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Co-expression and characterization of enterocin CRL35 and its mutant in *Escherichia coli* Rosetta

Abstract: Even though many sequences and structures of bacteriocins from lactic acid bacteria have been fully characterized so far, little information is currently available about bacteriocins heterologously produced by *Escherichia coli*. For this purpose, the structural gene of enterocin CRL35, *munA*, was PCR-amplified using specific primers and cloned downstream of *PelB* sequence in the pET22b (+) expression vector. *E. coli* Rosetta (DE3) pLysS was chosen as the host for production and enterocin was purified by an easy two-step protocol. The bacteriocin was correctly expressed with the expected intramolecular disulfide bond. Nevertheless, it was found that a variant of the enterocin, differing by 12 Da from the native polypeptide, was co-expressed by *E. coli* Rosetta in comparable amount. Indeed, the mutant bacteriocin contained two amino acid substitutions that were characterized by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and HPLC-electrospray (ESI)-Q-TOF tandem mass spectrometry (MS/MS) sequencing. This is the first report regarding the production of mutants of pediocin-like bacteriocins in the *E. coli* expression system.

Keywords: bacteriocins, heterologous expression, MALDI-TOF MS analysis, nanoHPLC-ESI MS/MS analysis

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1 Introduction

Bacteriocins are ribosomally-synthesized antimicrobial peptides produced by prokaryotes. Even though they are expressed by a wide range of bacteria, the specific peptides secreted by Lactic Acid Bacteria (LAB) are of special interest because they are generally recognized as safe for human health. To this purpose, bacteriocin-related toxicity has been investigated and no toxic effects has been reported so far [1, 2].

Bacteriocins produced by LAB can be classified in two main groups [3]: I) lanthionine-containing peptides, II) non-lantibiotic peptides. Both groups are in turn catalogued into subgroups. In particular, group II bacteriocins are categorized as: IIa) pediocin-like bacteriocins, IIb) two-component bacteriocins, IIc) cyclic bacteriocins and IId) peptides not assigned to any other subgroups. Subgroup IIa is the most widely studied, and the bacteriocins belonging to this group have awakened great interest for their possible biotechnological and medical applications in the near future [4–6]. Indeed, pediocin PA-1, the best-known subclass IIa peptide, is already being produced commercially in large quantities (ALTA 2431, Kerry Bioscience, Cork Ireland).

Enterocin CRL35 is a pediocin-like bacteriocin produced by *Enterococcus mundtii* CRL35, of which complete biosynthetic gene cluster has been cloned and sequenced [7]. This bacteriocin is mainly active against food-borne pathogen *Listeria monocytogenes*, other closely related LAB strains [8] and Herpes virus simplex type 1 and 2 [9]. Enterocin CRL35 also exhibits a synergistic effect with some clinical antibiotics, thus suggesting possible clinical applications in addition to its promising use as a food preservative [10].

The sequencing of numerous genomes has allowed the analysis of open reading frames related to the production of antimicrobial substances. Nevertheless, the purification at the peptide level still represents a key step for the elucidation of the molecular structure of antimicrobial compounds. As a matter of fact, preliminary attempts aimed to express the enterocin CRL35 in *E. coli* BL21 consistently showed the production of recombinant peptide(s) with a rather low specific activity. Therefore, the scope of the present work was to purify large amounts of pure recombinant enterocin CRL35 in order to accomplish further characterization by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) and HPLC-electrospray (ESI)-Q-TOF tandem MS (MS/MS).

2 Experimental Procedures

2.1 Strains and culture media

Bacteriocinogenic *E. coli* cells were cultured in Lauria-Bertani (LB) medium (Sigma, St Louis, MI, USA). Opportune antibiotics were added when needed. The natural enterocin CRL35 producer strain, *E. mundtii* CRL35 was grown in LAPTg [11], meanwhile the sensitive strain *L. innocua* 7 was grown in TSB medium supplemented with yeast extracts and 7.5 μg/ml nalidix acid [12]. The *E. coli* strains tested for overexpressing enterocin CRL35 as well as plasmids used in this study are listed in Table 1. Plasmids were amplified and maintained in *E. coli* DH5α. When appropriate, the following antibiotics were used in *E.coli* cultures: 50 μg/ml ampicillin; 30 μg/ml chloramphenicol; 15 μg/ml tetracycline.

2.2 *munA* cloning strategy

munA gene was PCR-amplified using the following primers: munAF3 5´ CATGCCATGGGTAAATACTACGGTAATGGA 3´ and munAR3 5´ CGGGATCCTTAACTTTTCCAACCAG 3´, which encoded a *Nco*I and *Bam*HI recognition sites respectively. The amplified fragment was purified by AccuPrep® PCR Purification Kit (Bioneer, Korea) and digested with the appropriated enzymes. Then, it was ligated into pET22b(+) vector (Novagen) linearized with the same enzymes. *E. coli* DH5α was transformed by

Table 1: Strains and plasmids

Strains and Plasmids	Genotype or relevant characteristics	Reference
Listeria innocua 7	enterocin CRL35 sensitive strain	INRA[1]
Enterococcus mundtii CRL35	enterocin CRL35 producer strain	CERELA[2]
Escherichia coli DH5α	F^-Φ80d*lacZ*ΔM15Δ(*lacZYA-argF*) U169*recA1 endA-1supE4 hsdR17*($r_k^-m_k^+$)*thi*-1*gyrArelA1* (Nal^R)	BRL[3]
Escherichia coli BL21(DE3) pLysS	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^R)	Novagen
Escherichia coli C41(DE3) pLysS	BL21(DE3)-derivative	(Miroux and Walker 1996)
Escherichia coli C43(DE3) pLysS	BL21(DE3)-derivative	(Miroux and Walker 1996)
Escherichia coli Rosetta(DE3) pLysS	F^- *ompT hsdS*B(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)	Novagen
Escherichia coli Origami(DE3)	Δ(*ara–leu)7697* Δ*lacX74* Δ*phoA PvuII phoR araD139 ahpC galE galK rpsL* F'[*lac+ lacI q pro*] (DE3) *gor522*::Tn*10 trxB* pLysS (Cam^R, Kan^R, Str^R, Tet^R)	Novagen
Escherichia coli Rosetta-gami 2(DE3)	Δ(*ara–leu)7697* Δ*lacX74* Δ*phoA PvuII phoR araD139 ahpC galE galK rpsL* (DE3) F'[*lac+ lacI q pro*] *gor522*::Tn*10 trxB* pRARE23 (Cam^R, Str^R, Tet^R)	Novagen
pET-22b(+)	Bacterial vector for protein expression in the periplasm, T7*lac* promoter, Amp^R	Novagen
pE35	pCR-Blunt II- Topo (Invitrogen) with enterocin CRL35 biosynthetic clustercloned	(Saavedra et al. 2004)
pEM05	pET-22b(+) with *munA* cloned	This work

