Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Combined effect of synthetic enterocin CRL35 with cell wall, membrane-acting antibiotics and muranolytic enzymes against *Listeria* cells

E. Salvucci, E.M. Hebert, F. Sesma and L. Saavedra

Centro de Referencia para Lactobacilos (CERELA-CONICET), San Miguel de Tucumán, Tucumán, Argentina

Keywords
antibiotic, bacteriocin, *Listeria*, monensin, mutanolysin.

Correspondence
Lucila Saavedra, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, San Miguel de Tucumán, Tucumán, Argentina, T4000ILC.
E-mail: lucila@cerela.org.ar

2010/1789: received 13 October 2009, revised 27 May 2010 and accepted 27 May 2010

doi:10.1111/j.1472-765X.2010.02879.x

Abstract

Aims: To evaluate the inhibition effectiveness of enterocin CRL35 in combination with cell wall, membrane-acting antibiotics and muranolytic enzymes against the foodborne pathogen *Listeria*.
Methods and Results: Synthetic enterocin CRL35 alone and in combination with monensin, bacitracin, gramicidin, mutanolysin and lysozyme were used in this study. Minimal inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) index assays were performed using *Listeria innocua* 7 and *Listeria monocytogenes* FBUNT as sensitive strains. Antibiotics showed positive interactions with the bacteriocin in both strains tested. On the other hand, when mutanolysin and enterocin CRL35 were added to resting cells in a buffer system, the lytic effect of mutanolysin was enhanced. However, the addition of mutanolysin showed no effect on the growth of *L. innocua* 7 cells in a culture medium. Moreover, mutanolysin allowed the overgrowth of *L. innocua* 7 cells to an OD similar to control cells in the presence of inhibitory concentration of enterocin CRL35. In contrast, the combination of lysozyme and enterocin CRL35 resulted in a 50% inhibition of the *L. innocua* 7 growth.
Conclusions: Based on our results, we conclude that the combination of synthetic enterocin CRL35 with some antibiotics is effective against *L. innocua* 7 and *L. monocytogenes* FBUNT cells, and more importantly the amount of these agents to be used was considerably reduced. The effectiveness of the combination of synthetic enterocin CRL35 with muramidases seems to depend on complex environments, and more detailed studies need to be performed to elucidate this issue.
Significance and Impact of the Study: Enterocin CRL35 represents a promising agent that not only can ensure the quality and safety of food but it can also be combined with several antimicrobial agents important in the medical field.

Introduction

Disease-causing micro-organisms that have become resistant to drug therapy are an increasing public health problem. 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 (Boucher *et al.* 2009; Savjani *et al.* 2009). Bacteriocins are cationic bioactive peptides that have attracted attention as potential substitutes or to be used in combination with antibiotics. The majority of lactic acid bacteria (LAB) produce bacteriocins that permeabilize the membrane and deplete the proton motive force (PMF) of sensitive cells or induce lysis-activating autolysins (Cotter *et al.* 2005). LAB bacteriocins have been successfully used to inhibit some food borne pathogens, but only nisin, a bacteriocin produced by certain strains of *Lactococcus lactis*, is approved by the Food and Drug Administration (FDA-USA) to be used as a preservative in food (Delves-Broughton 2005). Beyond this field, continuing attempts

© 2010 The Authors

are being made to find applications in veterinary science and medicine (Twomey *et al.* 2000).

Enterocin CRL35 is a well-characterized bacteriocin from subclass IIa that presents antilisterial and antiviral activity (Saavedra *et al.* 2004).

Previous studies demonstrated that the bacteriostatic activity of some clinical antibiotics was strongly increased in the presence of sublethal concentrations of enterocin CRL35 (Minahk *et al.* 2004).

In this work, we evaluated the effect of synthetic enterocin CRL35 on the antimicrobial activity of cell wall and membrane-acting antibiotics and muranolytic enzymes using our bacterial established model of *Listeria* cells.

