusion. The presence of enterocin CRL35 induced a clear inhibition of ethidium efflux, being a concentration dependent effect. The extrusion process was strongly impaired by inhibitory concentrations of bacteriocin, and sub-lethal concentrations, although less efficient, were still effective. The increase in ethidium fluorescence upon addition of enterocin CRL35, just as with nigericin, a selective proton gradient dissipator, is consistent

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**Figure 1.** Effect of enterocin CRL35 concentration on the viability of *L. innocua* 7 cells. Circles, 8 ng/mL enterocin CRL35; squares 4 ng/mL.; and triangles, 2 ng/mL.

**Figure 2(a).** Dissipation of the cell membrane potential (∆Ψ) upon the addition of lethal and sub-lethal concentrations of enterocin CRL35. Each assay was performed in duplicate.

**Figure 2(b).** Modification of intracellular pH after the addition of 2 ng/mL enterocin CRL35 concentrations. Each assay was performed in duplicate.

**Figure 3.** Effect of enterocin CRL35 concentration on the viability of *L. innocua* 7 cells. Circles, 8 ng/mL enterocin CRL35; squares 4 ng/mL.; and triangles, 2 ng/mL.

**Figure 4.** Effect of enterocin CRL35 on the uptake of leucine. Each assay was performed in duplicate.

**Figure 5.** Effect of enterocin CRL35 on the extrusion of ethidium bromide. Each assay was performed in duplicate.
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The effect of enterocin CRL35 on the growth rate of *L. innocua* was studied in the presence of ethidium bromide. As shown in Figure 6, neither the presence of 20 µM ethidium bromide nor 4 ng/mL enterocin CRL35 affected cell growth. However, when both agents were added simultaneously to the growth medium, a significant inhibitory effect was observed. The inhibition was more evident when the medium contained 20 µM ethidium bromide.

**Discussion**

Polycationic peptides provide a new structural class of highly active antimicrobial agents and offer a new resource for the development of novel antimicrobial agents. They are found in all living species and can have antiviral, antibacterial, antifungal or antiprotozoal activity *in vitro*. However, very few *in vivo* studies on cationic peptide action have been published. Despite this, it does seem that cationic peptides can show efficacy in animal models. Another approach would be the use of peptides with non-peptide antibiotics. Though there is little information in this area, some combinations have been reported to be successful. The mechanism of this synergic effect is not fully understood. Positive interaction of β-lactams with peptides might be the result of increased access of peptides to the cytoplasmic membrane following breakdown of the peptidoglycan. In other cases, the presence of peptides may permit the entrance of large antibiotics into the cells by permeabilizing the inner or outer bacterial membranes. The sequential inhibition of a given biosynthetic pathway has also been suggested.

Table 1. MICs of antibiotics in the presence or absence of enterocin CRL35

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg/L) without enterocin CRL35</th>
<th>MIC (mg/L) with 4 ng/mL enterocin CRL35</th>
<th>FIC index&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>20</td>
<td>15</td>
<td>0.9</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>50</td>
<td>50</td>
<td>1.53</td>
</tr>
<tr>
<td>Cefalexin</td>
<td>100</td>
<td>85</td>
<td>1.32</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>25</td>
<td>25</td>
<td>ND</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>5</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>3</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2</td>
<td>0.2</td>
<td>0.38</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5</td>
<td>1</td>
<td>0.4</td>
</tr>
</tbody>
</table>

ND, value not determined.
<sup>a</sup>FIC index <0.5 denotes synergy, ≥0.5 to <1.0, an additive effect and ≥1.0 to <4.0, indifference.