[1]INRA, Jouy-en-Josas, France. [2]CERELA: Centro de Referencias para Lactobacilos. Tucumán. Argentina [3]BRL, Bethesda Research Laboratories.

the calcium chloride transformation protocol [13] and plasmids purified by Wizard® Plus Minipreps DNA Purification kit (Promega). The resultant plasmid was called pEM05 (Table 1). Competent cells from each expression host strains listed above were prepared and transformed following the same protocol.

2.3 Recombinant enterocin CRL35 production

Enterocin CRL35 production was tested with all *E. coli* strains listed in Table 1 in order to find the best producer in LB broth. For this purpose, cells were grown in LB broth till OD= 0.8, then 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Genbiotech, Argentina) was added and cells were incubated 1 h at 37 °C with aeration. Anti-*Listeria* activities from supernatants, intracellular and periplasmic fractions were assayed by a modified spot-on-lawn assay [14]. After complete adsorption of each diluted sample (10 µL), TSB plates were covered with 5 ml of 0.6 % (w/v) agar inoculated with 10^6 cells of the sensitive strain *L. innocua* 7 (supplementary information for more details). Once *E. coli* Rosetta was chosen, the optimal concentration of inducer was selected by changing IPTG concentration from 0.1 to 2 mM. Afterward, bacteriocin synthesis was investigated in minimal media varying different parameters such as salt concentration, tryptone or yeast extract supplement, carbon source (glucose, glycerol), aeration and time of induction (supplementary information).

2.4 Recombinant enterocin CRL 35 purification procedure

When growth conditions in minimal medium were set up, bacteriocinogenic *E. coli* Rosetta cells were grown till mid-log phase in 100 ml of modified M9 medium. Cultures were induced with IPTG and after 150 min cells were pelleted at 10,000 x *g* for 15 min. Supernatants were precipitated with ammonium sulfate (70 % saturation) and the pellet was dissolved in deionized water. Afterward, sample was C_{18} reverse-phase HPLC fractionated using an ÄKTA-purifier chromatography system (GE Amersham Pharmacia) equipped with a µBonda Pack column (10 µm 300 x 4.6 mm Waters) and applying a non-linear gradient of acetonitrile. Effluents were UV monitored at 254 and 280 nm using a multiwave detector. The active fractions were pooled, dried under vacuum with a Savant speed-vac and finally sub-fractionated by electrophoresis, according to the typical protocol for small peptides [15]. Purified bacteriocin was quantified by measuring absorbance at 280 nm, based on the molar extinction coefficient of the synthetic enterocin CRL35 (Biosynthesis, TX, USA). HPLC purified enterocin (50 ng) was suspended in loading buffer without β-mercaptoethanol and kept 4 min in a boiling water bath. Thereafter, the gel was divided in two halves, one was stained with either SYPRO® Ruby Protein Gel Stain (Sigma) or Colloidal Coomassie Blue Brillant G-250 [16] to visualize the bands and the other half was fixed with methanol: acetic acid: water (40:10:50). After fixing, the gel was washed with sterile double distilled water and placed on a TSB agar plate. Then, it was covered with 10 mL of 0.6 % (w/v) agar inoculated with the sensitive strain and plate was incubated at 30 °C for 16 h.

2.5 Characterization of recombinant enterocin CRL35 by MS analysis

The HPLC active fraction was lyophilized and re-dissolved in 100 µL of 0.1% trifluoroacetic acid (TFA). Prior to MS analysis, an aliquot (10 µL) was further desalted by C_{18} Zip Tip® micro-columns (Millipore, Bedford, CA, USA). MALDI-TOF MS experiments were carried out on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N_2 laser (λ = 337 nm). Sinapinic acid (Sigma, 50 % acetonitrile (v/v) / 0.1 % TFA), was used as the matrix. The mass spectra were acquired in the reflector linear ion mode using the Delayed Extraction (DE) technology. The instrument operated at an accelerating voltage of 25 kV. External mass calibration was performed with a commercial standard peptide mixture (Sigma). Raw data were elaborated using Data Explorer 4.0 software purchased with the spectrometer.

2.6 Reduction and Cys-alkylation of enterocin CRL35

An aliquot (10 µL) of the HPLC fraction of enterocin CRL35 was ten-fold diluted with a denaturing/reducing buffer (6M guanidine HCl, 75 mM Tris, 1 mM EDTA, 10 mM DTT, pH 8.0) and incubated 1 h at 56 °C. After reduction, cysteines were alkylated 40 min at room temperature in the dark with iodoacetamide (three-fold molar excess with respect to DTT). Cys-alkylated enterocin was purified using a C_{18} Zip Tip® micro-columns, dried in a speed-vac and analyzed by MALDI-TOF MS.

2.7 Enzymatic proteolysis of enterocin CRL35

An aliquot of the enterocin CRL35 (10 µL) was diluted with 20 µl of 50 mM ammonium bicarbonate, pH 8.0, and incubated overnight at 37 °C with 0.2 µg of sequencing grade endo-proteinase Asp-N (Roche, Mannheim, Germany). Reaction was stopped by freeze-drying and peptides were reconstituted in 20 µL of 0.1 % TFA. Resulting peptides were analyzed by MALDI-TOF MS, using the same conditions above, except for the matrix that was α-cyano-4-hydroxycinnamic acid (Sigma, 10 mg/ml in 50 % acetonitrile / 0.1 % TFA, v/v). Mass values are reported as average MH^+, except where indicated. The remaining Asp-N hydrolyzed peptides were analyzed with nanoflow HPLC-ESI MS/MS.