Materials and methods

Sensitive strains and media

Listeria innocua 7 (Unitè de Recherches Laitières et Gènètique Apliqueè, INRA, Jouy-en-Josas, France) and *Listeria monocytogenes* FBUNT (Castellano *et al.* 2001), used as sensitive strains, were grown in Brain Heart Infusion (BHI) without aeration at 30 and 37°C, respectively (Saavedra *et al.* 2004).

Bacteriocin, antibiotics and muranolytic enzymes

Enterocin CRL35 was chemically synthesized (Bio-Synthesis, Lewisville, TX, USA), based on the amino acid sequence of the mature bacteriocin (Saavedra *et al.*2004). The purity of the synthetic peptide (>80%) was verified by mass spectrometry (matrix-assisted laser desorption ionization-time of flight) and high-performance liquid chromatography. Bacitracin, mutanolysin (Sigma-Aldrich, Bueuos Aires, Argentina) and lysozyme (USB Corporation) stock solutions were diluted in water. Stock solutions of gramicidin and monensin (Sigma-Aldrich) were prepared in 90% ethanol.

Minimal Inhibitory Concentration (MIC) and Fractional Inhibitory Concentration (FIC) assays

MIC of synthetic enterocin CRL35 and all antibiotics was determined by broth microdilution method against *L. innocua* 7 and *L. monocytogenes* FBUNT (Salvucci *et al.* 2007). Ranges of concentrations of the antimicrobials tested for *L. innocua* 7 were as follows: enterocin CRL35: 0·2–1·4 ng ml^{-1}, bacitracin 0·6–100 μg ml^{-1}, monensin 0·01–10 μg ml^{-1} and gramicidin 0·01–10 μg ml^{-1}. In the case of *L. monocytogenes* FBUNT, the ranges of concentrations were as follows: enterocin CRL35: 0·025–3·2-ng ml^{-1}, bacitracin 0·5–64 μg ml^{-1}, monensin 0·25–32 μg ml^{-1} and gramicidin 0·015–32 μg ml^{-1}. MIC was performed in duplicates with a 96-well microtitre plate. Positive growth controls were included in wells lacking the antimicrobial compounds tested. MIC was defined as the lowest dilution of antibiotic that inhibited growth of the indicator strain by 50% after 12 h of incubation respect to a control without antimicrobial. FIC index was determined by combining fixed concentrations of one antimicrobial with increasing concentrations of second antimicrobial tested and *vice versa*. The growth of the sensitive cells was followed by measuring the OD at 560 nm (OD_{560}) in 96-well microplate reader (Versamax; Molecular Devices, Sunnyvale, CA). The index was calculated as follows: FIC = (MIC bacteriocin in combination)/(MIC bacteriocin alone) + (MIC antibiotic in combination)/(MIC antibiotic alone). FIC values lower than 0·5 indicate synergism; 0·5–1: addition; 1–: indifference; and above 4: antagonism.

Mutanolysin lysis assay

Listeria innocua 7 cells in mid-exponential growth phase were harvested by centrifugation at 7000 ***g*** for 10 min, washed in 0·05 mol l^{-1} phosphate buffer (pH 7) and resuspended containing 0·012 mol l^{-1} glucose (control cells), 0·2 ng ml^{-1} enterocin CRL35, 150 U ml^{-1} mutanolysin or 0·2 ng ml^{-1} enterocin CRL35 plus 150 U ml^{-1} mutanolysin. The OD_{560} of the cell suspensions was monitored using a 96-well microplate reader (Versamax, Molecular Devices) for 4 h at 37°C.

