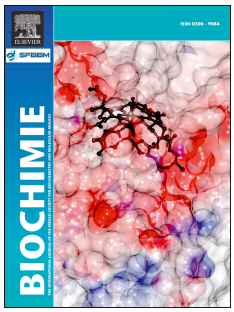


# The case for class II bacteriocins: a biophysical approach using "suicide probes" in receptor-free hosts to study their mechanism of action

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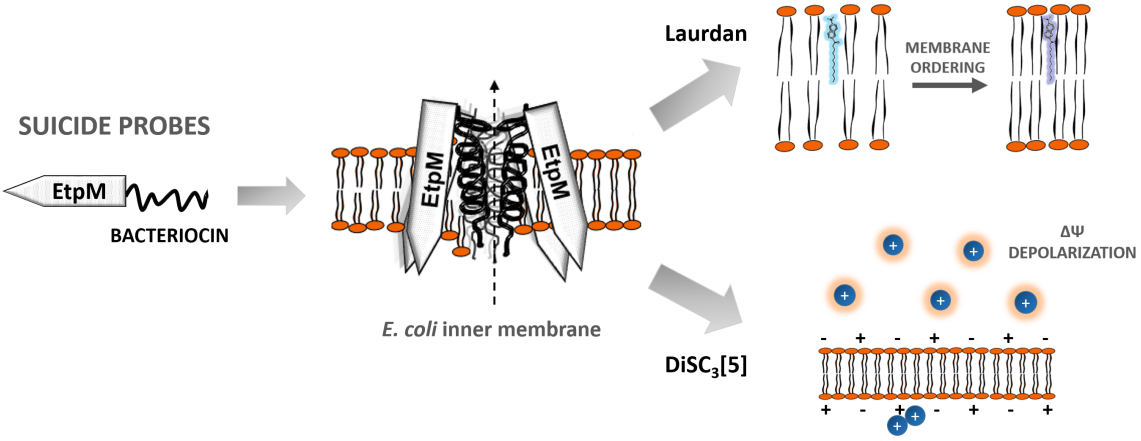
Class II bacteriocins are unmodified membrane-active peptides that act over a narrow spectrum of target bacteria. They bind a specific receptor protein on the membrane to form a pore, leading to membrane permeabilization and cell death. However, little is known about the molecular events triggering the pore formation after the bacteriocin recognizes the receptor. It is not clear yet if the pore is the same receptor forced into an open conformation or if the pore results from the bacteriocin insertion and oligomeric assembly in the lipid bilayer. In order to reveal which model is more suitable to explain the toxicity mechanism, in this work we use chimeric peptides, resulting from the fusion of the bitopic membrane protein EtpM with different class II bacteriocins: enterocin CRL35, pediocin PA-1 and microcin V. *E. coli* strains lacking the specific receptors for these bacteriocins were chosen as expression hosts. As these constructs display a lethal effect when they are heterologously expressed, they are called "suicide probes". The results suggest that, indeed, the specific receptor would act as a docking molecule more than as a structural piece of the pore, as long as the bacteriocin is somehow anchored to the membrane. These set of chimeric peptides also represent an *in vivo* system that allows to study the interaction of the bacteriocins with real bacterial membranes, instead of model membranes. Hence, the effects of these suicide probes in membrane fluidity and transmembrane potential were also assessed, using fluorescence spectroscopy. The data show that the different suicide probes are able to increase phospholipid order and depolarize the membranes of receptor-free bacterial cells.



# The case for class II bacteriocins: a biophysical approach using "suicide probes" in receptor-free hosts to study their mechanism of action.

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**Abstract**

Class II bacteriocins are unmodified membrane-active peptides that act over a narrow spectrum of target bacteria. They bind a specific receptor protein on the membrane to form a pore, leading to membrane permeabilization and cell death. However, little is known about the molecular events triggering the pore formation after the bacteriocin recognizes the receptor. It is not clear yet if the pore is the same receptor forced into an open conformation or if the pore results from the bacteriocin insertion and oligomeric assembly in the lipid bilayer. In order to reveal which model is more suitable to explain the toxicity mechanism, in this work we use chimeric peptides, resulting from the fusion of the

bitopic membrane protein EtpM with different class II bacteriocins: enterocin CRL35, pediocin PA-1 and microcin V. *E. coli* strains lacking the specific receptors for these bacteriocins were chosen as expression hosts. As these constructs display a lethal effect when they are heterologously expressed, they are called "suicide probes". The results suggest that, indeed, the specific receptor would act as a docking molecule more than as a structural piece of the pore, as long as the bacteriocin is somehow anchored to the membrane. These set of chimeric peptides also represent an *in vivo* system that allows to study the interaction of the bacteriocins with real bacterial membranes, instead of model membranes. Hence, the effects of these suicide probes in membrane fluidity and transmembrane potential were also assessed, using fluorescence spectroscopy. The data show that the different suicide probes are able to increase phospholipid order and depolarize the membranes of receptor- free bacterial cells.

**Keywords**

Laurdan; Microcin; Pediocin; Enterocin; Mechanism of action; Trans-membrane potential.

**Abbreviations**

MccV: microcin V. OD: optical density. TAT: twin arginine translocation. CFU: colony forming units. EDTA: ethylenediaminetetraacetic acid. Man-PTS: mannose phosphotransferase system. ΔΨ: transmembrane electric potential.

## 1. Introduction

Bacteriocins are membrane-active peptides displaying antimicrobial activity usually against phylogenetically related bacteria. Since many bacteriocins are produced by lactic acid bacteria generally recognized as safe (GRAS), these peptides represent a natural and sustainable alternative as antimicrobial agents in food and as a replacement for the traditional antibiotics used in medical practice [1–3]. Bacteriocins have been classified in two broad groups: class I and class II. Class I bacteriocins have post-translational

modifications and nisin is a prime example of this class, since is the only bacteriocin approved to be directly used in aliments as a pure additive [4]. Class II bacteriocins are a heterogeneous group of ribosomally synthesized peptides, divided into 5 subclasses (a, b, c, d, and e), that do not undergo post-translational modification beyond the cleavage of a leader peptide [1]. This class is of particular interest, as it would be more readily used for medical and biotechnological applications. The successful use of pediocin PA-1 in ALTA® 2341 (a commercial fermented product) as a biopreservative ingredient in the food industry [5] has largely fostered the research interest in this group.

It is accepted that these peptides have a specific receptor in the membrane of the target bacteria. Once the protein-protein interaction is established, hydrophilic pores are formed in the membrane. This leads to cytoplasmic components and ions efflux as well as dissipation of the transmembrane electrical potential [6–9]. However, it remains elusive the precise molecular events triggering the formation of the pore immediately after the bacteriocin recognize its receptor. Neither the stoichiometry of the pore complex is known. To date, two theoretical models have been developed according to different experimental approaches. The first model suggests that bacteriocins could induce conformational changes in the membrane receptor that would lead to the opening of an intrinsic channel. The second model, propose the receptor as a docking molecule positioning the bacteriocin closer to the plasma membrane. This event would allow the subsequent bacteriocin insertion and oligomeric assembly in the lipid bilayer to form the pore [10–13]. Very little consensus exists on the matter, since the details of these molecular interactions were not elucidated yet. The mannose phosphotransferase system (Man-PTS) of several Gram-positive bacteria, has been confirmed as the specific receptor for class IIa bacteriocins (also known as pediocin-like bacteriocins) such as enterocin CRL35 and pediocin PA-1 [14–16]. For microcin V (MccV), a class IId bacteriocin [1], the inner membrane protein SdaC from *E. coli* (also present in other Gram-negative bacteria) has been reported as the specific receptor [17].

The design of the chimeric genetic construct *etpM-cvaC* was published for the first time on 2004 by Gérard *et al* [18]. The resulting hybrid protein EtpM-MccV fuses the bitopic

membrane protein EtpM (also known as GspM) encoded by enterohemorrhagic *E. coli* serotype O157:H7 [19,20] with the MccV sequence at the C-terminal. In that study, the authors take advantage of the MccV bactericidal activity, using EtpM-MccV as an indicator to elucidate the topology and membrane insertion mechanism of EtpM. They demonstrated that this process is dependent on YidC, a system that specifically assists the insertion of integral membrane proteins [21–23]. Since MccV has a bactericidal effect only from the periplasm [24–26] their results confirmed the N (in) – C (out) EtpM topology, and suggested an EtpM-mediated, YidC-dependent translocation of MccV. In other words, when the expression of the chimeric gene *etpM-cvaC* is induced, the resulting hybrid protein, EtpM-MccV, would translocate the MccV portion to the periplasmic side of the membrane and it would remain anchored to the lipid bilayer through EtpM. As this construct has a lethal effect when expressed, it was called “suicide probe”. Interestingly, the *sdaC* mutant *E. coli* strain (resistant to exogenous MccV) is also killed when the expression of EtpM-MccV is induced. This mutant *E. coli* strain is a receptor-free host for MccV, as it does not express SdaC, the specific membrane receptor [17]. Therefore, SdaC does not seem to be essential for the MccV bactericidal activity when the peptide is brought into close contact to the inner membrane.

Although the original aim of EtpM-MccV as a suicide probe, was the study of an integral membrane protein insertion and topology, the concept was recently repurposed to assess a class II bacteriocin mechanism of action [27]. The gene encoding EtpM was fused to *munA*, the structural gene of enterocin CRL35. *E. coli* was selected as an expression host because this bacterium is naturally insensitive to pediocin-like bacteriocins, since their specific Man-PTS receptor is not present on its inner membrane [15] The resulting fusion, called EtpM-Ent35, was proven to be toxic for the expressing host cell. Thus, it was suggested that the specific receptor could be dispensable for the final step of membrane disruption. Several experiments and controls were carried out to verify that the bacteriocin portion is efficiently located towards the periplasm and remains bound to the inner membrane through EtpM, just as EtpM-MccV [27].

Based on the former evidence presented above, in this work we attempt to express the suicide probes "EtpM-bacteriocins" in receptor-free hosts to reveal and compare biochemical features of three different class II bacteriocins: enterocin CRL35, pediocin PA-1 and microcin V (**Figure 1A**).

