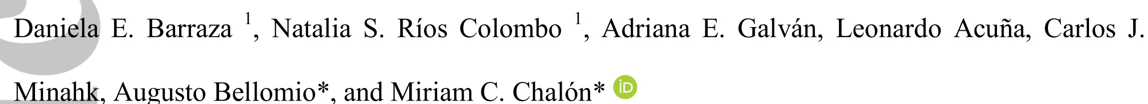
New insights into enterocin CRL35; mechanism of action and immunity revealed by heterologous expression in *Escherichia coli*



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SUMMARY

The role of the class IIa bacteriocin membrane receptor protein remains unclear and two different mechanisms have been proposed: the bacteriocin could interact with the receptor changing it to an open conformation or the receptor might act as an anchor allowing subsequent bacteriocin insertion and membrane disruption. Bacteriocin-producing cells synthesize an immunity protein that forms an inactive bacteriocin-receptor-immunity complex. To better understand the molecular mechanism of enterocin CRL35, the peptide was expressed as the suicidal probe EtpM-enterocin CRL35 in *Escherichia coli*, a naturally insensitive microorganism since it does not express the receptor. When the bacteriocin is anchored to the periplasmic face of the plasma membrane through the bitopic membrane protein, EtpM, *E. coli* cells depolarize and die. Moreover, co-expression of the immunity protein prevents the deleterious effect of EtpM-enterocin CRL35. The binding and anchoring of the bacteriocin to the membrane has demonstrated to be a sufficient condition for its membrane insertion. The final step of membrane disruption by EtpM-enterocin CRL35 is independent from the receptor, which means that the mannose PTS might not be involved in the pore structure. In addition, the immunity protein can protect even in the absence of the receptor.

KEYWORDS: BACTERIOCIN, ENTEROCIN CRL35, SUICIDE PROBE, ETPM

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INTRODUCTION

Class IIa bacteriocins, known as pediocin-like bacteriocins, are linear polypeptides with potent antilisterial activity, capable of killing target bacteria at concentrations in the sub-nanomolar range (Drider *et al.*, 2006). Since they are produced by lactic acid bacteria (LAB), these peptides have potential as antimicrobial agents in food. The successful use of pediocin PA-1 as biopreservative in the food industry has fostered the research on the mechanism of action of bacteriocins belonging to class IIa (Rodríguez *et al.*, 2002). In particular, enterocin CRL35 is produced by *Enterococcus mundtii* CRL35 (Farías *et al.*, 1996; Saavedra *et al.*, 2004) and displays a potent antilisterial activity. It seems to be clear at present that class IIa bacteriocins are essentially membrane-active peptides (Bruno and Montville, 1993). In fact, these peptides induce the leakage of potassium and phosphate ions as well as amino acids and other essential molecules. As a consequence, both components of the proton-motive force, $\Delta\psi$ and ΔpH, are dissipated and intracellular ATP is thus depleted (Bhunia *et al.*, 1991; Chikindas *et al.*, 1993). At first, class IIa bacteriocins were believed to interact through their positively charged amino acids with anionic phospholipids of the target bacterial cell. Then, an amphipathic portion of the bacteriocin would be inserted disturbing the membrane (Drider *et al.*, 2006). All pediocin-like bacteriocins have a very narrow spectrum of action, which is restricted to bacterial genera phylogenetically related to the bacteriocin producer strain. In 2001, Dalet et al. described that sensitivity to class IIa bacteriocins from LAB was associated with the mannose phosphotransferase system (man-PTS) expression in *Listeria monocytogenes* (Dalet *et al.*, 2001). Thereupon, the man-PTS complex was confirmed as the membrane specific receptor for many class II bacteriocins (Ramnath *et al.*, 2004). A phylogenetic analysis of 86 members of the man-PTSs family from a wide range of bacterial genera, groups the man-PTSs into three main clusters (groups I, II and III). Only members from group I could serve as receptors for class IIa bacteriocins, and the receptor efficiency varies in a pattern directly related to their phylogenetic positions (Kjos *et al.*, 2009). For that particular reason and because they are unable to cross the outer membrane, bacteriocins do not act on Gram-negative bacteria (Chalón et al., 2012). It is currently unclear how these bacteriocins permeabilize the membranes of sensitive cells and what the exact role of the receptor is in this process. Two possible mechanisms were proposed (Cotter *et al.*, 2005; Kjos *et al.*, 2011): i) the

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bacteriocin could induce a conformational change in the receptor that would lead to the opening of an intrinsic pore; or ii) the bacteriocin could employ the receptor as an anchor to bring the peptide closer to plasma membrane, allowing subsequent bacteriocin oligomerization and membrane insertion. The amphipathic C- terminal would insert into the membrane, leading to the pore formation.

An immunity protein encoded by the genetic systems of bacteriocins protects the producer strain against its own bacteriocin. There is a variation in the degree of sequence identity among the pediocin-like immunity proteins (Fimland *et al.*, 2002). While the N- terminal domain of immunity proteins is more conserved, significant differences are observed in the C-terminal region, containing several charged residues that are crucial for the specific recognition of cognate bacteriocins (Johnsen *et al.*, 2005). A decade ago, it was suggested that the immunity protein formed a strong ternary complex with the receptor protein and cognate bacteriocin, thereby preventing cells from being killed (Diep *et al.*, 2007). Experimental evidence suggests that the immunity protein-man-PTS complex only occurs in the presence of the extracellular bacteriocin. Thus, the immunity protein would not act by preventing the binding of the bacteriocin to its receptor, but rather by direct interaction with the man-PTS-bacteriocin complex. Therefore, it might block the process that leads to membrane permeabilization and cell death. These observations strongly support the first mechanism commented above, and became the generally accepted paradigm of the mechanism of action and immunity of class IIa bacteriocins. However, neither the stoichiometry of the ternary complex nor the way in which the proteins interact with each other is known.

The design of a suicide genetic construct, which expresses a chimeric protein EtpM-microcin V has previously been reported (Gérard et al., 2004). EtpM is a bitopic membrane protein belonging to the Type II secretion system (SstII) from enterohemorrhagic *Escherichia coli* serotype O157:H7 (Lim *et al.*, 2007), and microcin V (MccV, or colicin V) is a linear non-modified class IIa microcin secreted by *E. coli* (Gratia, 1925). MccV alters the permeability of the membrane requiring the membrane protein SdaC as a receptor (Yang and Konisky, 1984; Rodríguez and Laviña, 2003; Gérard *et al.*, 2005; Biéler *et al.*, 2010). The 5' of the MccV structural gene *cvaC*, was fused with the 3' of truncated *etpM* under the control of the P_{BAD} promoter. When the expression of *etpM-cvaC* chimeric gene is

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induced with arabinose, MccV is translocated to the periplasm but remains anchored in the inner membrane through EtpM. The bacteria expressing the chimeric gene die; hence the fusion protein is called "suicide probe". Interestingly, the co-expression of the immunity protein of MccV completely protects the host cell from the lethal effect of the suicide probe EtpM-MccV (Gerard *et al.*, 2004; Gérard *et al.*, 2005).

A complete understanding of the mechanism of membrane insertion of bacteriocins and microcins is crucial for the design and construction of new bacteriocins improved by genetic engineering (Acuña *et al.*, 2012). In this regard, several questions arise such as: can a class IIa bacteriocin from Gram-positive bacteria be active in Gram-negative bacteria? Is the presence of the membrane receptor in target cells the unique requirement for the bacteriocin mechanism of action? To answer these questions, we built a chimeric peptide (EtpM-Ent35) by fusing *etpM* with the structural gene of the class IIa bacteriocin, enterocin CRL35. Our results suggest that enterocin CRL35 fused with EtpM can form pores in the absence of the man-PTS complex in *E. coli* membrane. Therefore, the receptor would work just as an anchor on the target cell membrane. Furthermore we show that the immunity protein of enterocin CRL35 co-expressed with EtpM-Ent35 in *E. coli* is able to counteract the bactericidal effect of the suicide probe, even in the absence of its specific receptor.

EXPERIMENTAL PROCEDURES

Bacterial culture media and growth conditions- Bacteria and plasmids used are listed in Table 1. Primer sequences are listed in Table 2. Luria broth (LB) and TSB were purchased from Sigma Chemical Co (St. Louis, MO) and Britania, respectively. *Enterococcus mundtii* CRL35 was grown in LAPTg medium (g. L^{-1}: 15, peptone; 10, tryptone; 10, glucose; 10, yeast extract; 1, Tween 80). Solid media were prepared by adding agar to a final concentration of 1.5%. When required, 50 μg mL^{-1} ampicillin, 30 μg mL^{-1} chloramphenicol, glucose 0.6%, lactose 0.2% and arabinose 0.2% or 0.6% were added.