Results

The objective of this work was to evaluate the effect of synthetic enterocin CRL35 on the antimicrobial activity of classic cell wall or membrane-acting antibiotics (bacitracin, monensin and gramicidin) and muramidases (mutanolysin and lysozyme) using our bacterial established model of *L. innocua* 7 cells. In previous studies, we have shown that synthetic enterocin CRL35 presents a MIC of 1 ng ml^{-1} against *L. innocua* 7 (Salvucci *et al.* 2007). As shown in Table 1, combinatorial studies showed positive interactions between the bacteriocin and the antibiotics tested against *L. innocua 7*. The concentration of enterocin CRL35 that decreases to 50% the growth of sensitive cells was reduced to 0·6 ng ml^{-1} in the presence of bacitracin, monensin or gramicidin. This result represents a reduction of 40% of the bacteriocin MIC. Bacitracin showed the strongest antilisterial activity being four hundred times more active when combined with enterocin CRL35. MIC values for bacitracin alone and in combination were 50 and 0·12 μg ml^{-1}, respectively. Although monensin and gramicidin showed an enhanced effect when they were combined with enterocin CRL35, the

© 2010 The Authors

Table 1 MIC of enterocin CRL35 and antimicrobials against *Listeria* cells

	Listeria innocua 7			*Listeria monocytogenes* FBUNT		
Antimicrobials	MIC alone (ng ml^{-1})	MIC combined (ng ml^{-1})	FIC	MIC alone (ng ml^{-1})	MIC combined (ng ml^{-1})	FIC
Enterocin CRL 35	1	0·6		1·6	0·8	
Bacitracin	50000	125	0·60	32000	8000	0·75
Monensin	500	125	0·85	1000	250	0·75
Gramicidin	250	50	0·80	500	125	0·75

FIC index <0·5 denotes synergy, ≥0·5 to <1·0, an additive effect, ≥1·0 to <4·0, indifference and ≥4·0 antagonism. The results correspond to the average of two independent experiments.
MIC, Minimal inhibitory concentration; FIC, fractional inhibitory concentration index.

result was not as strong as the observed for bacitracin. Monensin and gramicidin added together with enterocin CRL35 were four and five times more active. MIC values, alone and in combination, were of 0·5–0·125 and 0·25–0·05 μg ml^{-1} for monensin and gramicidin, respectively. FIC indexes for all antibiotics showed an additive effect (Table 1). To evaluate whether this effect was also evident against pathogenic strains, the same set of experiments using *L. monocytogenes* FBUNT were performed (Table 1). In this case, the MIC of enterocin CRL35 alone resulted in 1·6 ng ml^{-1} that was reduced to 0·8 ng ml^{-1} when the peptide was combined with all the antibiotics. On the other hand, MIC values for monensin, bacitracin and gramicidin were 1, 32 and 0·5 μg ml^{-1}, respectively. These values were also reduced to 0·5, 8 and 0·12 μg ml^{-1} in combination with 0·8 μg ml^{-1} of bacteriocin, respectively.

Mutanolysin M1 is an extracellular enzyme, a 1,4-*N*, 6-O-diacetylmuramidase. This enzyme hydrolyses glycosidic bonds, mainly in the peptidoglycan structure. The addition of synthetic enterocin CRL35 enhanced the lytic effect of mutanolysin in a buffer system (Fig. 1). Enterocin CRL35 at levels of 2 ng ml^{-1} in combination with 150 U ml^{-1} of mutanolysin decreased the OD_{560} of sensitive strain approximately 0·2 units after 4 h of incubation (Fig. 1). However, mutanolysin did not show any effect on the sensitive strain when it was tested in culture medium. Moreover, *Listeria* cells grow at a similar growth rate than control cells, reaching the same final OD even in the presence of high concentrations of mutanolysin (1170 U ml^{-1}). These levels of mutanolysin also prevent the inhibitory action of 2 ng ml^{-1} of enterocin CRL35 (Fig. 2a).

To exclude a potential inhibition by a component of the culture media (BHI), lysozyme, another muramidase, was tested. Although lysozyme did not inhibit completely the growth of *Listeria* cells, the combination of 0·5 ng ml^{-1} of enterocin CRL35 plus 1000 μg ml^{-1} of lysozyme reduced the growth of *Listeria* cells to a 50% respect to the control (Fig. 2b).