The use of suicide probes represent an *in vivo* system that allows to evaluate interactions of bacteriocins with real bacterial membranes, as a tool to complement *in vitro* studies using model membranes. Hence, besides the role of the specific receptor in the pore structure, some aspects of the molecular mechanisms of these bacteriocins can be explored in living cells, such as the effects of bacteriocin insertion in transmembrane potential and membrane fluidity. We aim to provide a detailed comparative analysis of class II bacteriocins that share several characteristics, but act on different target bacteria.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and media

Bacteria and plasmids used in this work are listed in **Table 1**. Luria broth (LB) and tryptic soy broth (TSB) were purchased from Sigma Chemical Co (St. Louis, MO) and Britania (CABA, Argentina), respectively. Solid media were prepared by adding agar to a final concentration of 1.5%. When required, ampicillin, streptomycin and/or kanamycin were added to a final concentration of 50 μg $mL^{-1}$. Glucose and arabinose were added to a final concentration of 0.6 %.

**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 (rk¯mk+) *thi*-1 *gyr*A96 *rel*A1 *deo*R *pho*A | Promega |
| *E. coli* O157:H7 ATTC 700728 | *etpM* template | ATCC |
| *E. coli* MC4100 | *F- araD139 e(argF-lac) 205 l- rpsL150 ftbB5301* | CGSC |

| | | |
|---|---|---|
| | *relA1 deoC1 pstF25. Sm*$^r$. Sensitive to MccV. | |
| *E. coli sdaC* | MC4100 *ΔsdaC::km.* Resistant to MccV. | Acuña *et al*., 2012 [28] |
| *Listeria monocytogenes* FBUNT | Sensitive to enterocin CRL35 and pediocin PA-1 | FBQF, UNT |
| *Pediococcus acidilactici* LMGT 2351 | Pediocin PA-1 producer strain | LMGT NMBU |
| **Plasmid** | **Description** | **Reference** |
| p8760 | pBAD24 derivative with *tat-gfp-mut2* cloned, Ap$^r$ | Santini *et al*., 2001 [29] |
| pHK11 | pBR322 with the genetic system of MccV (source for the *cvaC* gene), Ap$^r$ | Gilson *et al*., 1987 [30] |
| p-etpM-gfp | pBAD24 derivative with *etpM -gfp-mut2* cloned, Ap$^r$ | This work |
| p-etpM | pBAD24 derivative with *etpM* gene cloned, Ap$^r$ | Barraza et al., 2017 [27] |
| p-tat-munA (p1707) | pBAD24 derivative with *tat-munA* gene cloned, Ap$^r$ | Barraza et al., 2017 [27] |
| p-etpM-munA | pBAD24 derivative with *etpM-munA* gene cloned, Ap$^r$ | Barraza et al., 2017 [27] |
| p-tat-pedA | pBAD24 derivative with *tat- pedA* gene cloned, Ap$^r$ | This work |
| p-etpM-pedA | pBAD24 derivative with *etpM- pedA* gene cloned, Ap$^r$ | This work |
| p-tat-cvaC | pBAD24 derivative *tat-cvaC* gene cloned, Ap$^r$ | This work* |
| p-etpM-cvaC | pBAD24 derivative with *eptM -cvaC* gene cloned, Ap$^r$ | This work** |

Sm$^r$, streptomycin resistant. Ap$^r$, ampicillin resistant. CGSC, *E. coli* Genetic Stock Center. ATCC: American Type Culture Collection. LMGT, Laboratory of Microbial Gene Technology. FBQF: Facultad de Bioquímica, Química y Farmacia; Universidad Nacional de Tucumán, Argentina. LMGT NMBU: Laboratory of Microbial Gene Technology, Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway. (*) p-tat-cvaC was built to be the same as p8754 by Ize *et al*., 2002[25]. (**) p-etpM-cvaC was built to be the same as p8792 by Gérard *et al*., 2004 [18].

## 2.2. Genetic constructions, cloning and cell transformation

The construction of the plasmids p-etpM, p-etpM-munA, and p-tat-munA was already described by Barraza *et al* [27]. The induction of these plasmids results in the expression of EtpM (a membrane protein of 170 amino acids), EtpM-Ent35 and RR-Ent35 respectively. Additionally, for this work, the same set of plasmids were built, using different bacteriocins structural genes instead of *munA*. The *cvaC* and *pedA* genes were amplified by PCR from pHK11 and colony PCR from *Pediococcus acidilatici* PA-1.0 LMGT 2351 respectively. Primer sequences are listed in **Table 2**. The amplified fragments were purified, digested by *Nhe*I and *Hind*III, and cloned into the corresponding sites of the plasmids p-etpM-gfp and p8760, also digested with the same enzymes. Then, *E. coli* DH5α cells were transformed by the classical calcium chloride protocol [31]. The plasmid p-etpM-gfp, contains a segment encoding the first 49 aminoacids of EtpM. The suicide probes were assembled by replacing the *gfp-mut2* gene by *cvaC* and *pedA*. The resulting constructions encode the chimeric proteins EtpM-MccV and EtpM-PedA1 where the EtpM segment is a truncated version of the original protein, but still contains the transmembrane segment with a periplasmic C-terminal fused to the bacteriocin portion **(Figure 1A)**. The plasmid p8760 contains a region encoding the twin arginine translocation (TAT) signal of TMAO reductase. As a consequence, when any of the bacteriocins structural genes is cloned, is preceded by a signal sequence (RR) that leads the peptide to the periplasm **(Figure 1B)**. In the resulting fusions called RR-MccV and RR-PedA1, as well as in RR-Ent35, the signal sequence is supposed to be removed by a membrane associated proteolytic enzyme after translocation to the periplasmic side [32].

The expression of these genetic fusions (EtpM-bacteriocins and RR-bacteriocins) are under the tight control of the $P_{BAD}$ promoter [33]. All the genetic constructions in the resulting plasmids were confirmed by DNA sequencing (CERELA, CCT-Tucumán, Argentina) and their expression was analyzed using medium containing either 0.6 % glucose or arabinose.

**TABLE 2. Primers**

| **Name** | **Sequence 5´- 3´** |
|---|---|
| *etpM*cor | CCTGAGAATTCACAATGAACGAGCTTAAAA |

| | |
|---|---|
| *etpM*nh1 | ACTGTGTTTTTTTCACGGCTAGCTACAGTC |
| *etpM*R*Hind*III | ATATAAGCTTTCAGCGACTAAGCG |
| *munA*F*Nhe*I | TGGCTAGCAAATACTACGGTAATGGAGT |
| *munA*R*Hind*III | CGAAGCTTTTAACTTTTCCAACCAGCTG |
| *pedA*F*Nhe*I | TGGCTAGCAAATACTACGGTAATGGGGT |
| *pedA*R*Hind*III | CGAAGCTTCTAGCATTTATGATTACCTTG |
| *cvaC*F*Nhe*I | GCTAGCGCTTCAGGGCGTGATATT |
| *cvaC*R*Hind*III | AAGCTTTTATAAACAAACATCACT |

GAATTC: *Eco*RI restriction site. AAGCTT: *Hind*III restriction site. GCTAGC: *Nhe*I restriction site.

### 2.3. Cell growing and viability assays

To evaluate the effect of the expression of the different fusions, strains were incubated with shaking at 37 °C in LB until $OD_{600}$ ~ 0.2. An aliquot of 200 µl of each culture was induced upon addition of arabinose 0.6% in a microplate well. OD measurements were performed in a microplate reader, at one hour intervals during four hours.

To assess how the suicide probes affect *E. coli* viability, all expressing strains were grown at 37°C in LB-glucose 0.6% until $OD_{600}$ ~ 0.3. Then they were harvested, washed and resuspended in M9 medium. Aliquots were log-diluted and plated onto LB medium with glucose 0.6 % and arabinose 0.6 %. The viability was determined by colony forming units CFU/ml counting after an overnight incubation at 37 °C.

### 2.4. Antimicrobial activity of cell extracts against sensitive strains

All *E. coli* strains were grown at 37°C in LB-glucose 0.6% until $OD_{600}$ ~ 0.3. Cells were collected by centrifugation, washed and resuspended in LB. The expression of EtpM, EtpM-bacteriocins and RR-bacteriocins was induced with arabinose 0.6 % for 30 minutes. Cells were centrifuged and resuspended in 20 mM Tris-HCl, 0.2% Triton X-100 pH 7.4. Cells were sonicated in an ice bath for 10 min and then boiled for 5 min. In addition, crude

187 extracts of EtpM-PedA1 and RR-PedA1 were precipitated with 70% ammonium sulfate. 10
188 µl of each cellular extract were spotted onto TSB or LB agar plates. When the drops were
189 completely dry, the plates were layered with 4 ml of 0.6% agar containing $10^7$ cells of *L.*
190 *monocytogenes* FBUNT or *E. coli* MC4100 respectively. The TSB plates were incubated
191 overnight at 30 °C and the LB plates at 37°C. Growth inhibition halos were examined to
192 visualize the antimicrobial activity.

193

194 **2.5. *E. coli* inner membrane fluidity measurements**

195 Changes in membrane fluidity were assessed using Laurdan (6-dodecanoyl-2-di-
196 methylaminonaphthalene) (Sigma-Aldrich, St. Louis, USA), a fluorescent molecule that
197 detects changes in membrane phase transitions (gel and liquid-crystalline) through its
198 sensitivity to the polarity of the environment in the bilayer. This phenomenon is a
199 consequence of water molecules associated to the membrane interface. Polarity changes
200 are detected by shifts in the Laurdan emission spectrum, and quantified by the excitation
201 generalized polarization, $GP_{exc}$ [34–38]. *E. coli* strains were grown in LB medium at 37°C
202 until $OD_{600}$ ~ 0.3. The strains were then induced with arabinose 0.6% for 30 minutes to
203 express the suicide probes and controls. Cells were collected by centrifugation, washed
204 and resuspended in 15 mM Tris-HCl, 5 mM EDTA, pH 7.4 to a final $OD_{600}$ ~ 0.1. EDTA was
205 added to permeabilize of the outer membrane, which is required for inner membrane
206 staining in Gram-negative bacteria. Laurdan was added from a 1 mM stock in
207 dimethylformamide, to a final concentration of 0.2 µM. Samples were incubated in the
208 dark at 37°C during 1.5 h with shaking. Laurdan-labeled samples were transferred to a 1-
209 cm quartz cuvette and the emission spectra (**Figure 2S**) were obtained at 37°C (excitation
210 wavelength: 350 nm; emission wavelengths: from 400 to 550 nm). The emission spectra
211 from blank samples (unlabeled cell suspensions) were subtracted to the respective sample
212 spectrum. $GP_{exc}$ from each spectrum was calculated using the equation: $GP_{exc} =$
213 $\frac{I\,440 - I\,490}{(I\,440 + I\,490)}$, where $I_{440}$ and $I_{490}$ are fluorescence intensities at 440 and 490 nm,
214 respectively. Fluorescence data were collected in an ISS PC1 fluorimeter equipped with

sample holder, magnetic stirring and temperature control by means of an external circulating bath (Cole Palmer).