Genetic constructions, cloning and cell transformation- The plasmid p1707 was a gift from Dr. Emilse Masias (INSIBIO, CONICET-UNT). It encodes for enterocin CRL35 structural gene *munA,* which is preceded by a twin arginine translocation (Tat) signal that leads the peptide to the periplasm,

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under the tight control of P_{BAD} promoter. The resulting peptide was called RR-Ent35 and its signal sequence is supposed to be removed by a membrane associated proteolytic enzyme after translocation to the periplasmic side (Palmer and Berks, 2012).

To obtain the suicide probe EtpM-Ent35, we replaced the sequence that encodes the Tat signal in p1707 with a truncated *etpM* gene (*etpM49*) (Gerard *et al.*, 2004), obtaining *etpM49-munA* under the control of P_{BAD}. The *etpM49* fraction was amplified from the strain *E. coli* O157:H7 by colony PCR, using primers etpmcor and etpmnh1 as forward and reverse, respectively (Gerard *et al.*, 2004). The amplified fragment was purified, digested with *Eco*RI and *Nhe*I. *E. coli* DH5α was transformed with the ligation mixture containing DNA ligase (New England Biolabs®), and both digested p1707 and *etpM49.* Transformed cells were plated onto LB ampicillin agar added with 0.6% glucose. Yielded colonies were streaked on LB plates containing arabinose 0.6% or glucose 0.6%. If the suicidal probe was correctly expressed, the cell would die in presence of arabinose. Hence, one colony which was unable to grow in the presence of arabinose was selected from the glucose supplemented plate to purify the plasmid named p-etpM-munA. The resulting construction codifies a chimeric protein $EtpM_{1-49}$-enterocin CRL35 (EtpM-Ent35) where $EtpM_{1-49}$ is a truncated version of EtpM but still contains the transmembrane segment.

In order to express the control protein EtpM, the complete *etpM* gene was amplified; the PCR product was digested with *Eco*RI and *Hin*dIII and then was ligated into p8760 vector (Guzman *et al.*, 1995). The resulting plasmid was called p-etpM.

The immunity gen, *munC,* was amplified from pE35 (Saavedra *et al.*, 2004) by PCR using the following primers: munCrXhoI and munCfNdeI. The amplified fragment were digested by *Xho*I and *Nde*I and cloned into the plasmid pACYCDuet-1 (Novagen). Therefore, immunity protein from enterocin CRL35 were under control of *lac*-promoter.

All the genetic constructions were confirmed by DNA sequencing (CERELA, CCT-Tucumán). *E. coli* BL21 strain (Novagen) was transformed with the plasmids described above by the classical calcium chloride transformation protocol(Sambrook and Russell, 2001).

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Antimicrobial activity of EtpM-Ent35- All strains described in the present work were grown in LB -glucose 0.6% at 37°C until exponential phase (OD_{600} ~ 0.6). Cells were collected by centrifugation, washed and resuspended in LB. The expression of the immunity protein was induced with lactose 0.02% for 1 hour and the fusion EtpM-Ent35 with arabinose 0.6 % for 5 minutes. Cells were centrifuged and resuspended in 20 mM Tris-HCl, 0.2% Triton X-100 pH 7.4. Then, cells were sonicated in an ice bath for 10 min. 10 µl of each cellular extract was spotted onto TSB or LAPTg agar plates. After the drops had dried, the plates were layered with 4 ml of 0.6% agar containing 10^7 cells of *L. innocua* 7. The plates were incubated overnight at 30 °C and examined for growth inhibition. When the antimicrobial activity was assayed against *E. coli* BL21, LB plates were employed and cells were grown at 37ºC overnight.

Subcellular fractionation. The bacterial strains were grown until OD_{600} = 0.6. At that point they were induced with 0.6% arabinose for 10 minutes at 37°C and the cultures were divided in two. Each half was centrifuged at 7600 x *g* for 2 minutes, the supernatant was saved and the pellet was resuspended in 100 mM Tris-HCl, 5 mM EDTA, pH 7.5. EDTA acts as an OM perturbing agent (Gray and Wilkinson, 1965). One of the samples was supplemented with protease inhibitor cocktail (Sigma ®) + PMSF 1 mM (Sigma ®) and the other with proteinase K (10 µg/ml). Then, cells were centrifuged and the supernatants were heated 10 minutes at 65ºC to inactivate proteinase K. This supernatant was separated as periplasm fraction. The pellets were washed, resuspended in 100 mM Tris-HCl pH 7.5 buffer and then cells were disrupted by sonication. After a centrifugation at 50,000 x *g* for 1 hour, the cytoplasm was removed and finally the membrane fraction was resuspended in 100 mM Tris-HCl pH 7.5 buffer with 0.2% Triton. Finally, the different subcellular fractions of each treatment were spotted on TSB agar plate covered with a *L. innocua* 7 lawn.

Suicide probe and immunity protein expression- In order to study the effect of the suicide probe expression in *E. coli* cells, we carried out different experiments. In the first one, a semi quantitative approach, control cells *E. coli* BL21 (p-etpM) as well as *E. coli* BL21 (p-etpM-munA) and *E. coli* BL21 (p-etpM-munA) (pmunC) cells were grown in LB-glucose 0.6% to an OD_{600} ~ 0.6. The cells were then collected by centrifugation and diluted in LB-glucose 0.6% (repressed) or LB-lactose

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0.02% (immunity induced). 10 µl of repressed cell suspensions were plated on LB agar supplemented with glucose 0.6% or L-arabinose 0.6%. In the same way, cells with pre-induced immunity expression were plated on LB agar supplemented with lactose 0.02% or lactose 0.02% + L-arabinose 0.6%. The plates were incubated overnight at 37 °C.

For the quantitative approach, all strains were incubated with shaking at 37 °C in LB-glucose 0.6% until OD_{600} ~ 0.3. They were washed and resuspended in LB with lactose 0.02% for 1 hour to induce the expression of MunC. The expression of the fusion EtpM-Ent35 was induced upon addition of arabinose 0.6% during 5 minutes. After that, cells were washed, and resuspended in 20 mM Tris-HCl buffer pH 7.4. Aliquots were log-diluted and plated onto LB medium and colony forming units (CFU) were counted after an overnight incubation at 37 °C.

A bacterial viability test was utilized as a complementary measurement. For that purpose, live/dead bacteria were quantified using the LIVE/DEAD® BacLightTM bacterial viability Kit from Invitrogen (Life Technologies Corporation). Cells were grown and induced as for CFU counting experiment. Aliquots of 1 ml of each cell suspension were centrifuged and resuspended in 20 mM Tris-HCl buffer pH 7.4 to obtain an OD_{600} ~ 0.1. Finally, the fluorophores SYTO 9 and propidium iodide were added from a pre-mixed solution and the fluorescence was determined. The excitation wavelength was set at 488 nm and emission was adjusted to 500 nm for SYTO 9 and 617 nm for propidium iodide. The samples were also observed with an epifluorescence microscope Olympus BX51. For this purpose, a number of fields were randomly selected for each sample, and cells were observed with a 100X objective lens and a numerical aperture of 1.4. The CFU/ml were determined before and after the induction of the suicide probe. Logarithmic dilutions of the cultures were made and spots were placed on LB plates.

Tricine SDS-PAGE: Electrophoresis were carried-out as previously described (Schägger, 2006) and the samples were treated with loading buffer without β-mercaptoethanol. For fusion protein activity the gel was fixed, and then washed successively with ethanol 70%, 30% and sterile bidistilled water. The gel was placed on TSB agar plate, covered with 10 ml of 0.6% (w/v) agar inoculated with 10^6

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cells of *L. innocua* 7 (Acuña *et al.*, 2012). The plate was incubated at 30 °C for 16 h and the inhibition zones were examined.

For immunity protein expression analysis, *E. coli* BL21 (pACyC-Duet) and *E. coli* BL21 (p-munC) strains were grown until OD_{600}~0.6, then induced with 0.01 mM IPTG or repressed with 0.6% glucose. A milliliter of each culture was centrifuged, washed, resuspended in loading buffer with β-mercaptoethanol and then boiled for 5 minutes. The expression of MunC immunity protein was further confirmed by Tris-Tricine SDS-PAGE electrophoresis revealed with Coomassie Blue. In Figure 1S a band of 11.05 kDa can be observed in lane 5 which corresponds to the calculated size of the immunity protein MunC. The band corresponding to MunC protein is not observed on the lane of repressed extracts nor the control expressing the empty plasmid. This confirms the correct expression of the MunC immunity protein.