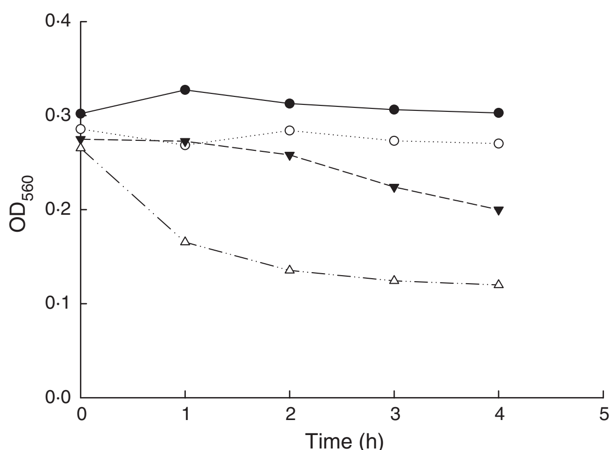

Figure 1 Effect of enterocin CRL35 alone and in combination with mutanolysin on the viability of *Listeria innocua* 7 cells. Cell viability (OD_{560}) was determined at the indicated times. Control cells (●), enterocin CRL35 2 ng ml^{-1} (○), mutanolysin 150 U ml^{-1} (▼) and enterocin CRL35 2 ng ml^{-1} plus mutanolysin 150 U ml^{-1} (△).The results correspond to the media of two independent experiments.

Discussion

Enterocin CRL35 is an antilisterial and antiviral class IIa bacteriocin (Wachsman *et al.* 1999; Saavedra *et al.* 2004). The purified peptide exhibits a dual mode of action, whereas 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 PMF (Minahk *et al.* 2000, 2004). This bacteriocin also presents a MIC of 8 ng ml^{-1} (Minahk *et al.* 2004) against *L. innocua* 7; however, the synthetic enterocin CRL35 showed eight times more antimicrobial activity (MIC of 1 ng ml^{-1}) against the same sensitive strain (Salvucci *et al.* 2007). Previous studies using the purified peptide demonstrated that at sublethal concentrations, enterocin

© 2010 The Authors

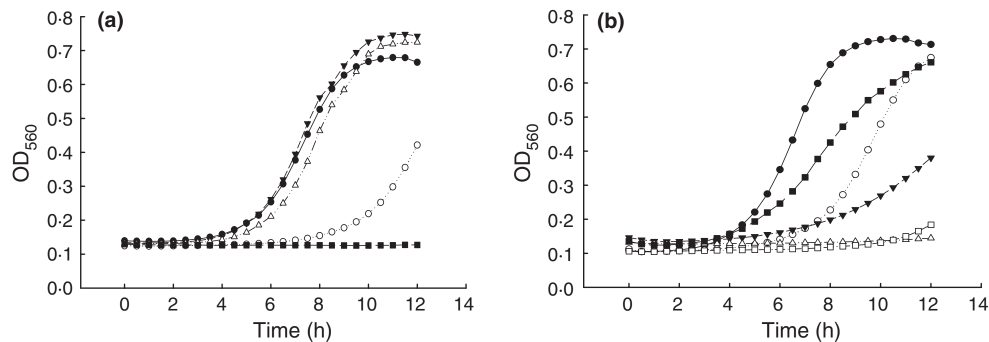

Figure 2 Effect of enterocin CRL35, mutanolysin and lysozyme on the growth of *Listeria innocua* 7. (a) Growth curves with enterocin CRL35 2 ng ml^{-1} (■), enterocin CRL35 1 ng ml^{-1} (○), mutanolysin 1170 U ml^{-1} (▼), mutanolysin 1170 U ml^{-1} + enterocin CRL35 2 ng ml^{-1} (△) and control without antimicrobials (●). (b) Growth curves with enterocin CRL35 0·5 ng ml^{-1} (○), enterocin CRL35 1 ng ml^{-1} (□), lysozyme 1000 μg ml^{-1} (■), enterocin CRL35 0·5 ng ml^{-1} + lysozyme 1000 μg ml^{-1} (▼), enterocin CRL35 1 ng ml^{-1} + lysozyme 1000 μg ml^{-1} (△) and control without antimicrobials (●).The results correspond to the media of two independent experiments.