### 2.6. *E. coli* inner transmembrane potential measurements

Cytoplasmic membrane depolarization was determined by using the potential sensitive dye $DiSC_3[5]$ (3,3 dipropyl thiacarbocyanine iodide) [39], purchased from Invitrogen (Life Technologies corporation). The dye aggregates within the polarized cytoplasmic membrane of energized cells, resulting in a decreased fluorescence emission [38]. When the membrane is depolarized, the fluorophore is released to the medium and fluorescence intensity increases because is no longer confined to the bilayer. Therefore, this experiment set out to measure fluorescence changes associated with membrane-medium distribution of the extracellularly applied dye, which is dependent on the transmembrane potential. The control cells expressing only EtpM as well as the cells expressing the fusion EtpM-bacteriocins and RR-bacteriocins were grown in LB medium at 37°C until $OD_{600}$ ~ 0.3. The fusions were then induced with arabinose 0.6% for 30 minutes. Cells were collected by centrifugation washed and resuspended in 50 mM HEPES-K, 5 mM EDTA, pH 7.4 to obtain an $OD_{600}$ ~ 0.1. Cell suspensions of each strain were transferred to a 1-cm quartz cuvette, and $DiSC_3[5]$ was added from a 0.3 mM stock in methanol to a final concentration 0.4 µM. The emission of fluorescence was monitored during 300 seconds at 37°C (excitation wavelength: 622 nm; emission wavelength: 667 nm).

### 2.7. Statistical analysis

Values presented are the average of at least three independent experiments. The data were statistically treated with one-way repeated measures analysis of variance (ANOVA) to determine the differences between the different conditions ($p<0.05$). Multiple comparisons were done by Tukey test using INFOSTAT statistical software (Facultad de Ciencias Agropecuarias, UNC, Argentina).

## 3. Results

### 3.1. Construction of hybrid fusions EtpM-bacteriocins (suicide probes) and controls.

In order to compare the effect of enterocin CRL35, pediocin PA-1 and MccV in *E. coli* when their specific receptors are missing, we used different suicide probes that were engineered using the EtpM protein as a membrane anchor. The genetic constructions were cloned under the $P_{BAD}$ promoter (see Materials and Methods). Upon arabinose induction [33], EtpM is driven to the cell membrane of *E. coli* and its C-terminus translocates to the periplasm alongside the bacteriocin that was fused to it, in a process mediated by the YidC complex (**Figure 1A**). Thus, the bacteriocin remains anchored to the cell membrane by EtpM on the periplasmic side [18,27].

The receptor- free hosts chosen to express the different suicide probes were *E. coli* MC4100 strain for enterocin CRL35 and pediocin PA-1, and the mutant strain *E. coli* MC4100 *sdaC* for MccV. Since both strains share the same genetic background, it is possible to compare the effects generated by each bacteriocin. We also considered two control systems (**Figure 1B**). On the one hand, it is well known that the over-expression of membrane proteins is often toxic to cells. To verify that the lethal effect is due to the bacteriocin portion and not due to the mere over-expression of a membrane protein, we used a system that expresses only the complete EtpM protein as a control. On the other hand, it was important to prove that the strains do not have an alternative inner membrane receptor other than Man-PTS or SdaC. This would mean that the expressing cells were in fact receptor-free for the tested bacteriocins. The twin arginine translocation system (TAT) was used for this purpose. We employed bacteriocins preceded by a twin arginine signal (RR-bacteriocins) that is recognized by the TAT secretion pathway which exports the bacteriocins to the periplasm of *E. coli*. This would confirm that bacteriocins are inactive on their respective host cells.

### 3.2. The suicide probes affect bacterial growth of strains lacking the specific receptors.

As shown in **Figure 2**, there is a clear inhibitory activity of the EtpM-bacteriocin fusions, whereas the control expressing only the complete membrane protein EtpM grow normally. As it can be seen from the growing curves, the three bacteriocins can be active in the absence of the specific receptor if they are somehow anchored to the membrane.

This results also suggest that the membrane insertion of EtpM-Ent35 and EtpM-PedA1 might be Man-PTS-independent, and EtpM-MccV insertion might be SdaC-independent as well. It seems that the sole insertion of the bacteriocin in the membrane would be sufficient for the antibacterial activity, allowing Gram-positive bacteriocins to be active against Gram-negative cells.

Additionally, when the twin arginine signal (RR) delivers the bacteriocins to the periplasm there is no inhibition on *E. coli* growing. This would mean that neither *E. coli* has a receptor for enterocin CRL35 (**Figure 2a**) or pediocin PA-1 (**Figure 2b**), nor *E. coli sdaC* has a receptor for MccV (**Figure 2d**). As expected, in *E. coli* MC4100 strain -which naturally expresses the SdaC receptor- MccV has a toxic effect from the periplasm (**Figure 2c**), either when is exported by the TAT system or when is anchored to the membrane through EtpM.

### 3.3. The bacteriocin portion of the suicide probes causes a differential effect on *E. coli* viability.

*E. coli* viability was assessed by CFU/ml counting on LB-glucose and LB-arabinose plates. **Figure 3A** depicts that there is no decrease in the number of colonies when the expression of EtpM-Ent35 is induced, but the size of each colony is affected, compared to the control that only expresses EtpM (**Figure 3B**). When EtpM-PedA1 is induced, the outcome is similar, but in this case, the colonies look even more transparent and smaller. Conversely, the expression of EtpM-MccV turns out to be completely lethal to their host strains *E. coli* MC4100 and *E. coli* MC4100 *sdaC*. In both cases, there is a total loss of viability since no colony grows on the arabinose plate. Thus, when MccV is anchored to the membrane, even in the absence of the SdaC receptor, there is a clear effect in growth and viability considering the controls. However, unlike enterocin CRL35 and pediocin PA-1, the loss of viability by MccV is total, since no colony grows after the induction (**Figure 3B**). Not only would the receptor be dispensable for the toxicity of the three peptides, but also it seems to exist a differential degree of toxicity that could be defined by the composition of the target membrane besides the presence of the specific receptor (See Discussion).

The receptor-free strains expressing the fusions RR-bacteriocins grow normally in LB medium with arabinose. This reinforces again, the concept that the peptides cannot be recognized by any other receptor of *E. coli*. As expected, RR-MccV is completely lethal from the periplasm when SdaC is present on the membrane.

### 3.4. Antimicrobial activity of crude extracts from *E. coli* strains expressing EtpM, EtpM-bacteriocins and RR-bacteriocins

To check whether the heterologous expression of EtpM-bacteriocins and RR-bacteriocins conserved their corresponding antimicrobial activity, the cellular extracts of *E. coli* expressing strains were assayed against *L. monocytogenes* FBUNT and *E. coli* MC4100. As expected, the extracts obtained from the control strain, which expresses EtpM, do not show antibacterial activity against *Listeria* and *E. coli* (**Figure 4**). It is observed that EtpM-Ent35 and RR-Ent35 are actually being expressed by *E. coli* after the induction, since samples from crude extracts display anti-listerial activity. In this case, heterologous expression in *E. coli* does not seem to affect the antimicrobial activity of the Ent35 portion (**Figure 4a**). In contrast, the inhibition halos obtained from EtpM-PedA and RR-PedA crude extracts can barely be noticed. Only when these crude extracts are concentrated by precipitation with $(NH_4)_2SO_4$ 70%, it appears a clear inhibitory activity (**Figure 2S**)**.** The possible causes of this results are deeply analyzed in the discussion.

In respect of EtpM-MccV, there is no inhibitory halo when it is expressed in *E. coli* MC4100 whereas the extract from the *sdaC* mutant does show activity (**Figure 4b**). As mentioned before, MccV is highly toxic for *E. coli* MC4100 both from the periplasm and when it is anchored to the membrane. It looks like the synthesis machinery of the cell is immediately affected by the toxicity of the peptide, which seems to be higher when the SdaC receptor is present than when is absent. This suggests that the receptor might not be a key factor in the pore formation, but it would play a role in making the pore structure more efficient to kill the target bacteria.

Regarding RR-MccV, anti-*E. coli* activity is observed in extracts obtained from both *E. coli* MC4100 and *E. coli* MC4100 *sdaC*. Since the mutant lacking the SdaC receptor is resistant to RR-MccV, it make sense that the inhibition halo from this strain extract is bigger than

the one from *E. coli* MC4100. This is a very interesting feature of the mutant that should be considered for large scale production of bacteriocins active against Gram- negative bacteria.

### 3.5. The suicide probes alter *E.coli* inner membrane fluidity.

Membrane fluidity of *E. coli* cells can be measured *in vivo* using the fluorescent probe Laurdan [40,41]. This molecule detects changes in membrane phase transitions (gel and liquid-crystalline) through its sensitivity to the polarity of the environment surrounding the bilayer [34–38]. Polarity changes are detected by shifts in the Laurdan emission spectrum, and that can be estimated by the excitation generalized polarization, $GP_{exc}$ (See Materials and Methods and **Figure2S**). In general terms, the higher the $GP_{exc}$ values, the closer the cytoplasmic membranes are to a gel phase (less fluid), while lower $GP_{exc}$ values mean that membranes are closer to a liquid crystalline phase (more fluid) [36,41,42]. **Figure 5** allows the straight comparison between the $GP_{exc}$ obtained for the different experimental conditions. In the case of cells expressing pediocin-like peptides (enterocin CRL35 and pediocin PA-1), $GP_{exc}$ values are statistically higher when the peptides are anchored to the membrane, in comparison to the control strain that only expresses EtpM. This means that the expression of EtpM-Ent35 and EtpM-PedA1 generates more rigid membranes. In contrast, these pediocin-like bacteriocins do not seem to significantly increase the $GP_{exc}$ values when they are exported to the periplasm (RR-bacteriocins). Therefore, they would not alter membrane fluidity (**Figure 5a and 5b**).