2.8 nanoHPLC-ESI MS/MS analysis of recombinant enterocin CRL35

Nanoflow HPLC separations were carried out with an Ultimate 3000 nano HPLC system (Dionex, Sunnydale, CA, USA) equipped with an autosampler. Asp-N (2 µL) digests of enterocin CRL 35 were loaded onto a C_{18} trapping cartridge (LC Packings, Dionex, USA) using a Famos autosampler (Dionex) and flushed 5 min at the flow rate of 5 µL/min by means of the loading pump. Separation was carried out using a commercial capillary column (PepMap, C18, 5 µm, 300 Å, 75 µm x 15 cm, LC Packings, Dionex) applying a linear gradient from 2% to 40% of solution B over 60 min, following 10 min of isocratic elution at 2% of solution B, at a constant flow rate of 300 nL/min. Solvent A was H_2O containing 0.1% formic acid; solvent B was 0.08% formic acid in 80% acetonitrile (v/v). Eluted peptides were analyzed on-line using an ESI-Q TOF Q-Star Pulsar instrument (Applied BioSystems, Foster City, CA, USA) equipped with a nanospray source (Protana, Denmark). The MS/MS fragmentation was performed in the information-dependent acquisition (IDA) mode. Precursor ions were selected for fragmentation using the following MS to MS/MS switch criteria: ions greater than m/z 400.0, charge states 2 to 4, intensity exceeds 15 counts, former target ions were excluded for 60 s and ion tolerance was 50.0 mmu. CID was used to fragment multiple charged ions, and N_2 was used as the collision gas. MS/MS spectra were manually assigned, with the aid of the GPMAW 5.1 software (Lighthouse data, Odense, Denmark) that calculates the mass of the peptides and expected fragment ions.

3 Results

3.1 Cloning strategy for *munA* gene

The plasmid pET22b(+) was chosen for heterologous expression for two reasons. First of all, it allows the cloning of the target gene downstream the sequence encoding the signal peptide of the periplasmic protein PelB. Therefore, the protein of interest would be exported by the *E. coli* sec pathway to the periplasm, guaranteeing an easier purification as compared to proteins purified from cytoplasmic material. On the other hand, the cloned gene is under the strong promoter T7*lac*. Once *munA* was directionally cloned into pET22b(+) vector, the nucleotide sequence was analyzed by the DNA sequencing service at CERELA (CCT-Tucumán/CONICET). It was confirmed that the DNA fragment had correctly been inserted and no frame shifts were observed. Thus, a variant of enterocin CRL35 with two extra amino acids i.e. methionine and glycine was designed. These two amino acids come from the *Nco*I sequence utilized for cloning. In fact, signal peptidase would cleave the PelB signal peptide just before methionine. The cloned *munA* gene has an AGA codon instead of AAA as the 22nd codon of the sequence, which implies the conservative substitution Lys22Arg. A similar finding was reported by Acuña et al. when designed a chimeric peptide combining enterocin CRL35 and microcin V [17].

3.2 Purification of enterocin CRL35

Enterocin CRL35 was precipitated from culture supernatant and passed through C_{18}-HPLC as described in Experimental Procedures. The weak broadened peak (n. 2) eluted at intermediate retention time of the HPLC chromatogram exhibited exclusive anti-*Listeria* activity (Figure 1). On the contrary, both the peak eluted at low percentages of acetonitrile and the “washing peak” with very high retention time were inactive. In particular, the hydrophobic HPLC peak n. 3 contained no MS detectable polypeptides, but polymeric species (polyethylene) most likely released from the chromatographic stationary phase. Chromatographic fractions were further analyzed by SDS-PAGE. No significant amounts of contaminating proteins were detected with either SYPRO® Ruby or Comassie G-250 staining: the culture supernatant showed just a few faint protein bands (data not shown), which were expected because the culture medium was prepared with yeast extracts and no other protein source. It has to be considered that *E. coli* is known to not excrete many proteins into the modified M9 medium [18]. Most of the peptides present in the supernatant belonged to the yeast extract since sterile modified M9 medium, analyzed as the control, exhibited a strictly related proteinaceous pattern. Although enterocin CRL35 could not be stained by the protocols used, its presence was demonstrated by an activity gel assay. Indeed, an inhibition zone because

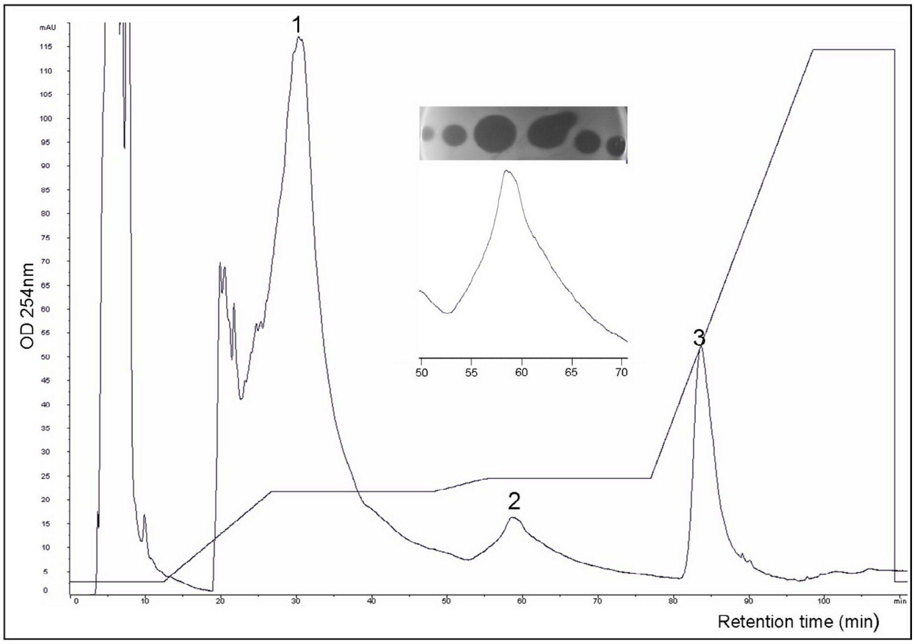

Figure 1: C_{18}-HPLC purification of Enterocin CRL35. The ammonium sulfate active fraction was dissolved in deionized water and fractionated as described. Elution was followed at OD= 254 nm while activity was analyzed in every fraction by the spot-on-lawn assay. The inset shows the anti-*Listeria* activity assay performed with peak n. 2. This result is representative of at least 10 independent replicate purifications.

of antimicrobial activity of enterocin CRL35 against *L. innocua* 7 at about 4.5 kDa clearly appeared as shown in Figure 2.