CRL35 presented a strong synergic effect with tetracycline, erythromycin and chloramphenicol, while in the presence of cell wall-acting antibiotics such as ampicillin and vancomycin no modification of MIC was observed (Minahk *et al.* 2004). However, if an antibiotic produces the breaking down of the peptidoglycan, cationic peptides such as bacteriocins might access more easily to the plasma membrane. Based on this hypothesis, we tested the widely used polypeptide bacitracin in combination with synthetic enterocin CRL35. We found that this combination was very effective, and the amount of antibiotic to be used was considerably reduced, as noted in MIC decreased. A possible explanation would be the dissipation of PMF caused by enterocin CRL35, which in turn hinders the function of the bacitracin-efflux transporters (Gauntlett *et al.* 2008).

Regarding the membrane-acting antibiotics, up to now only monensin was tested in combination with nisin as a way to improve the food intake in ruminants (Callaway *et al.* 1997). Because monensin is an ionophore that binds unspecifically to membrane (Duffield and Bagg 2000), we would expect a synergic effect with enterocin CRL35. However, the results showed only an additive effect possibly because of the lack of interaction between both antimicrobials.

We found that the combination of enterocin CRL35 with all antibiotics tested was also effective against the pathogenic strain, *L. monocytogenes* FBUNT. In addition, FIC indexes for each antibiotic alone (calculated as MIC antibiotic combine/MIC antibiotic alone) revealed that the presence of enterocin CRL35 produces an enhancement of their antimicrobial activity.

On the other hand, we demonstrate that a tolerance to mutanolysin confers protection against enterocin CRL35 in culture media. A possible explanation for this result might be attributed to a drug capture resistance mechanism, also described for certain strains of *Staphylococcus aureus* resistant to vancomycin. In these cells, cell wall turnover came to a halt, and the retention of cell wall material at the outer surface of the bacterium entraps the antibiotic at the periphery of the cells. This prevents the access of the drug molecules to the cell wall synthesis sites close to the plasma membrane (Sieradzki and Tomasz 2000). Another explanation may be a mechanism involving esteric blockages for enterocin CRL35 as a result of the ability of mutanolysin to link teichuronic, teichoic and lipoteichoic acids in certain ionic conditions (Valyasevi *et al.* 1991).

Because mutanolysin has a restricted hydrolytic specificity, we exclude the possibility that glycoproteins, membrane-bound lipoteichoic acids or accessory wall polysaccharides (Valyasevi *et al.* 1991) and the bacteriocin receptor manose phosphotransferase system (man-PTS), involved in the mode of action of bacteriocins subclass IIa (Diep *et al.* 2007), were hydrolysed. Further studies are being carried out to elucidate the exact mechanisms involved in this phenomenon. On the other hand, the fact that enterocin CRL35 enhances the effect of mutanolysin in buffer system could represent the increased rate of spontaneous autolysis (Mora *et al.* 2003).

The results of this work demonstrate the benefits of using mixtures of antimicrobials with synthetic enterocin CRL35 against *Listeria* cells over the use of individual agents. The combination of several antimicrobials might be an interesting strategy because the doses, the secondary effects and the resistance in some strains might be overcome. Enterocin CRL35 has demonstrated to be effective in combination with several antimicrobials, which constitutes the initial step in the research for future developments between biotechnology and medical fields.

© 2010 The Authors

Acknowledgements

This work was supported by grants from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) and ANPCyT (Agencia Nacional de Promoción Científica) PICT 0813.