In the presence of the SdaC receptor (**Figure 5c**), MccV considerably increases the $GP_{exc}$ values from the perisplasm (RR-MccV), although this effect is not as remarkable when MccV is anchored in the membrane (EtpM-MccV). This is in line with the high toxicity of EtpM-MccV when SdaC is present. As it was previously underlined, the synthesis of the peptide seems to be immediately affected and this would explain the low changes that this suicide probe generates in the membrane fluidity, besides the lack of anti-*E. coli* activity of the crude extract (**Figure 4b**),. Contrarily, in the absence of SdaC, both EtpM-

358 MccV and RR-MccV increase $GP_{exc}$ values with respect to the control that only expresses
359 EtpM (**Figure 5d**).

360 At a first glance, the results suggest that the decrease in membrane fluidity caused by all
361 the suicide probes could be due to the insertion of the bacteriocin portion in the bilayer.
362 In contrast, when the bacteriocins are exported to the periplasm, MccV is the only one
363 that significantly reduces the membrane fluidity in *E. coli* MC4100, both with and without
364 the receptor.

365 **3.6. The suicide probes dissipate *E. coli* inner membrane electric potential**.

366

367 As it was underlined before, class II bacteriocins dissipate the transmembrane electric
368 potential (ΔΨ) and the proton motive force (ΔpH) on sensitive cells [9]. For that reason,
369 we evaluated changes in the electric potential of *E. coli* membranes upon induction of the
370 different fusions by means of the membrane-potential sensitive fluorescent probe
371 $DiSC_3$[5] [39]. Changes on its fluorescence emission are dependent on the transmembrane
372 potential, resulting in a higher fluorescence intensity over time when the membrane is
373 depolarized [38]. **Figure 6** depicts the decrease of fluorescence intensity upon $DiSC_3$[5]
374 addition, in control strains expressing only EtpM as well as strains expressing the RR-
375 bacteriocins fusions because the membrane remains polarized. The exception is the case
376 of RR-MccV where ΔΨ of *E. coli* MC4100 is dissipated, when the SdaC receptor is present.
377 In this strain, as well as the strains expressing every EtpM-bacteriocins, $DiSC_3$[5]
378 fluorescence does not decrease in the same way as the controls, due to the depolarization
379 of their membrane. $DiSC_3$[5] fluorescence show that enterocin CRL35 and pediocin PA-1
380 manage to depolarize the membrane when they are anchored through EtpM, even in the
381 absence of the specific Man-PTS receptor (**Figure 6a and 6b**). Notably, these pediocin-like
382 bacteriocins are not active against *E. coli* membrane under natural conditions. Hence, this
383 is an interesting aspect to focus on, since they seem to be able to form a pore if they are
384 somehow anchored to the membrane.

385 Both in the presence and absence of the SdaC receptor, ΔΨ is markedly dissipated when
386 MccV is anchored to the membrane (**Figure 6c and 6d**). These results are in accordance

with those previously reported by Gérard [17], supporting that, just as Man-PTS for pediocin-like bacteriocins, SdaC would be dispensable for the MccV bactericidal activity when the peptide is brought into close contact to the inner membrane.

## 4. Discussion

Enterocin CRL35 and pediocin PA-1 are class IIa bacteriocins produced by Gram-positive bacteria and active against other Gram-positive bacteria. Through the years, different models of peptide-membrane interactions have been proposed for pediocin-like bacteriocins [11,13,43]. A requisite for their activity is the interaction with the membrane-associated Man-PTS subunits to finally form a membrane-located complex [1,44]. However, it has always been a great deal of debate whether the Man-PTS acts only as a docking molecule or if it is forced into an open conformation by binding of the bacteriocin.

On the other hand, MccV, a class IId bacteriocin, is produced by a Gram-negative bacterium and is active against other Gram-negative bacteria expressing the specific receptor protein SdaC [17]. Unlike pediocin-like bacteriocins, very little research exists on MccV structure-function relationship. However, these three antimicrobials share several characteristics, besides being membrane-active peptides. To name a few: 1) they are linear single peptides with a high proportion of glycine and alanine residues (small amino acids responsible for a high degree of conformational freedom); 2) they are produced as pre-bacteriocins holding an N-terminal leader peptide which is removed by proteolytic cleavage in a double glycine site; 3) this occurs during the secretion to the extracellular medium through an ABC transporter [1,11,28,45–49].

As mentioned above, it is unclear how the interaction between the bacteriocin and the receptor protein leads to pore formation. In this study we aimed to elucidate which model is more suitable to explain class II bacteriocins toxicity and what is the role of the receptor in this mechanism of action. Based on former reports, we grasped the previous design of hybrid proteins called suicide probes, where the membrane protein EtpM is fused with different bacteriocins. The construction is intended to insert the bacteriocin in the membrane via YidC, independently of the receptor. As a first evaluation of these suicide

probes functioning, we demonstrated that their expression turns out to be toxic for the receptor-free host. From these results we can draw a first conclusion: if these bacteriocins are somehow anchored to the inner membrane of *E. coli*, they are able to exert a bactericidal effect even in the absence of the specific receptor (**Figure 2**)**.** Even though it is clear that the specificity of each bacteriocin for their target bacteria is given by the membrane receptor, the suicide probes insinuate that the receptor would act as a docking molecule more than a structural part of the pore.

The EtpM protein (also known as GspM) is part of the inner membrane multimeric complex of the type II secretion system from *E. coli.* The stoichiometry and structural biology of this membrane complex are still uncertain or unknown [50]. The crystal structure of the periplasmic domain of $GspM^{EpsM}$, a homologous protein from *V. cholerae*, show a cleft between two subunits of a dimer in the crystals, indicating that a partner protein might bind at this site[51]. However, we do not know whether the truncated EtpM, without the periplasmatic domain, behaves as a monomer or if it eventually forms a multimeric complex when it is overexpressed in *E. coli* as a suicide probe. Though it has not been proposed for class II bacteriocins yet, it might be possible that the EtpM portion in the suicide probes, nucleates an oligomeric pre-assembly of the bacteriocins to finally form the killer complex in a subsequent step. This pre-pore intermediate might locate in the periplasmic surface of the membrane. The receptor could play a similar role pre-assembling the bacteriocins in the natural process of pore formation, although more experiments are necessary to confirm or rule out this possibility.

On other hand, the sensitivity of target cells to the antimicrobial effect of different class II bacteriocins, might be influenced not only by the receptor but also by the specific lipid composition of the membrane. In fact, there are reports of particular cases where resistant cells showing high Man-PTS expression and hypersensitive cells showing a decreased Man-PTS expression, present changes in lipid composition and other membrane properties [52–55]. As we can appreciate in **Figure 3**, the toxicity of EtpM-MccV is much higher than the probes based on pediocin-like bacteriocins. It is clear that EtpM-Ent35 and EtpM-PedA1 disturb the membrane integrity of Gram-negative bacteria

445 in spite of being Gram-positive bacteriocins. Evidence supporting this statement are the
446 bacterial growth inhibition they cause **(Figure 2a and 2b)** and the dissipation of
447 transmembrane potential they generate **(Figure 6a and 6b)**. Nonetheless, pediocin-like
448 bacteriocins may not insert properly into the plasma membrane of Gram-negative
449 bacteria because phospholipids are quite different from Gram-positive bacteria. In fact,
450 membranes of *L. monocytogenes* and lactic acid bacteria mainly contain anionic
451 phospholipids and branched chain fatty acids [56]. On the contrary, *E. coli* plasma
452 membrane contains mainly zwitterionic phospholipid [57]. This might be the reason why
453 there is a small or no decrease in CFU/ml for *E. coli*, when analyzing bacterial viability in
454 arabinose plates **(Figure 3Aa and 3Ab)**. In contrast, the lethal effect of EtpM-MccV is
455 absolute, since no colony grows upon arabinose induction.

456 It seems that the effects caused by the suicide probes vary in a strain-dependent way
457 because there is an apparent discrepancy in the resulting phenotypes when the host is a
458 different *E. coli* strain (see supplementary material, **Figure 1S**). EtpM-Ent35 and EtpM-
459 PedA1 decrease the number of CFU/ml in *E. coli* BL21 [DE3] by one order of magnitude.
460 However, unlike *E. coli* MC4100, the aspect of the colonies do not change at all. When
461 EtpM-MccV is induced in *E. coli* BL21 [DE3], the loss of viability is not absolute, but the
462 decrease in the CFU/ml is still highly significant (five orders). All in all, the toxicity of EtpM-
463 MccV is much more pronounced than the probes based on pediocin-like bacteriocins in
464 both BL21 [DE3] and in MC4100 *E. coli* strains. MccV could be forming a pore that is more
465 "efficient", in the membrane of *E. coli*. This is probably due to the fact that MccV is
466 naturally active on Gram-negative membranes, whereas enterocin CRL35 and pediocin PA-
467 1 are not able to act on Gram-negative bacteria in natural conditions. Thus, we conclude
468 that the membrane composition modulates bacteriocins activity and it could be a key
469 factor in the proper bacteriocin insertion and the final step of pore formation. This idea
470 also agrees with the fact that class IIa bacteriocins hold a random coil conformation in
471 water but they are able to acquire a structured form in a phospholipid-like environment,
472 such as TFE or DPC micelles [58–62]. It is generally described that the N-terminal domain
473 of the peptides folds into a β-sheet structure that is located at the membrane/water

474 interface whereas the C-terminal portion is embedded in the lipid bilayer as a helix. These
475 structural arrangements upon contact with membrane mimetics support the idea of a
476 direct phospholipid-peptide interaction that is beyond the presence of a receptor [43].

477 Returning to the receptor role, there might be other features that should be considered.
478 For instance, **Figure 4b** illustrates how the crude extracts of *E. coli* expressing EtpM-MccV
479 show inhibition halos or not depending on the presence of the SdaC receptor in the
480 producer strains. This might be explained by the self-toxicity of this suicide probe, that
481 seems to be higher when SdaC is present than when is absent. Due to this extreme
482 toxicity, it is possible that the synthesis machinery of the cell is immediately affected.
483 Although we established that the receptor might not be an essential factor in the pore
484 formation, this assay suggests that SdaC would play a role in making MccV more effective
485 to kill target cells. Still, these assumptions must be further explored and studied.