Table 2 summarizes the purification process. It can be observed that a 75-fold purification from M9 culture supernatants was achieved, considering that the starting concentration was around 0.5 mg/ml protein as determined by the Lowry method [19]. On the other hand, 7 % of the initial activity was recovered after two steps of purification. Some experiments required culture supernatants to be boiled 10 to 20 minutes to precipitate large proteins and improve the purification procedure. However, results showed no changes in purification yields.. This latter result was expected since most of the contaminants are small peptides, which generally lack elements of tertiary structure and, therefore, are not affected by temperature.

3.3 Mass spectrometry

The MALDI-TOF analysis of the HPLC active peak exhibited a couple of dominant signals, with nearly comparable intensity, at m/z 4507.0 and 4519.0 (Figure 3A). The first signal is compatible with that expected for enterocin CRL35. The second one was deemed to correspond to a variant form of enterocin CRL35 that differs by 12 Da from the native bacteriocin, probably due to mutation(s) arisen after transformation in *E. coli* Rosetta. Interestingly,

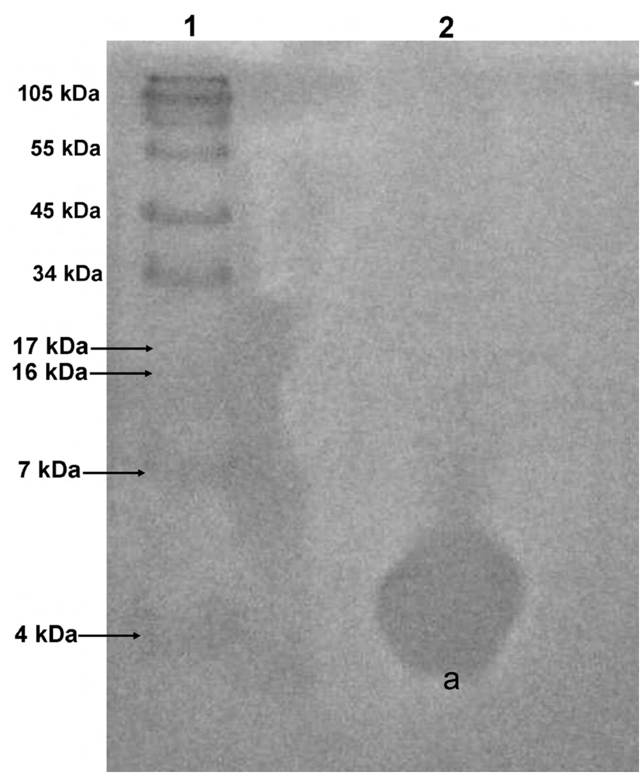

Figure 2: Recombinant enterocin CRL35 visualized by activity gel. The active HPLC fractions were pooled and concentrated. The broad range molecular weight markers were a mixture of standard pre-stained proteins. Inhibition zone (a) was observed after 16 h of incubation at 30 °C. This result is representative of three independent experiments.

Table 2. Purification of recombinant enterocin CRL35.

	Total protein (mg)	Total activity (AU)	Specific activity (AU/mg)	Yield (%)	Purification
Supernatant	534	1,000	1.87	100	1
$(NH_4)_2SO_4$ fraction	8	150	18.75	15	10
Peak 1 HPLC	0.5	70	140	7	75

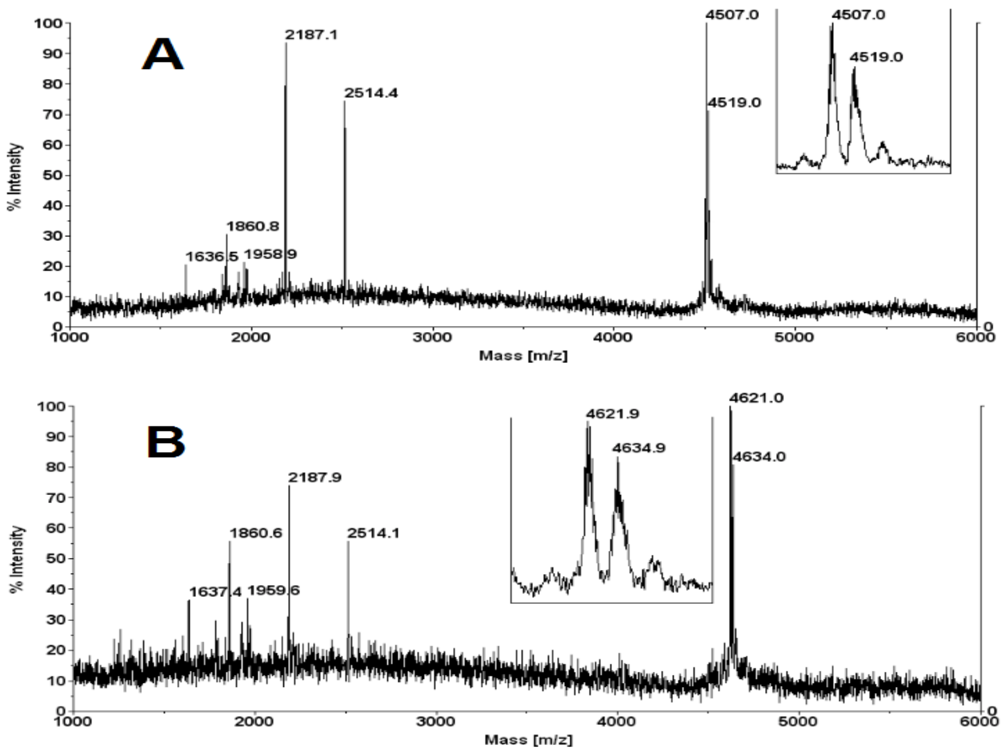

Figure 3: MALDI-TOF MS analysis of the HPLC purified active fraction either before (A) or after (B) reduction/Cys-alkylation by iodoacetamide. The measured mass of the reduced enterocin demonstrates that the two cysteines are engaged in an intramolecular disulfide bond, thus highlighting the ability of the selected *E. coli* strain to correctly process the bacteriocin, at the post-synthetic level. The magnified views in the insets evidence the presence of two enterocin isoforms differing by 12 Da. The minor signals at lower *m/z*, most likely due to interfering medium peptides, were unaffected by Cys-alkylation. No oligomeric forms of enterocin CRL35 were observed at higher *m/z* (not shown).