Cell membrane permeabilization measurements- Cytoplasmic membrane permeabilization was determined by using the potential sensitive dye 3,3 dipropyl thiacarbocyanine iodide ($DiSC_3$[5]) (Wu *et al.*, 1999), purchased from Invitrogen (Life Technologies corporation). The dye concentrates in the cytoplasmic membrane of energized cells, resulting in self-quenching of its fluorescence. The control cells expressing only EtpM as well as the cells expressing the fusion EtpM-Ent35 and the fusion peptide plus the immunity protein MunC were grown in LB medium at 37°C to exponential phase (OD_{600} ~ 0.2). The expression of MunC was induced with lactose 0.02 % for 1 hour. Then cells were collected by centrifugation and resuspended in 50 mM HEPES, 5 mM EDTA, pH 7 to obtain an OD_{600} ~ 0.1. The cell suspension was incubated at 37°C with 0.4 µM $DiSC_3$ [5] until the dye uptake was maximal (as indicated by a stable reduction of fluorescence). The expression of the fusion EtpM-Ent35 was induced with arabinose 0.6%. The emission of fluorescence was monitored at 667 nm as a function of time (excitation emission wavelength: 622 nm).

RESULTS

Expression of the suicide probe in E. coli

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In order to evaluate a possible effect of enterocin CRL35 in *E. coli*, we constructed a suicide probe, using EtpM as a membrane anchor for enterocin CRL35 (Gérard *et al.*, 2004). EtpM is a bitopic membrane polypeptide of 170 residues. Using the TMHMM server, it is predicted that the first 18 residues are located in the cytoplasm, the following 23 residues form a transmembrane α-helix and the C-terminal 129 residues are located in the periplasm (Krogh *et al.*, 2001). The genetic construction was cloned under the P_{BAD} promoter (see experimental procedures). Upon arabinose induction (Santini *et al.*, 2001), EtpM is driven to the cell membrane of *E. coli* and its C-terminus is translocated to the periplasm alongside the bacteriocin that was fused to it, in a process mediated by YidC protein (Gerard *et al.*, 2004). Thus, the bacteriocin remains anchored to the cell membrane by EtpM on the periplasmic side. If the expression of the suicide probe turns out to be lethal to the host cell, it would indicate that the bacteriocin could be inserted into the inner membrane.

To select a colony with a correctly cloned suicide construction, twenty colonies obtained from the transformation plate were picked and streaked on LB supplemented with glucose 0.6% (repressed) or arabinose 0.6% (induced). Most of the clones do not grow in the presence of arabinose (Figure 1A). One of them was selected to purify the plasmid p-etpM-munA. This preliminary experiment proves that the synthesis of the chimera EtpM-Ent35 is lethal for the host cells. The action of EtpM-Ent35 in *E. coli*, even in the absence of a specific receptor, suggests that enterocin CRL35 would only require to be brought to the membrane surroundings in order to exert its activity.

Anti-Listeria activity of EtpM-Ent35 fusion

To check whether the suicide probe possess antimicrobial activity by itself, the cellular extracts of different *E. coli* strains expressing EtpM, EtpM-Ent35, RR-Ent35 and co-expressing EtpM-Ent35 and MunC were assayed against *L. innocua* 7 and *E. coli* BL21. It is observed that the cell extracts from *E. coli* BL21 p-etpM-munA, *E. coli* BL21 p1707 and cells co-expressing the toxic fusion and the immunity protein show inhibition halos against *L. innocua* 7. In contrast, the cellular extract obtained from the control strain, which expresses EtpM does not show antimicrobial activity (Figure 1B). These results indicate that the addition of EtpM at the N-terminus of enterocin CRL35 does not affect the antimicrobial activity of the bacteriocin. When the antimicrobial activity against *E. coli* is assayed,

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no inhibition halo is observed. As we show below, if enterocin CRL35 is not anchored in the membrane, it cannot affect *E. coli.*

To confirm the expression of the suicide probe we carried out a Tris-Tricine SDS-PAGE. Electrophoretic comparison of synthetic enterocin CRL35 and the membrane fraction from strains *E. coli* BL21 (p-etpM-munA) and BL21 (p-etpM-munA) (p-munC) are shown in Figure 2A. Two bands with antimicrobial activity against *L. innocua* 7 are observed (lanes 1 and 2). It is possible that a proteolytic cleavage of EtpM-Ent35 occurs, resulting in a low molecular mass enterocin CRL35 derivative peptide.

The EtpM-Ent35 fusion translocates the Ent35 portion to the periplasm

In order to confirm the location of the suicide probe we compared the antimicrobial activity of subcellular fractions of the strain expressing EtpM-Ent35. EDTA permeabilized cells were treated with proteinase K or PMSF plus protease inhibitor cocktail as a control. Then the proteinase activity was inactivated by heating 10 minutes at 65 ºC, as described in experimental procedures. It was previously pointed out that EDTA allows permeabilization of the OM. As a consequence, proteinase K would be able to reach the periplasm and inactivate enterocin CRL35 only if EtpM-Ent35 were efficiently translocated to the periplasm and the enterocin CRL35 portion were actually placed in the periplasmic face of the membrane. Indeed, after the arabinose induction, only the membrane fraction has antimicrobial activity confirming that EtpM-Ent35 is in fact bound to the membrane. Besides, proteinase K treated samples loses antimicrobial activity (Figure 2B) supporting the concept that enterocin CRL35 portion is located towards the periplasm.

Enterocin CRL35 is not active on E. coli

It is well established that class IIa bacteriocins produced by LAB cannot pass across the Gram negative bacteria outer membrane (OM) and therefore they are not active on *E. coli* (Stevens *et al.*, 1991; Chalón *et al.*, 2012). However, it has been suggested that the combination of some pediocin-like bacteriocins and OM-disrupting agents may be active on Gram-negative bacteria (Naghmouchi *et al.*, 2011; Adler *et al.*, 2011).

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To prove that enterocin CRL35 does not have an IM receptor and is therefore inactive on *E. coli*, we employed the strain *E. coli* BL21 (p1707) where RR-Ent35 could be exported to the periplasm by the TAT secretion system upon the induction with arabinose (see Experimental Procedures). *E. coli* cells carrying the plasmid p1707 grow normally in LB medium, both repressed with glucose 0.6% and induced with arabinose 0.6 % (Figure 2S). Moreover, *E. coli* cells with altered OM by osmotic shock are unaffected when the purified enterocin CRL35 is added to the medium (see Supplementary Material, Figure 3S). Therefore, we conclude that enterocin CRL35 is not active on *E. coli* when it is delivered to the periplasm in both, inside-out and outside-in ways. These results further confirm that there is not a receptor on *E. coli* IM for enterocin CRL35. It is important to note that RR-Ent35 is in fact being expressed by *E. coli* BL21 since, after the addition of the inducer, samples from culture crude extracts display anti-listerial activity (Figure 1B).

Protective effect of the immunity protein

If the killing effect of the suicide chimera comes from the bactericidal activity of the bacteriocin, the co-expression of the immunity protein should protect the host cells. As it can be observed in Figure 3A, control cells *E. coli* BL21 (p-etpM); *E. coli* BL21 (p-etpM-munA) as well as the strain *E. coli* BL21 (p-etpM-munA) (p-munC) grow normally in LB agar plates supplemented with glucose 0.6%. However, the growth of cells expressing the EtpM-Ent35 fusion is significantly affected when they are plated onto LB agar containing arabinose, while the control cells are not affected. Moreover, the basal production of the immunity protein in the plates supplemented only with arabinose is apparently sufficient to observe a protective effect on the strain expressing both the chimera and the immunity protein. This could be explained by the well-known "leaky" nature of the *lac* promoter (Müller *et al.*, 1996). In fact, CFU are similar in plates supplemented only with arabinose or in arabinose-lactose plates. Results show that enterocin CRL35 immunity protein is capable of protecting cells from the lethal effect of the suicide probe even in the absence of the specific receptor. This evidence strongly supports the notion that the membrane perturbation and the bacterial death induced by the fusion peptide is exclusively associated with the bacteriocin part of the chimera.