486 Regarding the anti-*Listeria* inhibition of EtpM-PedA and RR-PedA (**Figure 4a**), the low
487 activity of the crude extracts could be explained by different factors. First of all, we did not
488 include in the constructions the genes *pedC* (encoding for the accessory protein PedC) and
489 *pedD* (enconding for an ABC transporter) that express the pediocin PA-1 secretion
490 machinery. Both genes are believed to be necessary for the optimal activity of the peptide
491 when is produced by *E. coli* [63]. Particularly, PedC protein is described to be necessary for
492 correct formation of disulfide bridges in pediocin-like bacteriocins that contain four
493 cysteine residues (e.g. pediocin PA-1) instead of two (e.g. enterocin CRL35). The formation
494 of incorrect disulfide bridges results in nearly inactive peptides [64–67]. On the other
495 hand, *etpM-pedA* and *tat-pedA* fusions are cloned in a pBAD plasmid derivative that is a
496 high copy level vector. For some reason, heterologous production of active pediocin PA-1
497 against *Listeria* was only achieved using a low copy level vector and activity become
498 undetectable at the medium-copy state [63]. Nonetheless, when pediocin PA-1 is
499 anchored to *E. coli* inner membrane through EtpM, bacterial growth, colonies aspect and
500 transmembrane potential is widely affected **(Figures 2b, 3Ab, 3B and 6b)**. Although the
501 possible causes of pediocin PA-1 loss of anti-listeria activity are beyond the scope of this
502 work, it is interesting to notice that the heterologous expression in *E. coli* is probably

affecting the bacteriocin ability to recognize the bacterial target more than its ability to form the pore.

Plenty research exists on interaction between antimicrobial peptides and model membranes. Although *in vitro* approaches are very useful, these models somehow demand to simplify an extremely complex and dynamic system such as the biological membrane. The use of suicide probes represent an *in vivo* approach that allows to study interactions of bacteriocins with real bacterial membranes, as a resource to complement *in vitro* studies performed in model membranes.

The present work focus on how the bacteriocin insertion affects bacterial membrane properties like fluidity and transmembrane potential, through the use of polarity-sensitive and potential-sensitive fluorophores, respectively. Laurdan, for instance, is one of the most popular fluorescence probes deemed to work extremely well not only in model membranes but also in living cells [35,36,40]. As depicted in **Figure 5**, the expression of all the suicide probes tend to increase the $GP_{exc}$ values. This points out an arrangement of the membrane lipids towards the gel phase, ergo, a decrease in the membrane fluidity. Once again, these results suggest that a receptor-independent interaction is taking place between the peptides and the membrane, inducing the phospholipids ordering. This make complete sense if we consider that the peptides are being inserted in the membrane and a pore structure is being formed, where conformational changes are likely to happen in both the peptides and the bilayer. It also should be noted that *E. coli* have the ability to adjust membrane lipid composition and, thus, to control membrane fluidity when they are subjected to stressful changes [68,69]. It is possible that the homeostatic mechanisms contribute to the alteration of lipid order in response to the processes associated with the simultaneous depolarization of the bilayer (**Figure 6**) and the deleterious effect on the cell. It is worthy to note how MccV is able to induce phospholipid ordering on *E. coli* inner membrane, even when it is not anchored by EtpM or by SdaC (**Figure 5d**). This suggests that there might be a peptide-membrane affinity that, while not sufficient to depolarize the membrane or kill the target cell, allows a receptor-independent interaction of the bacteriocin with the bilayer inducing ordering of the lipids.

Due to their association with the Man-PTS it has been described that pediocin-like bacteriocins alter sugar transport functionality causing a cell fitness impairment [14]. Since SdaC is a putative serine transporter [70], there is a possibility that bacteriocins in general could have additional effects, such as blocking nutrients uptake (either sugars or amino acids). Evidence supporting this notion brings about potential ecological and evolutionary roles for bacteriocins, as bacteria expressing these molecules could use them against similar bacteria, competing for specific nutritional sources [71,72]. That is to say, the environmental implications for bacteriocins production might be broader than expected, and this should point to possible future directions of research [43,73].

## 5. Conclusion

The use of suicide probes demonstrate that different class II bacteriocins are potentially toxic as long as they are anchored to the membrane, either through their natural receptor, or through an artificial anchor such as EtpM. Hence, the receptor would be more likely to act as a docking molecule bringing the bacteriocin closer to the membrane, allowing the peptide insertion to produce cell damage. Suicide probes also demonstrate how membrane composition plays an important role in modulating cells sensitivity to the bacteriocin.

It is clear that further studies should be carried out for a larger number of class II bacteriocins to reach more definite conclusions. Nonetheless we propose the use of suicide probes as an approach to gain more information about the toxicity processes and mechanisms in living cells, as it was done in this work. It might be plausible to wager on the use of additional fluorescent probes to investigate modifications in other membrane biophysical properties. Moreover, this system should be seriously considered for the study of other membrane-active peptides, even using alternative bacterial hosts to analyze different membrane environments.

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## Figure legends

**Figure 1A. Model for expression and insertion of the suicide probes in *E. coli*.** The expression of the suicide probes (EtpM-bacteriocin) would result in the anchorage of the bacteriocin portion on the inner membrane of *E. coli*. The EtpM region (N-terminal) would

be inserted by YidC [18] and the bacteriocin region (C-terminal) would be translocated towards the periplasm. Once anchored in the membrane; the bacteriocin would penetrate from the periplasm and disrupt the membrane forming an oligomeric pore. The dissipation of the electrochemical gradient and outflow of small organic molecules would trigger the cell death. All the cloned genes for this work are under the control of the $P_{BAD}$ promoter, induced by arabinose and repressed by glucose [33].

**Figure 1B. Model for expression of EtpM and RR-bacteriocins in *E. coli*.** Left: The plasmid p-etpM hold the complete *etpM* gene encoding the complete EtpM protein. Right: The twin arginine translocation (TAT) secretion pathway exports the bacteriocins to the periplasm of *E. coli*. The plasmids encode the bacteriocin structural gene, preceded by a twin arginine translocation (*Tat*) signal that leads the peptide to the periplasm. The resulting peptides are called RR-bacteriocins and their signal sequence (RR) is supposed to be removed by a membrane associated proteolytic enzyme after translocation to the periplasmic side [32].

**Figure 2. Bacterial growth in liquid LB medium upon arabinose induction.** The graphs represent the optical density (OD) over time of *E. coli* MC4100 (a, b, c) and MC4100 *sdaC* (d) expressing: **a)** EtpM-Ent35 (dotted line) and RR-Ent35 (dashed line); **b)** EtpM-PedA-1 (dotted line) and RR-PedA1 (dashed line); **c)** EtpM-MccV (dotted line) and RR-MccV (dashed line); and **d)** EtpM-MccV (dotted line) and RR-MccV (dashed line). The control strain expressing only EtpM is represented with a solid line.

**Figure 3A. Bacterial viability in solid LB medium.** Bacterial CFU/ml counting was performed in solid LB-glucose (dark grey bars) and LB-arabinose (light grey bars). The graphs represent *E. coli* MC4100 (a, b, c) and MC4100 *sdaC* (d) expressing: **a)** EtpM, EtpM-Ent35 and RR-Ent35; **b)** EtpM, EtpM-PedA1 and RR-PedA1; **c)** EtpM, EtpM-MccV and RR-MccV; and **d)** EtpM, EtpM-MccV and RR-MccV. Each bar is the mean obtained from 3

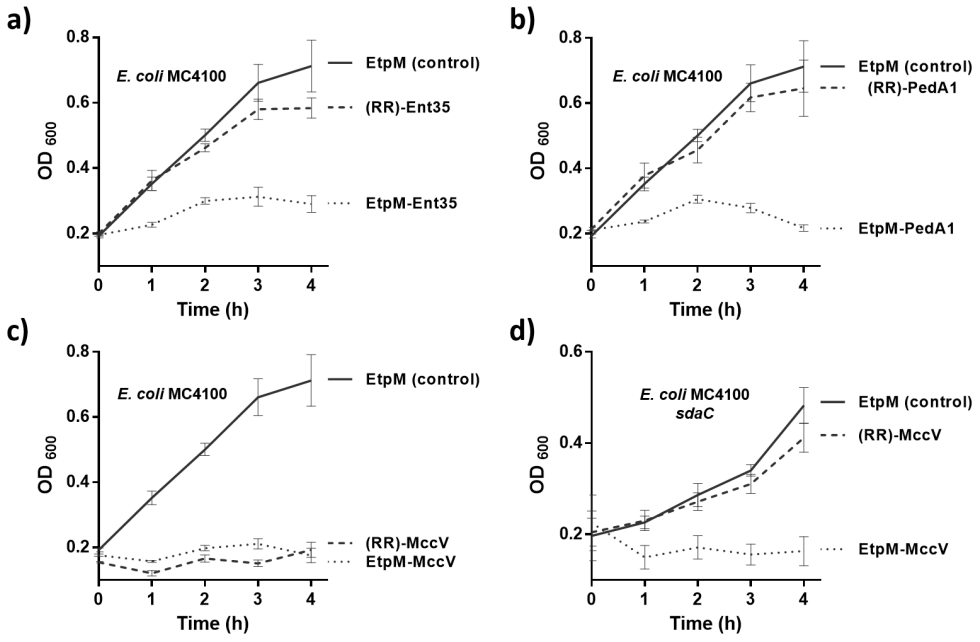
818 independent experiments; error bars represent the standard error of the mean. Asterisks
819 (*) indicate statistically significant difference according to Tukey test (p value < 0.0001).
820
821 **Figure 3B. Colony forming units of *E. coli* strains MC4100 and MC4100 *sdaC* growing on**
822 **LB-arabinose plate**. The strains expressing the different fusions were grown in LB-glucose
823 until $OD_{600}$ ~ 0.3. Then, they were harvested, washed and resuspended in M9 medium.
824 Each culture was log-diluted and plated onto LB-arabinose and LB- glucose (not shown).
825
826 **Figure 4. Antimicrobial activity of crude extracts from expressing strains.** 15 µl of crude
827 bacterial extracts obtained after arabinose induction were spotted and tested against
828 indicator strains *L. monocytogenes* FBUNT **(a)** and *E. coli* MC4100 **(b)**.
829
830 **Figure 5. Effect of the fusions on *E. coli* inner membrane fluidity ($GP_{exc}$).** Previously
831 induced strains were washed and labeled with Laurdan at 37°C. The graphs depict $GP_{exc}$
832 calculated for *E. coli* MC4100 strains expressing **a)** EtpM, EtpM-Ent35, RR-Ent35 **b)** EtpM,
833 EtpM-PedA-1, RR-PedA1 **c)** EtpM, EtpM-MccV, RR-MccV and *E. coli* MC4100 *sdaC*
834 expressing **d)** EtpM, EtpM-MccV, RR-MccV. Each bar is the mean obtained from six
835 independent experiments; error bars represent the standard error of the mean. Asterisks
836 (*) indicate statistically significant differences according to Tukey test. P values are: * ≤
837 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.
838
839 **Figure 6. Effect of the fusions on *E. coli* inner membrane electric potential**. Previously
840 induced strains were washed and resuspended in HEPES-EDTA buffer. $DiSC_3[5]$ addition
841 was made at time 30 seconds (indicated by arrows) and fluorescence was registered over
842 time at 37°C. Excitation and emission wavelengths were set at 622 and 667 nm
843 respectively. The data shown are representative of at least three separate and
844 independent assays. The graphs depict $DiSC_3[5]$ fluorescence intensity of *E. coli* MC4100
845 strains expressing **a)** EtpM, EtpM-Ent35, RR-Ent35 **b)** EtpM, EtpM-PedA-1, RR-PedA1 **c)**