both signals were shifted at *m/z* 114 Da higher (57 Da / carbamidomethyl group) after cys-carbamidomethylation, confirming that enterocin CRL35 contained two cysteins formerly engaged in a disulfide bond (Figure 3B). Such an outcome demonstrated the ability of the selected *E. coli* strain to correctly express and to post-synthetically process the bacteriocin, which preserved the expected folding ruled by the intramolecular disulfide bond. The minor signals appearing at lower *m/z* were unaffected by Cys-alkylation.

The enterocin CRL35 was expected to be split roughly in the middle by Asp-N, which specifically cleaves proteins at the N-terminal side of aspartate residues. Thus, the unreduced polypeptide was incubated with Asp-N and hydrolyzed peptides were analyzed again. The MALDI-TOF MS analysis showed three main signals: at *m/z* 1892.0, which matches the theoretical mass of 1-18 enterocin CRL35 containing an intramolecular disulfide bond and the $M+H^+$ 2628.3 and 2640.4 (monoisotopic) of nearly equal intensity belonging to the 19-45 peptide (Figure 4 and inset). The presence of these two peptides differing by 12 Da demonstrated that the possible amino acid substitutions between the enterocin variants were confined to the 19-45 region.

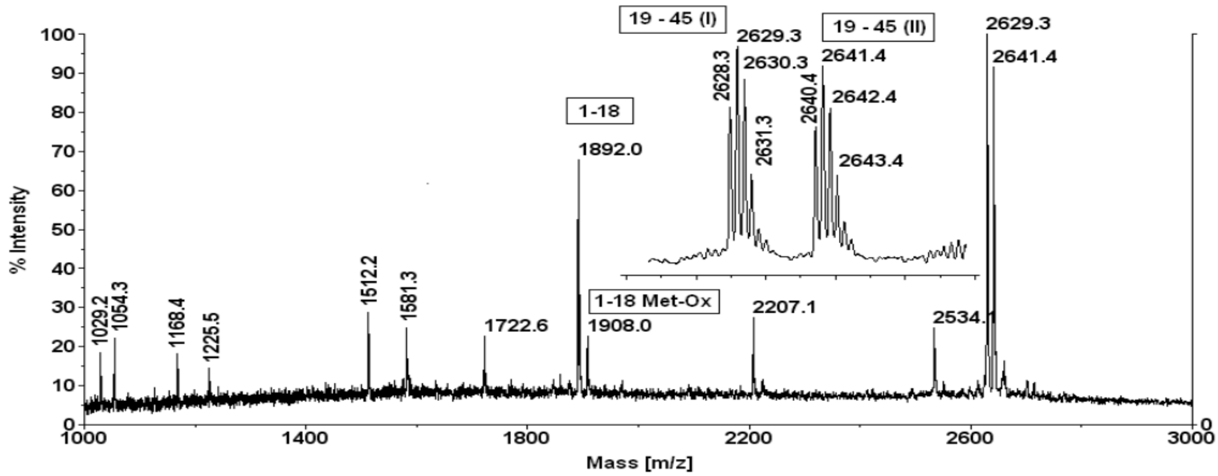

Figure 4: MALDI-TOF MS analysis of the Asp-N digest of the HPLC peak no. 2. Peptide signals were assigned according to the predicted sequence of enterocin CRL 35. The 1-18 peptide (*m/z* 1892.0) is flanked by a smaller signal with +16 Da (*m/z* 1908) due to oxidized Met1. The dominant signals were M+H$^+$ 2628.3 and 2640.4 (monoisotopic) of nearly equal intensity, which arose from the native and variant 19-45 enterocin peptide.