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In a quantitate assay, colony forming units were analyzed upon induction of the suicide probe during 5 minutes with arabinose, as well as the co-expression of the fusion peptide and the immunity protein. Figure 3B shows that cell count drops from 5.8 10^8 to 1 10^8 which represented an 82.7% decrease in viability. This result is backed up by the LIVE/DEAD® BacLight™ bacterial viability Kit (Figure 3D). Actually, we find 40% viable cell decrease upon induction of the chimera. Interestingly, MunC is shown to fully protect cells from the deleterious effect of EtpM-Ent35 by these two different approaches.

Fluorescence microscopy was used to visualize the effect of the different protein expression using LIVE/DEAD® BacLightTM. Figure 3E shows *E. coli* BL21 strains after induction with arabinose, which express only EtpM (control), EtpM-Ent35 and both EtpM-Ent35 and MunC. The suicide probe expression disturbs the membrane permeability, so bacteria emit red fluorescence (dead cells). As regards the immunity protein, the strain co-expressing EtpM-Ent35 and MunC increases the number of green cells compared to the strain that only expresses the suicide probe. The assay using LIVE/DEAD BacLight kit shows good correlation with the viable CFU count on LB agar plates (Figure 3C).

Effect of the EtpM-Ent35 fusion on E. coli transmembrane potential

Like other class IIa bacteriocins, enterocin CRL35 dissipates the electric potential ($\Delta\psi$) and the proton motive force (ΔpH) on sensitive cells (Minahk *et al.*, 2000). Therefore, we studied changes in the electric potential of *E. coli* cells upon induction of the fusion peptide using the membrane-potential sensitive fluorescent probe $DiSC_3[5]$. After the addition of arabinose, $\Delta\psi$ is markedly dissipated in cells expressing EtpM-Ent35, showing an increase in the fluorescence signal over time, as it can be seen in Figure 4. The cells that co-express both the suicide probe and the immunity protein, show much less pronounced transmembrane potential dissipation, probably due to MunC effect, which might obliterate the suicidal activity of the fusion. In contrast, no significant changes are observed in the fluorescence intensity in control cells that only express the membrane anchor (EtpM). The fact that cells expressing MunC undergo some dissipation of the membrane potential without significant changes in cell viability is not a contradictory finding. In effect, Masias et al. have reported that

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$\Delta\psi$ dissipation is a necessary factor in the enterocin CRL35 mechanism of action but it is not sufficient for promoting cell death (Masias *et al.*, 2015). As a matter of fact, $\Delta\psi$ changes induced by an antimicrobial peptide primarily indicates that the membrane has been targeted and a membrane-peptide interaction has taken place. The degree of $\Delta\psi$ dissipation is a better indication of how detrimental this interaction turns out to be.

DISCUSSION

The expression of a suicide probe in *E. coli*, based on the membrane protein EtpM and a bacteriocin from a Gram-positive bacterium, reveals new insights into the class IIa bacteriocins mechanism of action. Nowadays, it is well established that class II bacteriocins require a receptor in the plasma membrane of the target cell to exert their antimicrobial effects (Duquesne *et al.*, 2007; Cotter *et al.*, 2013) . However, the precise role of the receptor protein and the nature of the pore that is formed are still unknown. It is clearly shown that enterocin CRL35 is not able to exert any bactericidal effect on *E. coli* cells when it is expressed and targeted to the periplasm. This result shows that *E. coli* membrane does not possess a receptor for the bacteriocin and, as a consequence, RR-Ent35 does not exhibit any antimicrobial activity.

This fact could be explained mainly by two reasons: on the one hand, bacteriocins belonging to class IIa cannot cross the outer membrane, hence they are unable to reach their ultimate target, the plasma membrane (Chalón *et al.*, 2012). On the other, Gram-negative bacteria do not express the mannose permease belonging to group I, the receptor for class II bacteriocins produced by Gram-positive bacteria (Kjos *et al.*, 2009). Moreover, even if some membrane proteins might act as receptors and the bacteriocin could cross the OM, bacteriocins may not insert properly into the plasma membrane of Gram-negative bacteria because phospholipids are quite different. In fact, membranes of *L. monocytogenes* and LAB mainly contain anionic phospholipids such as phosphatidylglycerol and cardiolipin. Phosphatidilethanolamine only represents 8% of total phospholipids (Crandall and Montville, 1998). In addition, *L. monocytogenes* is characterized by branched chain fatty acids (Mastronicolis *et al.*, 2005). On the contrary, *E. coli* plasma membrane contains up to 70% of the zwitterionic phospholipid phosphatidylethanolamine and the combination of phosphatidylglycerol and

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cardiolipin represents just one third of total phospholipids (DeChavigny *et al.*, 1991). As it has been demonstrated in this work, the probe disturbs the membrane integrity of Gram-negative bacteria in spite of its composition. Thus, enterocin CRL35 has the potential to be employed as a broad range food biopreservative if it is properly engineered.

On the other hand, if the bacteriocin is somehow anchored to the IM, it is able to exert a bactericidal effect, suggesting that one of the roles of the receptor might be just as a mere docking molecule, bringing closer the bacteriocin to the plasma membrane in order to get the peptide inserted and produce cell injury (Figure 5A).

It has been previously reported that class IIa bacteriocins are able to acquire a structured form if they are placed in a phospholipid-like environment such as DPC micelles (Fregeau Gallagher *et al.*, 1997; Uteng *et al.*, 2003). Indeed, the bacteriocins hold a random coil conformation in water, but a helical structure is induced when they get attached to micelles, which would contribute to the membrane permeabilization.

Based on this former evidence, is logical to think that, when EtpM anchors enterocin CRL35 to the periplasmic side of the membrane, the proximity of the peptide to the lipid bilayer would induce a conformational change of the bacteriocin that would lead to the formation of a pore, disturbing the integrity of the membrane, all in the absence of man-PTS.

As it was previously mentioned, a bacteriocin producer strain also synthesizes a specific immunity protein, named MunC for enterocin CRL35 (Saavedra *et al.*, 2004). Previous reports in literature demonstrated that the addition of immunity proteins from class IIa bacteriocins to the extracellular medium, does not affect the antimicrobial activities of the corresponding bacteriocins (Nissen-Meyer *et al.*, 1993; Quadri *et al.*, 1995). Therefore, it was concluded that bacteriocin-immunity protein interaction did not exist or this interaction was weak (Drider *et al.*, 2006). Diep et al. previously demonstrated that class II bacteriocins associate with the membrane receptor and the immunity protein in a bacteriocin producer strain, forming a ternary complex in which the activity of the bacteriocin can be counteracted (Diep *et al.*, 2007). Here we show that the protective effect of the immunity protein, MunC, neutralizes EtpM-Ent35 activity and it might block the pore structure in this condition. This mechanism occurs in the absence of the membrane receptor, suggesting a direct

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bacteriocin-immunity protein interaction. Even though Diep's conclusion is that man-PTS is a necessary component for bacteriocins mechanism of action, the fact that the immunity protein acts only if the bacteriocin is present, supports this conclusion. We hypothesize that immunity protein could recognize and bind class IIa bacteriocins in a membrane-inserted form (Figure 5B and 5C).

In conclusion, the present paper shows evidence that EtpM-Ent35 might form functional pores on *E. coli* membrane. The fusion peptide is able to dissipate the membrane electric potential once anchored to the plasma membrane, in spite of lacking a specific receptor (Figure 5A). We propose a new model for enterocin CRL35 mechanism of action and immunity based on experimental evidence (Figure 5B). It is possible that class IIa bacteriocins produced by Gram-positive bacteria just need to stablish an intimate contact with the target membrane to get inserted. Subsequently, the bacteriocin itself would be able to form a pore in the absence of the receptor. Indeed, immunity protein would not require the presence of the receptor either in order to recognize the bacteriocin pore and prevent its deleterious action. This is the first *in vivo* evidence indicating that the receptor could not be involved in the pore structure but rather it would act as an anchor molecule. This observation has great importance for the designing of bacteriocins with biotechnological applications, as food biopreservative or with potential use in the pharmaceutical industry.

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AUTHORS' CONTRIBUTIONS: C.J.M., A.L., M.C.CH. and A.B. designed the study. D.E.B., M.C.CH. N.S.R.C and A.E.G. performed the experiments. C.J.M., A.L., M.C.CH., A.B. D.E.B. and

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N.S.R.C. analyzed the data. C.J.M., N.S.R.C., M.C.CH. and A.B wrote the manuscript. All authors read and approved the final manuscript.

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Table 1. Strains and plasmids

Strain	Description	Reference
E. coli DH5α	F⁻ ϕ80lacZΔM15 *rec*A1 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *sup*E44 *hsd*R17 ($r_k^- m_k^+$) *thi*-1 *gyr*A96 *rel*A1 *deo*R *pho*A	Promega
E. coli BL21 [DE3]	*E. coli* BF$^-$ *ompT hsdS*(rB$^-$mB$^-$) *gal dcm* λ(DE3)	Novagen
E. coli O157:H7 ATTC 700728	*etpM* template	ATCC
Listeria innocua 7	Sensitive to enterocin CRL35	INRA (France)
Enterococcus mundtii CRL35	Enterocin CRL35 producer strain	CERELA culture collection
Plasmid	**Description**	**Reference**
pACYCDuet-1	Expression vector, Cm^r	Novagen®
p8760	pBAD24 with GFP cloned, Ap^r	(Santini *et al.*, 2001)
p-etpM	p8760 with *etpM* gene cloned and without GFP, Ap^r	This work
pE35	pCR-Blunt II- Topo (Invitrogen) with enterocin CRL35 biosynthetic cluster cloned	(Saavedra *et al.*, 2004)
pmunC	pACYCDuet-1 with munC gene cloned,Cm$_r$	This work
p1707	p8760 with munA gene cloned Ap^r	A gift from Masias E.
p-etpM-munA	p1707 with *etpM* gene cloned, Ap	This work

553 Cm^r, chloramphenicol resistant; Ap^r, ampicillin resistant. CGSC, *E. c*oli Genetic Stock Center.
554 INRA: Unité de Recherches Laitiéres et Génetique Appliqueé. ATCC: American Type Culture
555 Collection. CERELA: Centro de Referencias para Lactobacilos, Tucumán. Argentina. LMGT,
556 Laboratory of Microbial Gene Technology. FBQF: Facultad de Bioquímica, Química y Farmacia;
557 Universidad Nacional de Tucumán, Argentina.
558

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Table 2. Primers

Name	Sequence 5´- 3´
munAFNheI	TGGCTAGCAAATACTACGGTAATGGA
munARHindIII	CGAAGCTTTTAACTTTTCCAACCAG
munCFNdeI	GGTGGTCATATGAGTAATTTAAAGTGGTTTTC
munCRXhoI	GGTGGTCTCGAGCTAATATCCATATCTAATATTAG
etpmcor	CCTGAGAATTCACAATGAACGAGCTTAAAA
etpmnh1	ACTGTGTTTTTTTCACGGCTAGCTACAGTC

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FIGURE LEGENDS

FIGURE 1. A) Selection of *E. coli* DH5α EtpM-Ent35 expressing clones. After transformation with the ligation mixture containing the plasmid p1707 and *etpM49* digested, twenty colonies were plated on LB-glucose 0.6 % (above) or LB- arabinose 0.6 % (below). The plates were incubated at 37 °C overnight. Control: *E. coli* DH5α (p1707). **B) Antimicrobial activity of probe expressing strains.** 20 µl of crude extracts obtained after induction with arabinose 0.6 % where spotted and tested against of *L. innocua* 7 (above) and *E. coli* BL21 (below). EtpM: *E. coli* BL21 (p-etpM); EtpM-Ent35: *E. coli* BL21 (p-etpM-munA); EtpM-Ent35/MunC: *E. coli* BL21 (p-etpM-munA) (pMunC); and RR-EtpM: *E. coli* BL21 (p1707), respectively.

FIGURE 2. Expression and cellular localization of EtpM-Ent35. A) Biological activity revealed by Tris-Tricine SDS-PAGE. Membranes were purified by ultracentrifugation and proteins were separated by electrophoresis. Fixed gel was placed on TSB and covered with soft agar inoculated with *L. innocua* 7. The picture shows the zones of growth inhibition by the membrane fraction from arabinose induced cells. Lane 1: EtpM-Ent35 expressing membranes, lane 2: EtpM-Ent35/MunC expressing membranes and lane 3: synthetic enterocin CRL35 (MM: 4.29 kDa). Calculated molecular mass for EtpM-MunA: 10.35 kDa. **B) Subcellular localization of EtpM-Ent35 expressed in *E. coli* BL21.** A culture of *E. coli* BL21 expressing EtpM-Ent35 was harvested, washed and permeabilized with EDTA. Then it was split and each half was treated with proteinase K (PK) or PMSF plus protease inhibitor cocktail, respectively. Then the different subcellular fractions of each treatment were obtained and 10 µl of each one were spotted on TSB agar plate and covered with a *L. innocua* 7 lawn. 1) supernatant, 2) periplasm, 3) cytoplasm and 4) membrane.

FIGURE 3. **Viability of strains expressing EtpM-Ent35 and immunity protein.** The strains *E. coli* BL21 (p-etpM-munA); *E. coli* BL21 (p-etpM-munA) (pmunC) and control *E. coli* BL21 (p-etpM) were grown in LB-glucose 0.6% until OD_{600} ~ 0.6. Then they were harvested, washed and resuspended in LB. **A)** Logarithmic dilutions of each culture were made and 10 µl of each dilution were spotted on plates with glucose 0.6 %, arabinose 0.6 % or arabinose 0.6 % + lactose 0.02 %. **B** and **C)** Effect of 5 minutes arabinose induction on cellular viability. Cultures were induced with lactose during 1 h and then with arabinose 0.6% for 5 minutes. Cells were washed and resuspended in

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20 mM Tris-HCl buffer pH 7.4. The viability was estimated by CFU/ml counting on LB plates. Results are representative of three independent replications. Different letters indicate significant differences according to Tukey's test, p <0.0001. **D)** Quantification by using LIVE/DEAD® BacLight™ bacterial viability Kit. Results represent the average of three independent experiments ± standard deviations. Different letters indicate significant differences according to Tukey's test, p <0.0001. **(E)** Fluorescence microscopy images of live and dead cells after 5 minutes induction with arabinose as described in figure 3B.

FIGURE 4. Effect of EtpM-Ent35 in cell membrane electric potential. The expression of the fusion and the immunity protein was induced with arabinose 0.6 % and lactose 0.02 %, respectively. $DiSC_3$[5] was added at a final concentration of 0.4 μM and fluorescence was registered over time. Excitation and emission wavelengths were set at 622 and 667 nm respectively. This data is representative of three separate studies.

FIGURE 5. A) Model for expression of EtpM-Ent35 in *E. coli*. The expression of EtpM-Ent35 fusion peptide could result in the insertion of enterocin CRL35 in the membrane of *E. coli*. EtpM is translocated and inserted by YidC (Gérard et al., 2004). Once EtpM is anchored in the membrane; enterocin CRL35 portion may penetrate from the periplasm and disrupt the membrane. *etpM-munA* is under control of the P_{BAD} promoter, induced by arabinose and repressed by glucose. OM: outer membrane, IM: inner membrane, PG: peptidoglycan. **B) Model for EtpM-Ent35 and MunC mechanism of action in *E. coli*.** The suicide probe EtpM-Ent35 is translocated to *E. coli* periplasm. Then enterocin CRL35 would be inserted in the membrane forming an oligomeric pore. The dissipation of the electrochemical gradient and outflow of small organic molecules would trigger the cell death. When immunity protein MunC is co-expressed (right panel), it would recognize the inserted bacteriocin in the membrane forming a bacteriocin-immunity protein complex preventing cell death. **C) Proposed model for enterocin CRL35 mechanism of action and immunity under physiological conditions.** The bacteriocin would bind to IIC and IID subunits of man-PTS system to be anchored in the proximity of the cellular membrane. To date, man-PTS is the only receptor described for class II bacteriocins (Kjos et al., 2009, 2010). Once anchored, the bacteriocin could

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form the oligomeric pore as described above. In a bacteriocin producer cell (right), the immunity protein would interact with the oligomeric pore forming a receptor-bacteriocin-immunity protein complex, as described by Diep *et al*. (Diep et al., 2007). The immunity protein would directly interact with the pore formed by the bacteriocin and indirectly with the receptor to form the ternary complex. OM: outer membrane, IM: inner membrane, PG: peptidoglycan, I: immunity protein, R: receptor, CW: cell wall, CM: cell membrane.

SUPPLEMENTARY FIGURE LEGENDS

FIGURE 1S. **Expression of MunC upon induction with IPTG.** Lanes 1) Protein Ladder, 2) repressed pACYCDuet-1 (Glucose 0.6%), 3) induced pACYCDuet-1 (0.01 mM IPTG), 4) repressed pmunC (Glucose 0.6%), 5) induced pmunC (0.01mM IPTG). Calculated molecular mass for MunC: 11.05kDa.

FIGURE 2S. Expression of RR-Ent35. *E. coli* BL21 transformed with p-etpM-munA and p1707 were grown in LB until OD_{600} ~ 0.6. Then the expression of EtpM-Ent35 and RR-Ent35 was induced with arabinose 0.6%. The cultures were washed, resuspended in 20 mM Tris-HCl buffer pH 7.4 and logarithmic dilutions were made. **A)** Viable counts and **B)** 10 µl of each dilution were spotted in LB-agar plates. Results are representative of four independent replications. Different letters indicate significant differences according to Tukey's test, $p < 0.0001$.

FIGURE 3S: Viability of permeabilized *E. coli* cells. Viability of permeabilized *E. coli* MC4100 after treatment with 50 AU/mL of enterocin CRL35, microcin V, Ent35-MccV or MccV-Ent35, respectively. The control was performed without bacteriocin. Data are graphed as percentage of viability with respect to the control. Results are representative of four independent replications. Different letters indicate significant differences according to Tukey's test, $p < 0.0001$.

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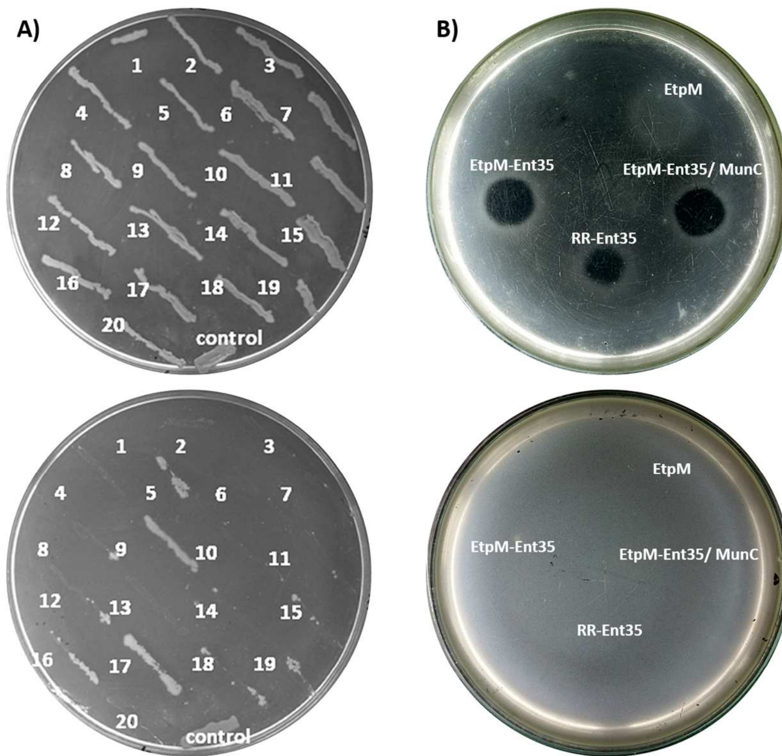

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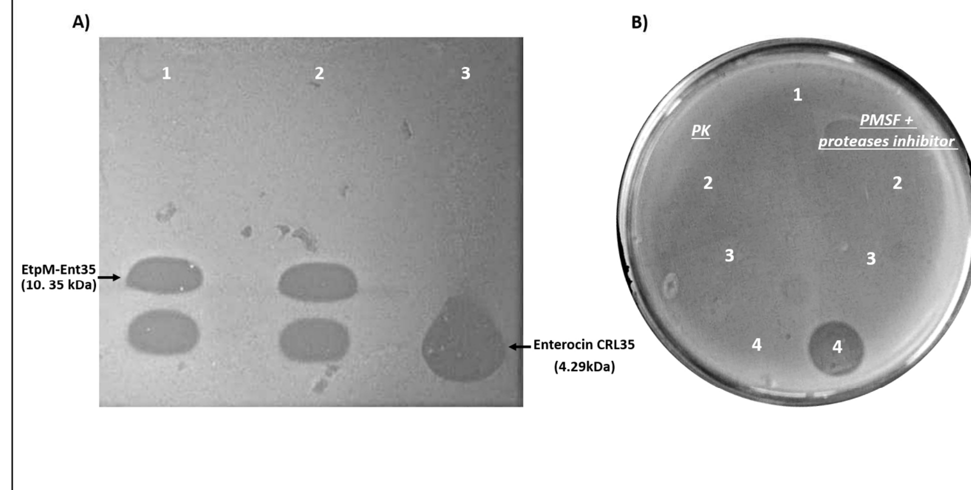

FIGURE 2. Expression and cellular localization of EtpM-Ent35. A) Biological activity revealed by Tris-Tricine SDS-PAGE. Membranes were purified by ultracentrifugation and proteins were separated by electrophoresis. Fixed gel was placed on TSB and covered with soft agar inoculated with *L. innocua* 7. The picture shows the zones of growth inhibition by the membrane fraction from arabinose induced cells. Lane 1: EtpM-Ent35 expressing membranes, lane 2: EtpM-Ent35/MunC expressing membranes and lane 3: synthetic enterocin CRL35 (MM: 4.29 kDa). Calculated molecular mass for EtpM-MunA: 10.35 kDa. B) Subcellular localization of EtpM-Ent35 expressed in *E. coli* BL21. A culture of *E. coli* BL21 expressing EtpM-Ent35 was harvested, washed and permeabilized with EDTA. Then it was split and each half was treated with proteinase K (PK) or PMSF plus protease inhibitor cocktail, respectively. Then the different subcellular fractions of each treatment were obtained and 10 μl of each one were spotted on TSB agar plate and covered with a *L. innocua* 7 lawn. 1) supernatant, 2) periplasm, 3) cytoplasm and 4) membrane.

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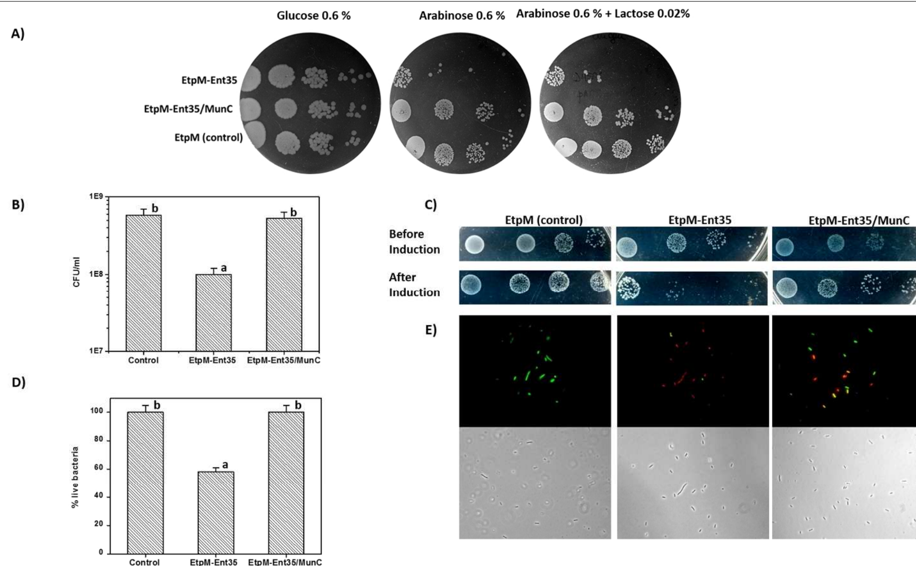

FIGURE 3. Viability of strains expressing EtpM-Ent35 and immunity protein. The strains *E. coli* BL21 (p-etpM-munA); *E. coli* BL21 (p-etpM-munA) (pmunC) and control *E. coli* BL21 (p-etpM) were grown in LB-glucose 0.6% until OD_{600}~ 0.6. Then they were harvested, washed and resuspended in LB. A) Logarithmic dilutions of each culture were made and 10 µl of each dilution were spotted on plates with glucose 0.6 %, arabinose 0.6 % or arabinose 0.6 % + lactose 0.02 %. B and C) Effect of 5 minutes arabinose induction on cellular viability. Cultures were induced with lactose during 1 h and then with arabinose 0.6% for 5 minutes. Cells were washed and resuspended in 20 mM Tris-HCl buffer pH 7.4. The viability was estimated by CFU/ml counting on LB plates. Results are representative of three independent replications. Different letters indicate significant differences according to Tukey's test, $p < 0.0001$. D) Quantification by using LIVE/DEAD® BacLightTM bacterial viability Kit. Results represent the average of three independent experiments ± standard deviations. Different letters indicate significant differences according to Tukey's test, $p < 0.0001$. (E) Fluorescence microscopy images of live and dead cells after 5 minutes induction with arabinose as described in figure 3B.

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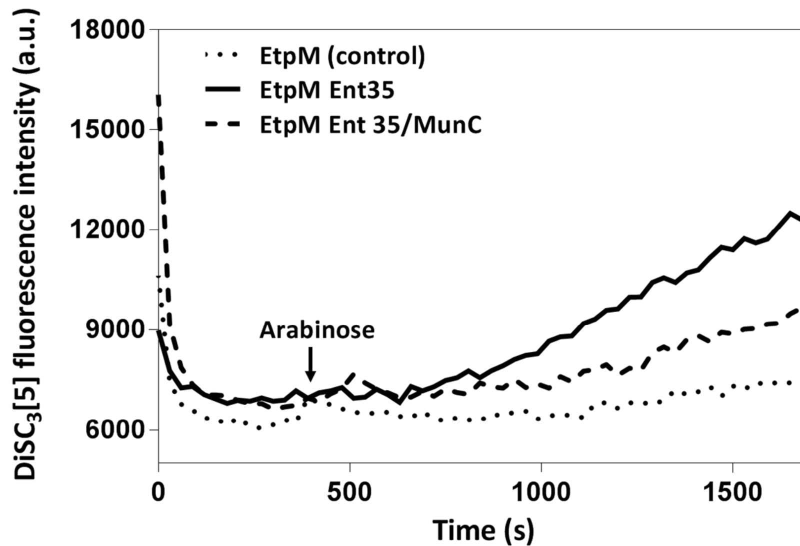

FIGURE 4. Effect of EtpM-Ent35 in cell membrane electric potential. The expression of the fusion and the immunity protein was induced with arabinose 0.6 % and lactose 0.02 %, respectively. $DiSC_3[5]$ was added at a final concentration of 0.4 μM and fluorescence was registered over time. Excitation and emission wavelengths were set at 622 and 667 nm respectively. This data is representative of three separate studies.

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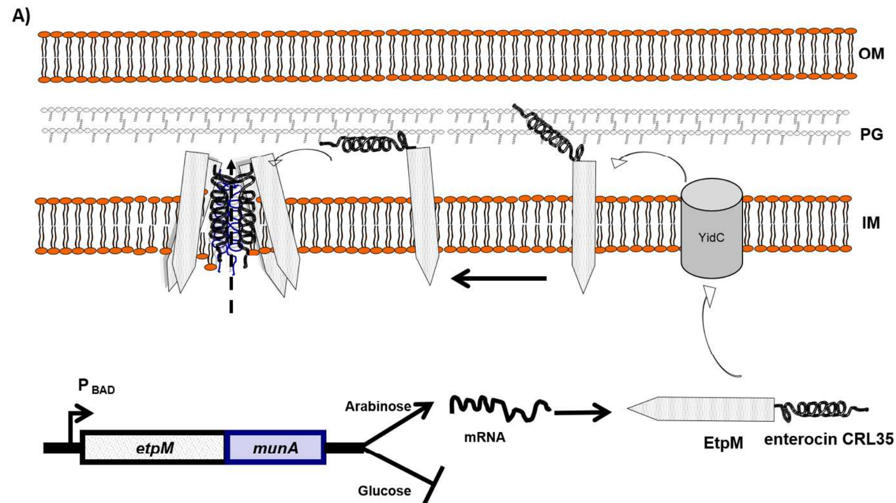

FIGURE 5. A) Model for expression of EtpM-Ent35 in *E. coli*. The expression of EtpM-Ent35 fusion peptide could result in the insertion of enterocin CRL35 in the membrane of *E. coli*. EtpM is translocated and inserted by YidC (Gérard et al., 2004). Once EtpM is anchored in the membrane; enterocin CRL35 portion may penetrate from the periplasm and disrupt the membrane. etpM-munA is under control of the PBAD promoter, induced by arabinose and repressed by glucose. OM: outer membrane, IM: inner membrane, PG: peptidoglycan.

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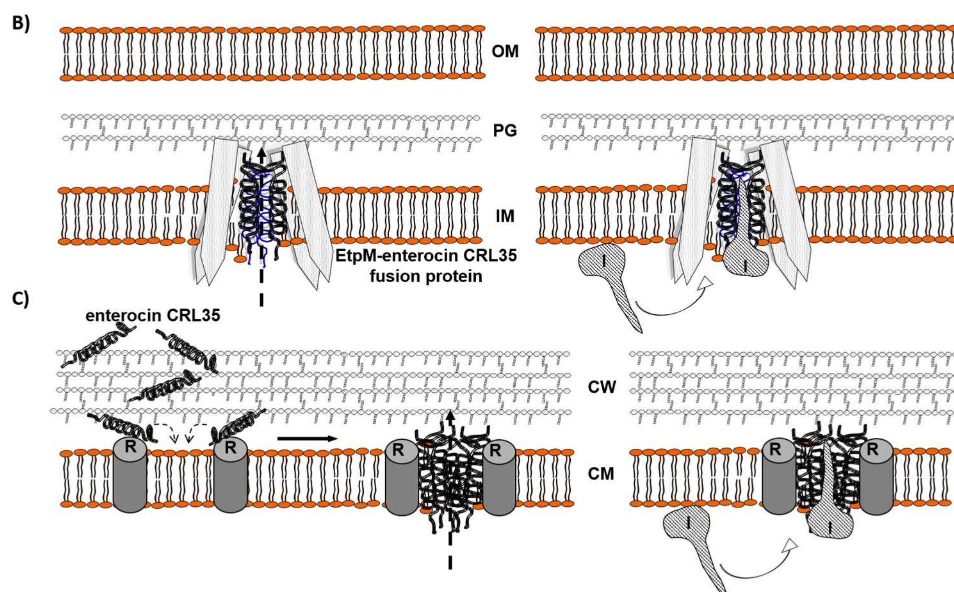

FIGURE 5. B) Model for EtpM-Ent35 and MunC mechanism of action in *E. coli*. The suicide probe EtpM-Ent35 is translocated to *E. coli* periplasm. Then enterocin CRL35 would be inserted in the membrane forming an oligomeric pore. The dissipation of the electrochemical gradient and outflow of small organic molecules would trigger the cell death. When immunity protein MunC is co-expressed (right panel), it would recognize the inserted bacteriocin in the membrane forming a bacteriocin-immunity protein complex preventing cell death. C) Proposed model for enterocin CRL35 mechanism of action and immunity under physiological conditions. The bacteriocin would bind to IIC and IID subunits of man-PTS system to be anchored in the proximity of the cellular membrane. To date, man-PTS is the only receptor described for class II bacteriocins (Kjos et al., 2009, 2010). Once anchored, the bacteriocin could form the oligomeric pore as described above. In a bacteriocin producer cell (right), the immunity protein would interact with the oligomeric pore forming a receptor-bacteriocin-immunity protein complex, as described by Diep et al. (Diep et al., 2007). The immunity protein would directly interact with the pore formed by the bacteriocin and indirectly with the receptor to form the ternary complex. OM: outer membrane, IM: inner membrane, PG: peptidoglycan, I: immunity protein, R: receptor, CW: cell wall, CM: cell membrane.

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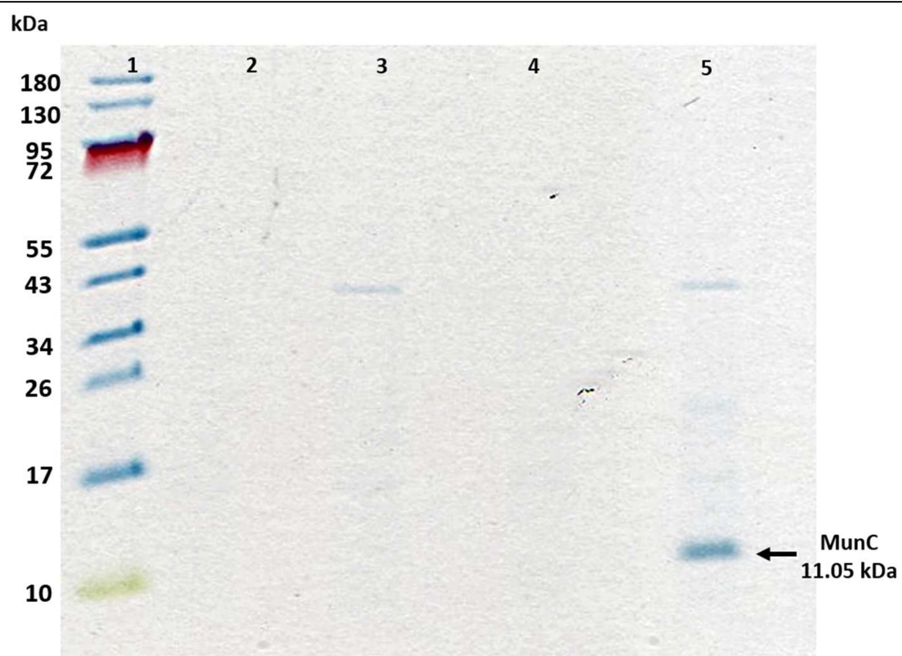

FIGURE 1S. Expression of MunC upon induction with IPTG. Lanes 1) Protein Ladder, 2) repressed pACYCDuet-1 (Glucose 0.6%), 3) induced pACYCDuet-1 (0.01 mM IPTG), 4) repressed pmunC (Glucose 0.6%), 5) induced pmunC (0.01mM IPTG). Calculated molecular mass for MunC: 11.05kDa.

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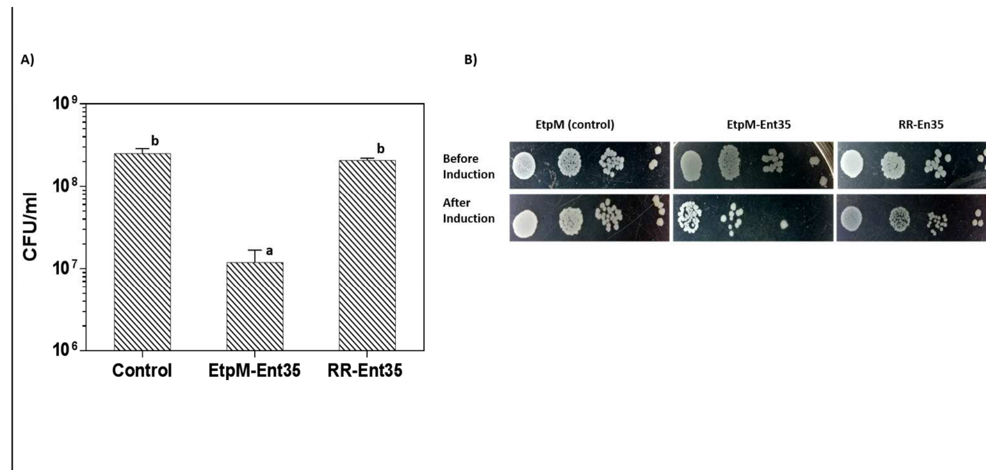

FIGURE 2S. Expression of RR-Ent35. *E. coli* BL21 transformed with p-etpM-munA and p1707 were grown in LB until OD_{600} ~ 0.6. Then the expression of EtpM-Ent35 and RR-Ent35 was induced with arabinose 0.6%. The cultures were washed, resuspended in 20 mM Tris-HCl buffer pH 7.4 and logarithmic dilutions were made. A) Viable counts and B) 10 µl of each dilution were spotted in LB-agar plates. Results are representative of four independent replications. Different letters indicate significant differences according to Tukey's test, $p < 0.0001$.

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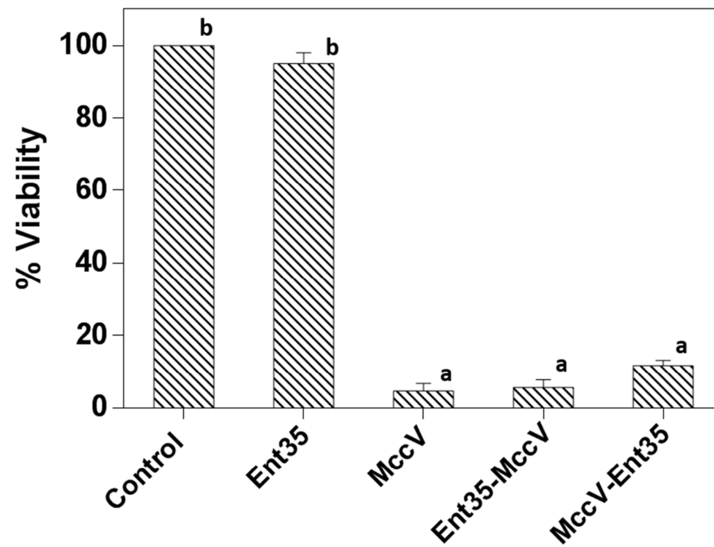

FIGURE 3S: Viability of permeabilized *E. coli* cells. Viability of permeabilized *E. coli* MC4100 after treatment with 50 AU/mL of enterocin CRL35, microcin V, Ent35-MccV or MccV-Ent35, respectively. The control was performed without bacteriocin. Data are graphed as percentage of viability with respect to the control. Results are representative of four independent replications. Different letters indicate significant differences according to Tukey's test, $p < 0.0001$.

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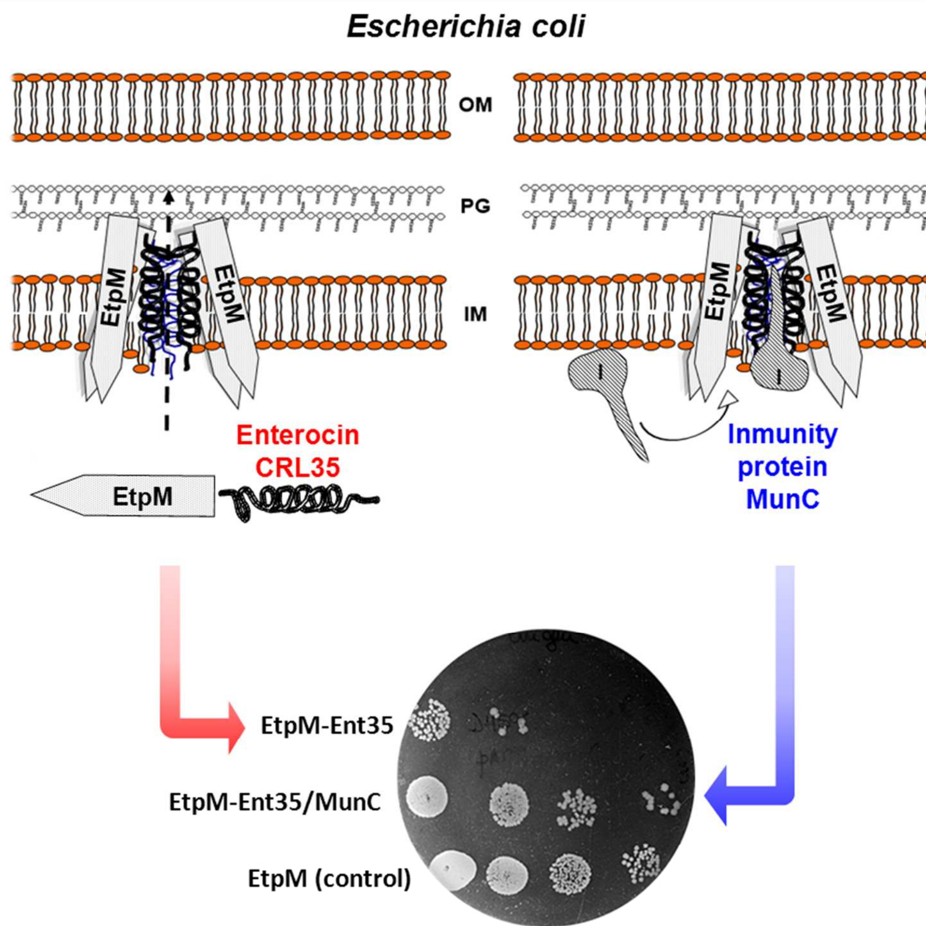

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Escherichia coli cells are naturally insensitive to the bacteriocin enterocin CRL35.

When the bacteriocin is anchored to the periplasmic face of the plasma membrane through the bitopic membrane protein, EtpM, the bacteria depolarize and die and the co-expression of its immunity protein prevents the deleterious effect of EtpM-enterocin CRL35.

The binding and anchoring of the bacteriocin to the membrane has demonstrated to be a sufficient condition for its membrane insertion.

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