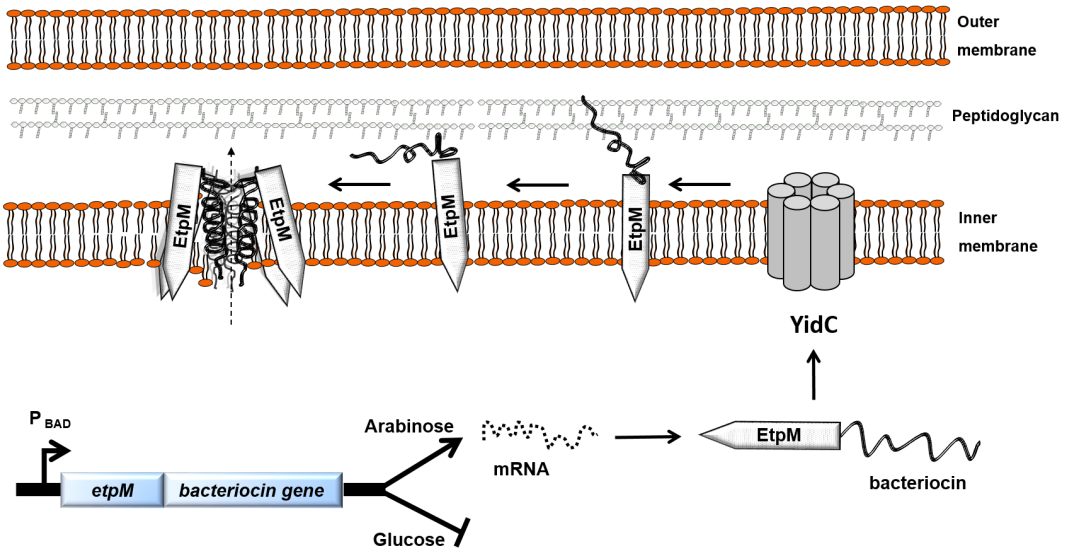
EtpM, EtpM-MccV, RR-MccV and *E. coli* MC4100 *sdaC* expressing **d)** EtpM, EtpM-MccV, RR-MccV.

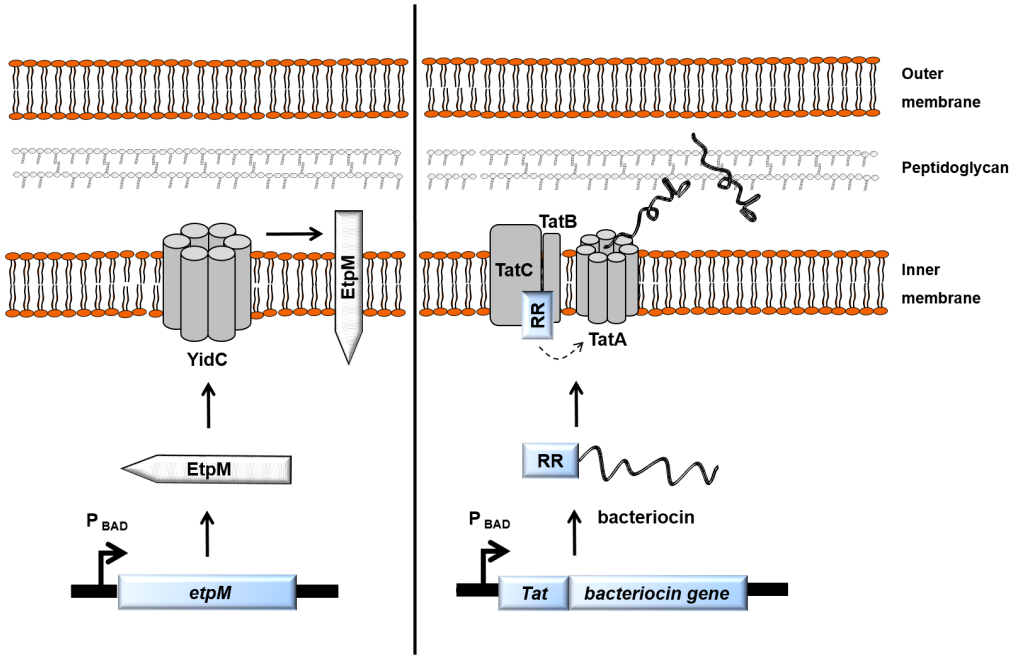
**Figure 1S. a) Colony forming units of *E. coli* strains BL21 [DE3] growing on LB-arabinose plate**. BL21 [DE3] strains expressing the different fusions were grown in LB-glucose until $OD_{600}$ ~ 0.3. Then, they were harvested, washed and resuspended in M9 medium. Each culture was log-diluted and plated onto LB-arabinose and LB- glucose (not shown). **b) Bacterial viability in solid LB medium.** Bacterial CFU/ml counting was performed in solid LB-glucose (dark grey bars) and LB-arabinose (light grey bars). The graphs represent *E. coli* BL21 [DE3] expressing EtpM, EtpM-bacteriocins and RR-bacteriocins. Each bar is the mean obtained from 3 independent experiments. Error bars represent the standard error of the mean. Asterisks (*) indicate statistically significant differences according to Tukey test. P values are: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

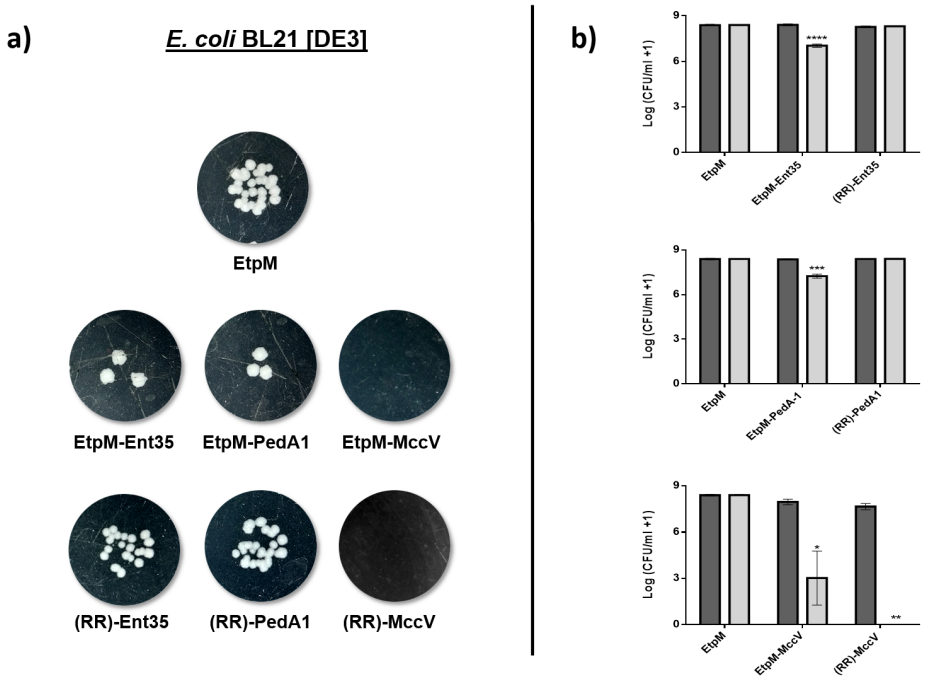
**Figure 2S. Antimicrobial activity of precipitated extracts from strains expressing EtpM, EtpM-PedA1 and RR-PedA1.** After an overnight precipitation with 70% ammonium sulfate, 15 µl of each sample were spotted and tested against *L. monocytogenes* FBUNT.

**Figure 3S. Normalized emission spectra of Laurdan excited at 350 nm in labeled *E. coli* cells.** Previously induced strains were washed and labeled with Laurdan at 37°C. The graphs depict emission spectra of Laurdan-labeled *E. coli* MC4100 strains expressing **a)** EtpM, EtpM-Ent35, RR-Ent35 **b)** EtpM, EtpM-PedA1, RR-PedA1 **c)** EtpM, EtpM-MccV, RR-MccV and *E. coli* MC4100 *sdaC* expressing **d)** EtpM, EtpM-MccV, RR-MccV. The data shown are representative of six separate and independent assays.