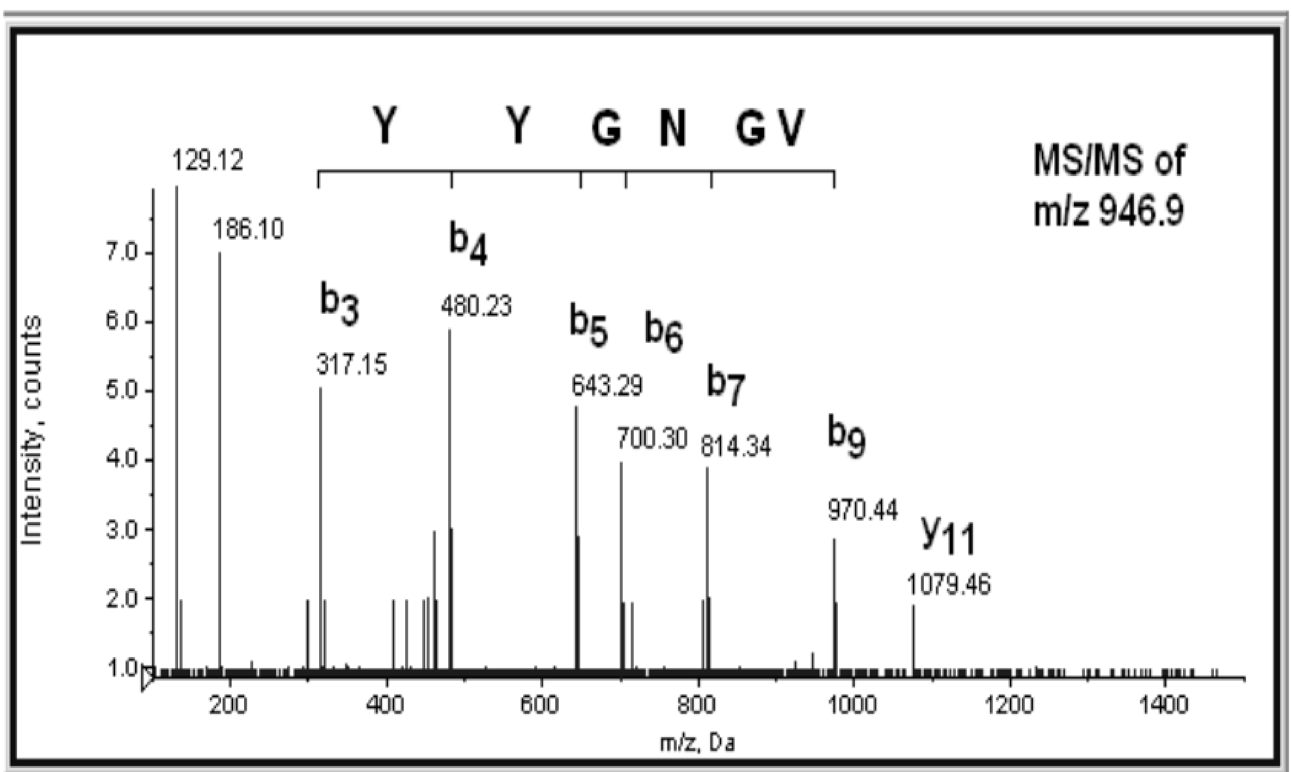

Figure 5: MS/MS confirmation of the 1-18 peptide showing a six-amino acid internal sequence. Additional internal fragments also occur.

When analyzed by nanoHPLC-ESI-Q-TOF MS/MS, the collision induced MS/MS fragmentation of the doubly-charged ion *m/z* 946.9 (Figure 5) confirmed the correct identification of the 1-18 peptide. A partial internal sequence including the expected YGNV motif of enterocin CRL35, was inferred (Figure 5). However, the 1-18 sequence could not be completely reconstructed, probably owing to the internal disulfide bridge that limited the fragmentation of the proximal amino acids. On the other hand, the nanoLC-ESI Q-TOF MS/MS analysis confirmed the presence of two peptides eluting in two closely overlapping HPLC peaks (triply-charged ions *m/z* 876.81 and 880.81, M+H$^+$ 2628.3 and 2640.40) which corresponded to the 19-45 peptide of the native and mutant enterocin CRL35, respectively. The collision induced MS/MS fragmentation of the *m/z* 876.81 and 880.81 ions (Fig 6A and 6B) allowed to reconstruct significant part of the peptide sequences, demonstrating the effective integral expression of enterocin CRL35 by *E. coli* Rosetta and the simultaneous production of a mutant form carrying two amino acid substitutions, namely proline instead of glycine at position 21 and lysine replacing the arginine 22 (P21-K22 à G21-R22). The expected mass difference for such a double replacement is 12 Da that also matches the difference recorded between the unhydrolyzed variants. In order to confirm this finding, plasmid DNA was purified from 5 ml culture of bacteriocinogenic *E. coli* Rosetta, 2.5 h after induction with IPTG, PCR-amplified using T7 promoter and terminator

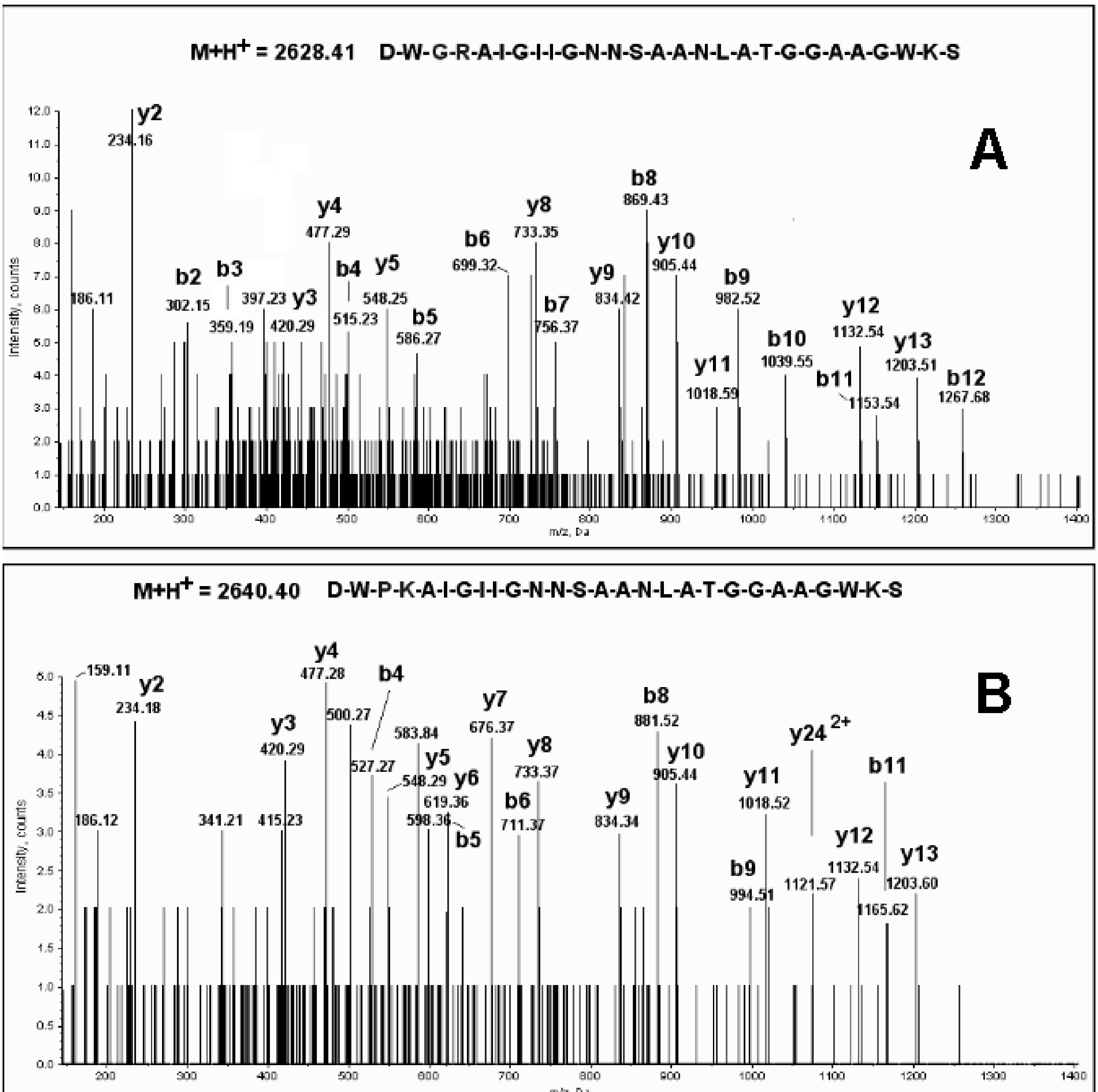

Figure 6: MS/MS collision induced decay (CID) of the triply-charged ions m/z 876.81 (A) and 880.81 (B). The sequence reconstruction of the y- and b- fragmentation series allowed inferring the two peptide sequences. The peptides differ by a double amino acid substitution: G21-R22 à P21-K22.