References

Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E. Jr, Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B. *et al.* (2009) Bad bugs, no drugs: no ESKAPE! an update from the infectious diseases society of America. *Clin Infect Dis* **48**, 1–12.

Callaway, T.R., Carneiro De Melo, A.M. and Russell, J.B. (1997) The effect of nisin and monensin on ruminal fermentations *In vitro*. *Curr Microbiol* **35**, 90–96.

Castellano, P., Farías, M.E., Holzapfel, W. and Vignolo, G. (2001) Sensitivity variations of *Listeria* strains to the bacteriocins, lactocin 705, enterocin CRL35 and nisin. *Biotechnol Lett* **23**, 605–608.

Cotter, P.D., Hill, C. and Ross, P. (2005) Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* **3**, 777–778.

Delves-Broughton, J. (2005) Nisin as a food preservative. *Food Aust* **57**, 525–527.

Diep, D.B., Skaugen, M., Salehian, Z., Holo, H. and Nes, I.F. (2007) Common mechanisms of target cell recognition and immunity for class II bacteriocins. *PNAS* **104**, 2384–2389.

Duffield, T.F. and Bagg, R.N. (2000) Use of ionophores in lactating dairy cattle: a review. *Can Vet J* **41**, 388–394.

Gauntlett, J.C., Gebhard, S., Keis, S., Manson, J.M., Pos, K.M. and Cook, G.M. (2008) Molecular analysis of BcrR, a membrane-bound bacitracin sensor and DNA-binding protein from *Enterococcus faecalis*. *J Biol Chem* **283**, 8591–8600.

Minahk, C.J., Farías, M.E., Sesma, F. and Morero, R.D. (2000) Effect of enterocin CRL35 on *Listeria monocytogenes* cell membrane. *FEMS Microbiol Lett* **192**, 79–83.

Minahk, C.J., Dupuy, F. and Morero, R.D. (2004) Enhancement of antibiotic activity by sub-lethal concentrations of enterocin CRL35. *J Antimicrob Chemother* **53**, 240–246.

Mora, D., Musacchio, F., Fortina, M.G., Senini, L. and Manachini, P.L. (2003) Autolytic activity and pediocin induced lysis in *Pediococcus acidilactici* and *Pediococcus pentosaceus* strains. *J Appl Microbiol* **94**, 561–570.

Saavedra, L., Minahk, C., Ruiz Holgado, A.P. and Sesma, F. (2004) Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH_2-terminal sequence. *Antimicrob Agents Chemother* **48**, 2778–2781.

Salvucci, E., Saavedra, L. and Sesma, F. (2007) Short peptides derived from the NH_2-terminus of subclass IIa bacteriocin enterocin CRL35 show antimicrobial activity. *J Antimicrob Chemother* **59**, 1102–1108.

Savjani, J.K., Gajjar, A.K. and Savjani, K.T. (2009) Mechanisms of resistance: useful tool to design antibacterial agents for drug – resistant bacteria. *Mini Rev Med Chem* **9**, 194–205.

Sieradzki, K. and Tomasz, A. (2000) Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*. *J Bacteriol* **179**, 2557–2566.

Twomey, D., Wheelock, A., Flynn, J., Meaney, W., Hill, C. and Ross, P. (2000) Protection against *Staphylococcus aureus* mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin, lacticin 3147. *J Dairy Sci* **83**, 1981–1988.

Valyasevi, R., Sandine, W.E. and Geller, B.L. (1991) A membrane protein is required for bacteriophage c2 infection of *Lactococcus lactis* subsp. *lactis* C2. *J Bacteriol* **173**, 6095–6100.

Wachsman, M.B., Farías, M.E., Takeda, E., Sesma, F., de Ruiz Holgado, A.P., de Torres, R.A. and Coto, C.E. (1999) Antiviral activity of enterocin CRL35 against herpesviruses. *Int J Antimicrob Agents* **12**, 293–299.

© 2010 The Authors