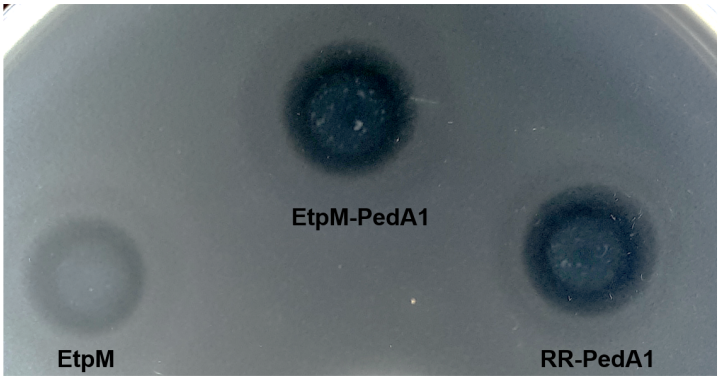

a)
E. coli MC4100
EtpM (control)
(RR)-Ent35
EtpM-Ent35
OD 600
Time (h)
b)
E. coli MC4100
EtpM (control)
(RR)-PedA1
EtpM-PedA1
OD 600
Time (h)
c)
E. coli MC4100
EtpM (control)
(RR)-MccV
EtpM-MccV
OD 600
Time (h)
d)
E. coli MC4100
sdaC
EtpM (control)
(RR)-MccV
EtpM-MccV
OD 600
Time (h)

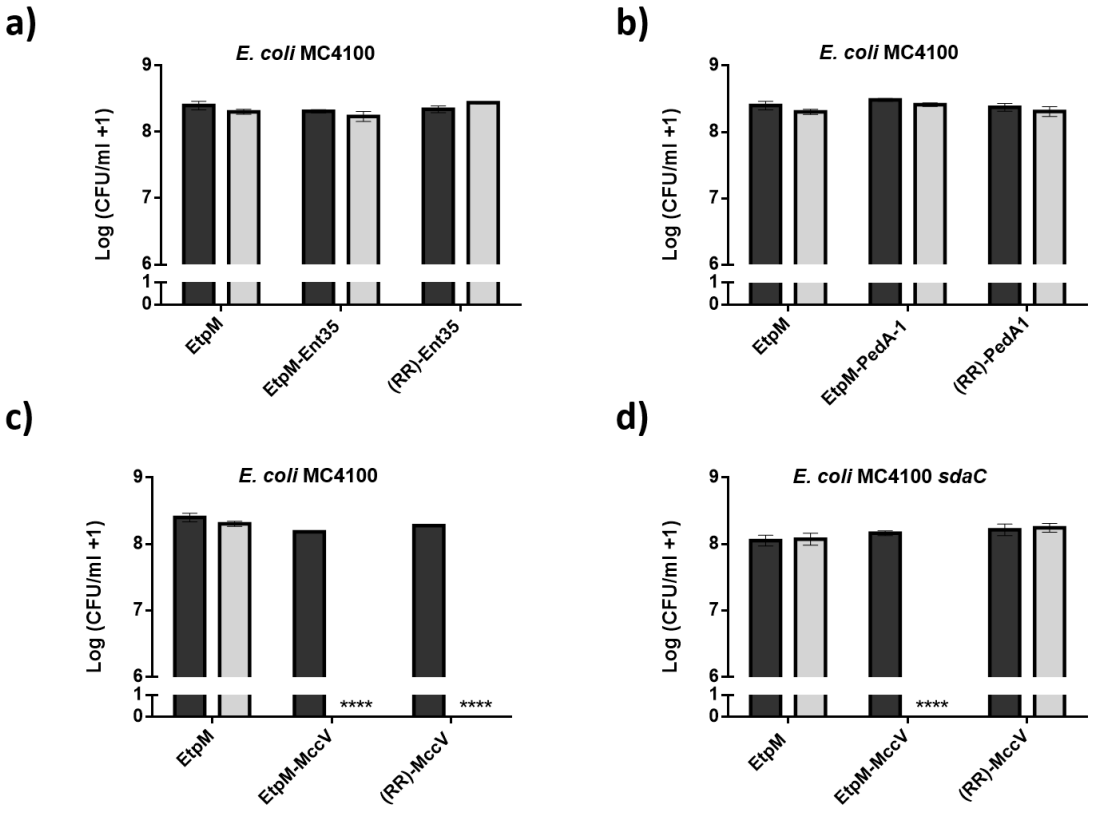

Outer
membrane
Peptidoglycan
Inner
membrane
EtpM
EtpM
EtpM
EtpM
YidC
P BAD
etpM
bacteriocin gene
Arabinose
Glucose
mRNA
EtpM
bacteriocin

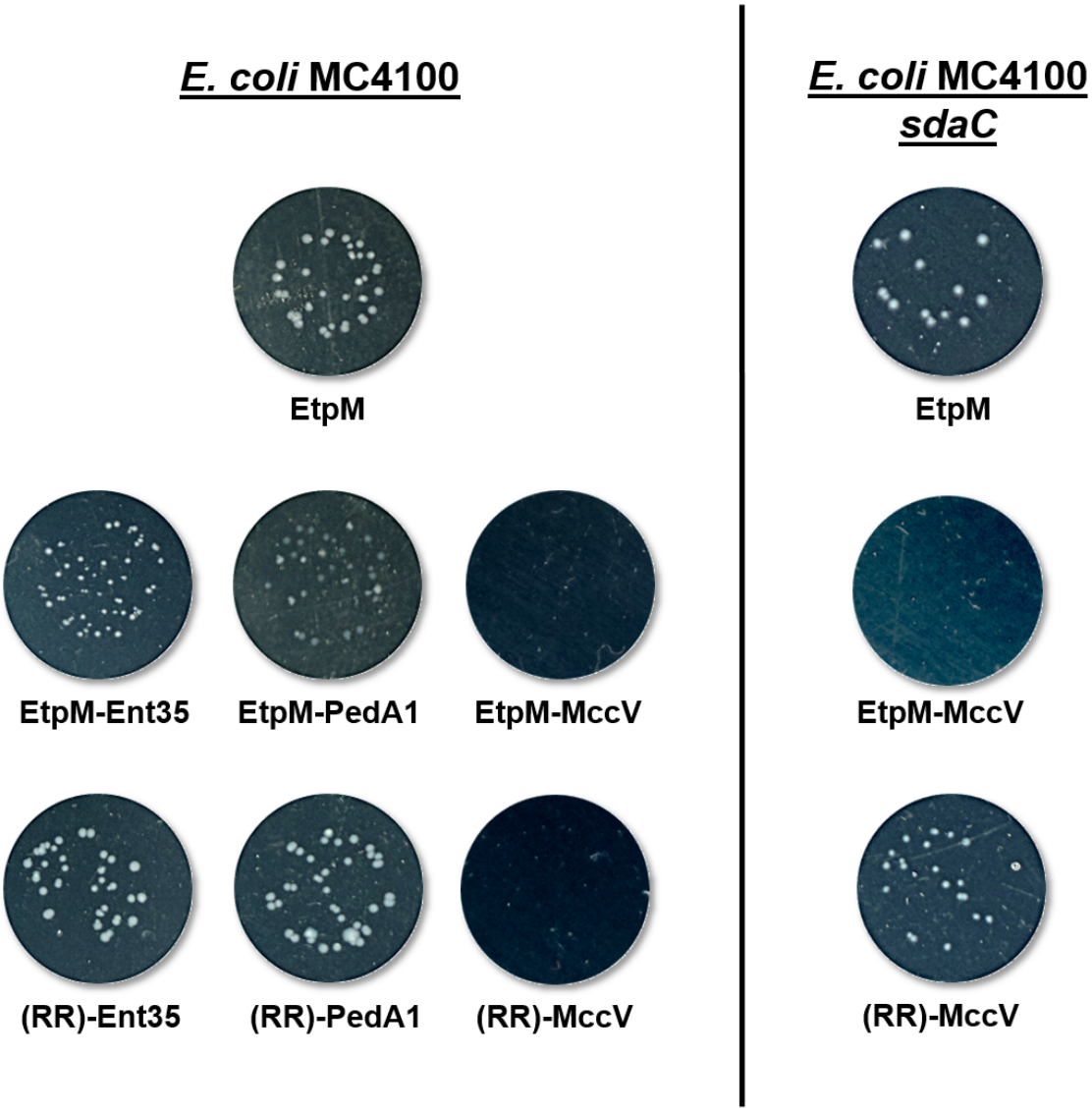

Outer membrane
Peptidoglycan
Inner membrane
EtpM
YidC
TatB
TatC
RR
TatA
EtpM
P BAD
etpM
RR
P BAD
bacteriocin
Tat
bacteriocin gene

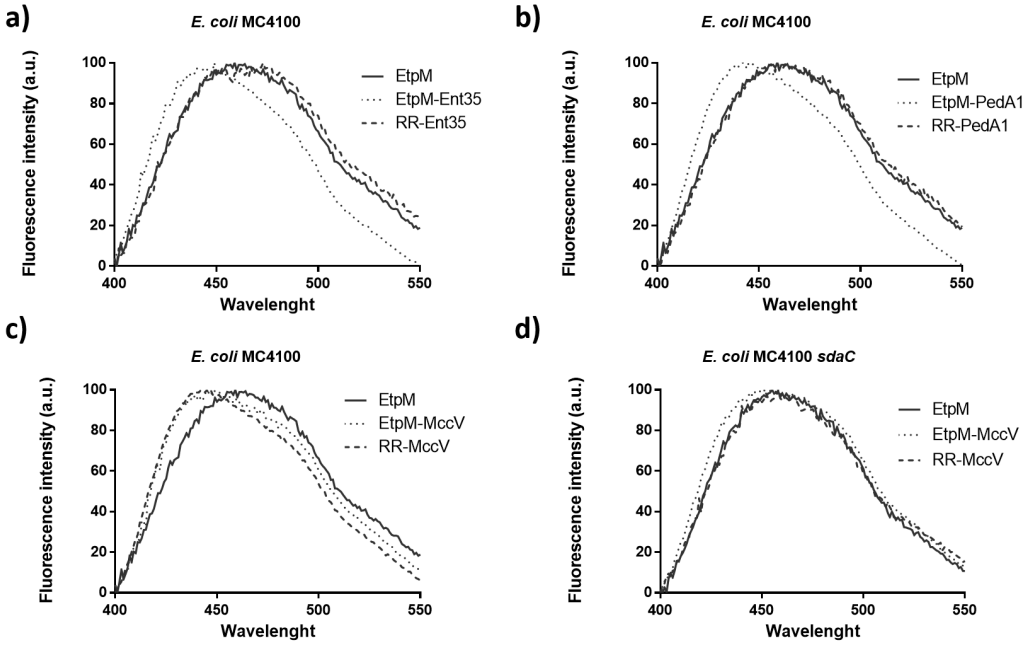

a)
E. coli BL21 [DE3]
EtpM
EtpM-Ent35
EtpM-PedA1
EtpM-MccV
(RR)-Ent35
(RR)-PedA1
(RR)-MccV
b)
Log (CFU/ml +1)
EtpM
EtpM-Ent35
(RR)-Ent35
EtpM-PedA-1
(RR)-PedA1
EtpM-MccV
(RR)-MccV