primers and *munA* gene was thus sequenced. It was found that the 21st codon of *munA* was present as the expected GGA (Gly) but also as CCA, which encodes for proline. In the same trend, AGA was found to be the 22nd codon but AAA was also detected, which implies the mutation R22K. Therefore, the double amino acid substitution in this new enterocin CRL35 variant is the result of a triple base mutation in the *munA* structural gene present in the pEM05 plasmid. Based on these results, it is probably that two subpopulations of *E. coli* Rosetta are present i.e. one group of cells that produces the expected enterocin CRL35 variant (Lys22Arg mutation and two extra amino acids at the N-terminus), and cells that express another variant 12 Da larger.

4 Discussion

One of the first attempts for the heterologous expression of bacteriocins in *E. coli* was reported 15 years ago by Miller et al., who fused the pediocin PA-1 structural gene with the maltose-binding protein and used a periplasmic leaky *E. coli* host [20]. The main scope of that pioneering work was not to over-express pediocin PA-1 for further structural and activity studies but to investigate the secretion process of that peptide. In fact, their approach had several pitfalls such as a leaky synthesis even in the absence of IPTG. In addition, the fusion protein could not be cleaved in order to release the mature bacteriocin upon secretion [20]. However, this study clearly showed that *E. coli* was indeed a good host for LAB bacteriocins and that they could be secreted via the sec machinery.

In this regard, it should be noted that there are several bacteriocins from lactic bacteria that can be exported by the general secretion pathway such as hiracin JM79 [21], divergicin A [22], acidocin B [23] and enterocin P [24]. Moreover, enterocin P was already successfully expressed in *E. coli* and exported by its own signal peptide, which was recognized by the general secretion machinery of *E. coli* [25]. Nowadays, there is a growing number of reports on the heterologous expression of bacteriocins in *E. coli*. Each approach has its own advantages and disadvantages [25–32].

In the present work, enterocin CRL35 has been expressed fused to the C-terminus of PelB signal sequence. Even though the peptide was thought to be confined to the periplasm, enterocin was mainly recovered from the culture supernatant. No extra manipulation of *munA* was needed since the host strain contained universal tRNAs, thus guaranteeing optimal synthesis of this heterologous peptide. The purification protocol presented here allowed a straightforward purification of the bacteriocin from the medium. However, it was found that the final sample had lower specific activity as compared to the pure synthetic peptide [7]. In fact, peptide was obtained at a final concentration of approximately 100 μM, considering 0.5 mg of pure bacteriocin per liter, but the activity was similar to that displayed by 10 μM of synthetic peptide (data not shown).

The converging indications of 1) the exact matching of the peptide MW as confirmed by MALDI-TOF analysis, 2) the specific cleavage by Asp-N, which releases the predicted fragments with the exact theoretical masses, 3) the presence of two cysteine residues that are engaged in a disulfide bond (confirmed by the analysis after reduction/alkylation) and 4) the identity of MS/MS fragment ions, undoubtedly prove that our enterocin CRL35 variant was indeed produced by *E. coli* Rosetta (with the Lys22Arg substitution as pointed out in the Results section). However, the MS studies also identified an additional enterocin CRL35 variant that had two amino acid substitutions with respect to the starting enterocin i.e. Gly21Pro and Arg22Lys. Since the *munA* sequence in pEM05 plasmid was verified and no errors were found, the mutations leading to those substitutions had to arise in one subpopulation of *E. coli* Rosetta . The mutant peptide may be responsible for the low specific antimicrobial activity detected. Even though enterocin CRL35 does not behave as a suicide probe, its production may pose a problem for *E. coli*, such as the partial dissipation of the proton-motive force. Indeed, a slight decrease in *E. coli* Rosetta survival was observed upon induction with either IPTG or lactose. Since enterocin CRL35 was mainly recovered from the extracellular medium rather than the periplasmic compartment, it can be argued that enterocin has to permeate the outer membrane of *E. coli* perhaps by partial disruption of the periplasmic leaflet of the outer membrane. This would imply that a similar disruption of the inner membrane may take place as well. Anyways, mutations that lead to less harmful or inactive heterologous proteins are not uncommon for BL21 derivatives. Consistently, the enterocin CRL35 variant we characterized has a proline instead of glycine, which might imply a major change in the bacteriocin tridimensional structure upon binding to membranes. As was previously shown for leucocin A and carnobacteriocin B2, C-terminus of pediocin-like bacteriocins may form a helical structure just after the disulfide bond [33, 34]. The occurrence of a Pro at the position 22 might definitively prevent the functional bacteriocin folding upon interaction with membranes. It can be speculated that the mutant enterocin would disrupt the pore structure at the *Listeria* membrane, which is thought to be the key step in the bacteriocin mechanism of action. This is the first time that a mutant of pediocin-like bacteriocins co-expressed in *E. coli* is reported. More strikingly, two amino acids were changed in the enterocin variant as a consequence of three point mutations in the enterocin CRL35 structural gene, which is a highly unusual event. This outcome suggests the possibility of carrying out detailed relevant structure-function studies.

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Supplementary information

Selection of the best host for heterologous expression of enterocin CRL35

A number of possible hosts for heterologous expression of enterocin CRL35 were tested in order to select the most suitable strain for bacteriocin production. Several *E. coli* host were selected: *E. coli* BL21 is a protease-defective strain widely used for heterologous expression. *E. coli* C41 and *E. coli* C43 are BL21 derivatives that were isolated as clones that efficiently expressed toxic proteins (*1*). On the other hand, *E. coli* Rosetta is another BL21-derived strain that has a plasmid encoding rare

E. coli codons, allowing the normal synthesis of heterologous proteins from almost all sources without needing further genetic manipulations. *E. coli* Origami strain is a double mutant in thioredoxin reductase and glutathione reductase with an oxidizing cytoplasm that allows disulfide bond formation. Finally, *E. coli* Rosetta-gami 2 is a combination between Origami and Rosetta strains, thus displaying both features: a universal translation and an oxidizing cytoplasmic environment. Interestingly, anti-*Listeria* activity was present not only in the periplasm fractions but also mainly in the culture supernatants as though the peptide could permeate the outer-membrane in all the strains tested. However, important differences in enterocin CRL35 production were found among them (Fig. S1).

The fact that *E. coli* Rosetta and *E. coli* Rosetta-gami 2 were the best strains is not surprising since they are able to synthesize proteins from all sources with no differences because of "rare" codons. In fact, enterocin CRL35 gene has the codon GGA repeated four times as well as the codon AGA, as it was mentioned above. Both codons are infrequent in *E. coli* and this issue would be a potential problem for heterologous expression of this bacteriocin in *E. coli* (*2*). Even though *E. coli* Rosettagami 2 is a suitable strain for producing enterocin CRL35, it has a major drawback: its growth rate is extremely slow (*3*).

Moreover, since this peptide is secreted into the periplasm and eventually into the culture medium, there is no need for assuring intracellular disulfide bond formation. For these reasons, *E. coli* Rosetta was chosen as the producing strain.

It is important to stress that even though no important changes in cell survival were found during enterocin CRL35 expression, a slight decrease in cell viability was seen for all producing strains (data not shown). As it can be seen in Fig. S2, the optimal concentration of IPTG was 1 mM.

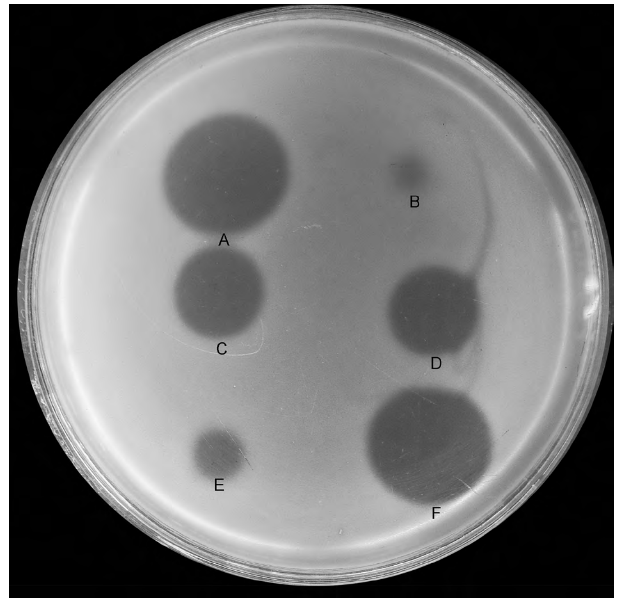

Figure S1: Expression of enterocin CRL35 in different *E. coli* strains. Cells were grown in LB medium till mid-log phase at 37 °C. Then, they were induced with 0.5 mM IPTG and supernatants were collected by centrifugation. 10 µL of different dilutions of each sample were spotted onto TSB plates and *L. innocua* 7 was seeded into soft agar. A: *E. coli* Rosetta, B: *E. coli* C41, C: *E. coli* Origami, D: *E. coli* BL21, E: *E. coli* C43, F: *E. coli* Rosetta-gami 2. The plate shown is representative of at least 5 independent experiments.

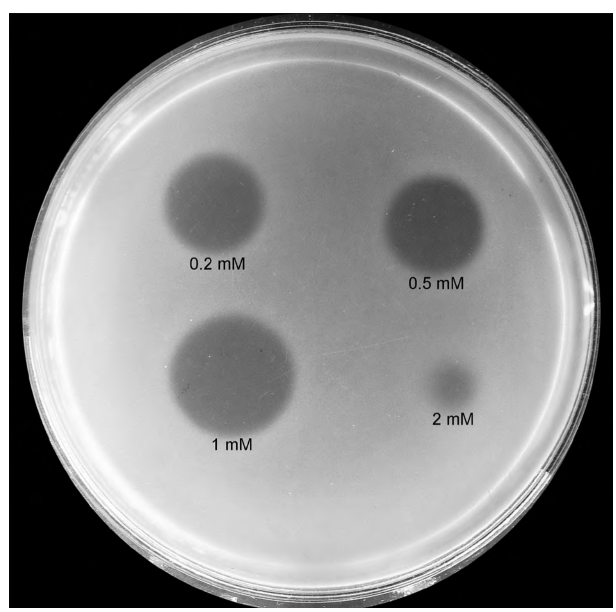

Figure S2: Induction of enterocin CRL35 synthesis. *E. coli* Rosetta was grown in LB till mid-log phase and induced with 0.2, 0.5, 1 and 2 mM IPTG. Culture supernatants were collected by centrifugation and inhibition halos were visualized as described in materials and methods by the spot-on-lawn assay. The plate shown is representative of at least 5 independent experiments.

Because enterocin CRL35 was able to cross the outer-membrane and therefore reach the culture medium, the purification had to be focused in this fraction. Enterocin production was tested in M9 (Sigma) as well as in MT medium (*4*). Since no significant difference was found between these two media, M9 was selected because M9 base can be obtained ready to use from the purchaser. Supplements such as tryptone, yeast extract and NaCl were added at different final concentrations and strains were induced with 1 mM IPTG. Finally, the chosen condition for enterocin CRL35 expression in M9 medium was: 0.6 % glycerol, 0.1 % yeast extract, 1 % NaCl, 1 mM IPTG with an induction time of 150 minutes. 37 °C turned out to be better than the usual 30 °C for heterologous expression.

Even though anti-*Listeria* activity was always higher in LB medium, the modified M9 medium allowed an acceptable production. In addition, it was found that supplementation of 10 mM EDTA and 0.05 % tween 20 at the time of induction significantly increased the final yield. It can be hypothesized that these chemicals would enhance the permeation of enterocin CRL35 through the outer-membrane of *E. coli* (*5*).

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