Figure 2. Dissipation of cell membrane electric potential (a), and pH gradient (b) induced by enterocin CRL35. Solid line, 8 ng/mL of enterocin CRL35 and dashed line, 4 ng/mL. The addition of nigericin (Nig) and valinomycin (Val) is denoted by arrows. The results are representatives of five independent experiments.
In the present work, we studied the effect of sub-lethal concentrations of enterocin CRL35, a cationic peptide, on the activity of antibiotics. At these concentrations, the peptide induces a significant membrane gradient dissipation without appreciable cell death. This result would be in apparent disagreement with our previous paper. A plausible explanation is that membrane depolarization is necessary but not sufficient to produce cell death, and another concentration-dependent step, not described at present may be implicated. It was recently described that pleurocidin and derivatives which are antimicrobial peptides from eukaryotic organisms lost their ability to damage cell membranes at sub-lethal concentrations, whilst maintaining their capacities to inhibit macromolecular synthesis. This is a different behaviour to enterocin CRL35, and not a general mechanism for antibiotic cationic peptides.

We found here that the bacteriostatic activity of three clinical antibiotics, erythromycin, chloramphenicol and tetracycline, was strongly increased in the presence of sub-lethal concentrations of enterocin CRL35. Additionally, we showed for the first time that these antibiotics are excluded from Listeria cells by active extrusion systems depending on the proton gradient. These results indicate that the mechanism by which the cationic peptide increases the effectiveness

Figure 3. Effect of erythromycin on the membrane proton gradient. Solid line, 1 \( \mu \text{M} \) nigericin; dashed line, 10 \( \mu \text{g/mL} \) erythromycin and dotted line, 3 \( \mu \text{g/mL} \) erythromycin. The results are representatives of three independent experiments.

Figure 4. Effect of enterocin CRL35 on the leucine uptake by L. innocua 7 cells. Enterocin CRL35 was added at the following concentrations: circles, no addition of peptide; triangles, 4 ng/mL and diamonds, 8 ng/mL. The uptake curves in the presence of 1 \( \mu \text{M} \) valinomycin or nigericin overlap with the latter concentration of bacteriocin tested.

Figure 5. Inhibition of the active ethidium bromide efflux by enterocin CRL35. Solid line, 8 ng/mL; dotted line, 4 ng/mL and dashed line, no addition of peptide. The curve obtained with 1 \( \mu \text{M} \) nigericin overlaps with the highest concentration of enterocin CRL35 tested.

Figure 6. Effect of ethidium bromide and sub-lethal concentration of enterocin CRL35 on the L. innocua 7 growth. Open circles, 20 \( \mu \text{M} \) ethidium bromide; filled circles, 4 ng/mL enterocin CRL35; squares, 10 \( \mu \text{M} \) ethidium bromide and 4 ng/mL enterocin CRL35; triangles, 20 \( \mu \text{M} \) ethidium bromide and 4 ng/mL enterocin CRL35.
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of these antibiotics is through dissipation of the proton gradient responsible for the extrusion of these compounds. This hypothesis is strongly supported by the observations that: (i) the activity of those antibiotics that are not affected by the proton gradient were not affected by enterocin CRL35; (ii) ethidium bromide and amino acids such as leucine which traverse the membrane using the proton motive force were affected by the peptide in a manner similar to certain antibiotics.

As far as we know, this is the first time this type of synergic mechanism has been described. The possibility that enterocin CRL35 at sub-lethal concentrations modifies membrane permeability and thus increases the entrance of antibiotics into cells may be dismissed since CRL35 did not act synergistically with ciprofloxacin or nalidixic acid. Further, as cefalexin, ampicillin and vancomycin act at cell wall level without intracellular targets, enterocin CRL35 does not enhance their activities at least in the range of concentrations tested.

In conclusion, enterocin CRL35 could be used as an adjuvant to antibiotic therapy. At high concentrations, the peptide is a bactericidal and antiviral agent, therefore enterocin CRL35 may have a promising future in clinical applications. Further studies including toxicity, stability and immunogenicity tests will be necessary in order to determine the viability of this bacteriocin as an antibiotic compound with clinical use. A few cationic peptide antibiotics have already entered clinical trials, some of them with relative success, and cationic peptides have excellent potential as a novel antibiotic class.

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References