***L. monocytogenes* FBUNT**

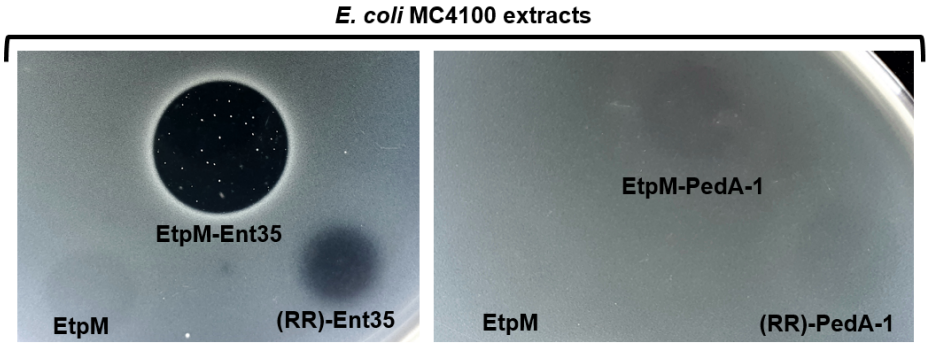



a)
E. coli MC4100
Log (CFU/ml +1)
EtpM
EtpM-Ent35
(RR)-Ent35
b)
E. coli MC4100
Log (CFU/ml +1)
EtpM
EtpM-PedA-1
(RR)-PedA1
c)
E. coli MC4100
Log (CFU/ml +1)
****
****
EtpM
EtpM-MccV
(RR)-MccV
d)
E. coli MC4100 sdaC
Log (CFU/ml +1)
****
EtpM
EtpM-MccV
(RR)-MccV

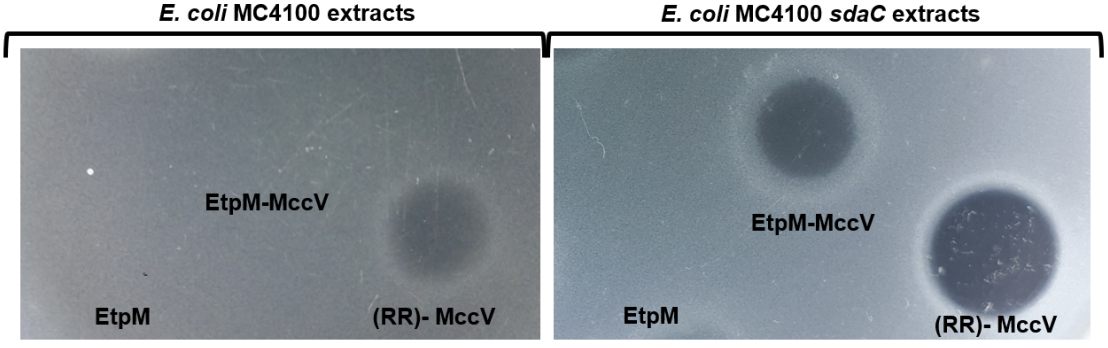

E. coli MC4100
E. coli MC4100 sdaC
EtpM
EtpM
EtpM-Ent35
EtpM-PedA1
EtpM-MccV
EtpM-MccV
(RR)-Ent35
(RR)-PedA1
(RR)-MccV
(RR)-MccV

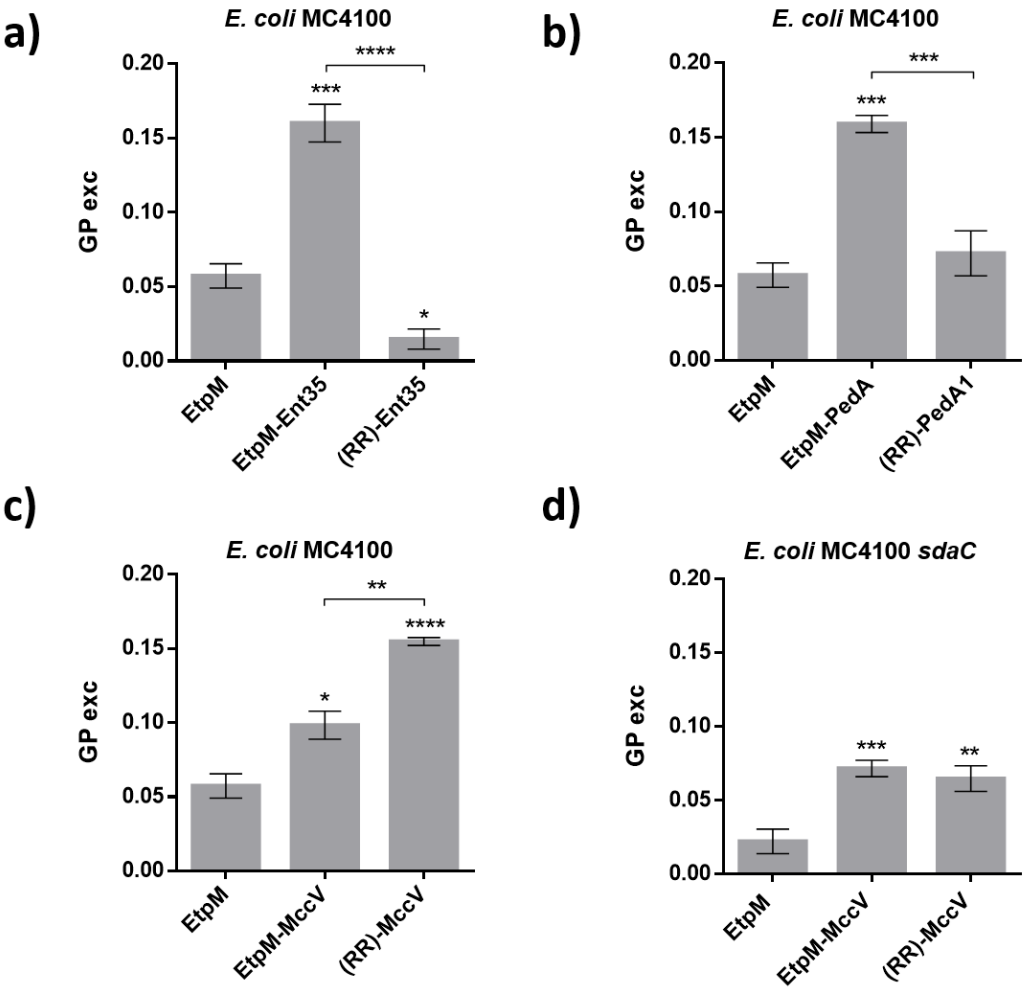

a)
E. coli MC4100
Fluorescence intensity (a.u.)
Wavelenght
EtpM
EtpM-Ent35
RR-Ent35
b)
E. coli MC4100
Fluorescence intensity (a.u.)
Wavelenght
EtpM
EtpM-PedA1
RR-PedA1
c)
E. coli MC4100
Fluorescence intensity (a.u.)
Wavelenght
EtpM
EtpM-MccV
RR-MccV
d)
E. coli MC4100 sdaC
Fluorescence intensity (a.u.)
Wavelenght
EtpM
EtpM-MccV
RR-MccV

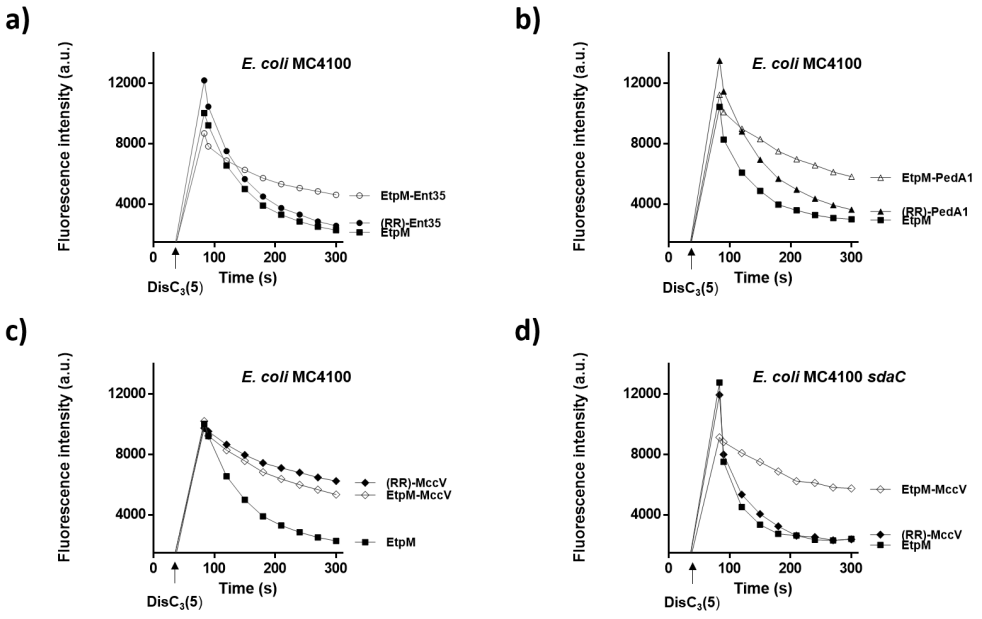
a) *L. monocytogenes* FBUNT lawn




b) *E. coli* MC4100 lawn




a)
E. coli MC4100
GP exc
0.20
0.15
0.10
0.05
0.00
****
***
*
EtpM
EtpM-Ent35
(RR)-Ent35
b)
E. coli MC4100
GP exc
***
***
EtpM
EtpM-PedA
(RR)-PedA1
c)
E. coli MC4100
GP exc
**
****
*
EtpM
EtpM-MccV
(RR)-MccV
d)
E. coli MC4100 sdaC
GP exc
***
**
EtpM
EtpM-MccV
(RR)-MccV


a)
E. coli MC4100
Fluorescence intensity (a.u.)
12000
8000
4000
0
100
200
300
Time (s)
DisC3(5)
EtpM-Ent35
(RR)-Ent35
EtpM
b)
E. coli MC4100
Fluorescence intensity (a.u.)
EtpM-PedA1
(RR)-PedA1
EtpM
c)
E. coli MC4100
Fluorescence intensity (a.u.)
(RR)-MccV
EtpM-MccV
EtpM
d)
E. coli MC4100 sdaC
Fluorescence intensity (a.u.)
EtpM-MccV
(RR)-MccV
EtpM

**Highlights**

- Suicide probes are hybrid peptides aimed to study bacteriocins mechanism of action
- Suicide probes are toxic for *E. coli* even in the absence of the specific receptor
- The receptor would act as an anchor allowing the bacteriocin assembly in the bilayer
- Membrane composition might be an important factor for bacteriocin activity.
- Bacteriocins insertion affects bacterial membrane fluidity and membrane potential

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**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: