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A new hybrid bacteriocin, Ent35–MccV, displays antimicrobial activity against pathogenic Gram-positive and Gram-negative bacteria

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ABSTRACT

Bacteriocins and microcins are ribosomally synthesized antimicrobial peptides that are usually active against phylogenetically related bacteria. Thus, bacteriocins are active against Gram-positive while microcins are active against Gram-negative bacteria. The narrow spectrum of action generally displayed by bacteriocins from lactic acid bacteria represents an important limitation for the application of these peptides as clinical drugs or as food biopreservatives. The present study describes the design and expression of a novel recombinant hybrid peptide combining enterocin CRL35 and microcin V named Ent35–MccV. The chimerical bacteriocin displayed antimicrobial activity against enterohemorrhagic *Escherichia coli* and *Listeria monocytogenes* clinical isolates, among other pathogenic bacteria. Therefore, Ent35–MccV may find important applications in food or pharmaceutical industries.

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1. Introduction

Bacteriocins are ribosomally synthesized bacterial antimicrobial peptides [1]. Many food pathogens as well as opportunistic bacteria that cause food spoilage are inhibited by these peptides. Particularly, bacteriocins from lactic acid bacteria (LAB) are being employed as antimicrobial agents for foods. In this regard, nisin and pediocin PA-1 were approved as food biopreservatives [2]. Actually, nisin was approved as food antimicrobial agent in 1969 by FAO/WHO Expert Committee on Food Additives and its use is permitted in over 50 countries nowadays. Biopreservation methods are being developed to respond to the market demand of fresh and minimally processed foods with longer shelf-life. One of the critical limitations to the use of LAB bacteriocins as biopreservative agents is the narrow spectrum of action they display. As a matter of fact, pediocin PA-1 is inactive against Gram-negative bacteria and nisin has to be employed in combination with chemical or physical treatments that disrupt the outer membrane (OM) when used to inhibit Gram-negative pathogens [3,4]. Pathogenic strains of *Escherichia coli* can cause different foodborne illnesses. The most

common of them is gastroenteritis. Moreover, Shiga toxin-producing *E. coli* is a foodborne pathogen causing alarming outbreaks. A possible alternative to control Gram-negative in foods is the use of antimicrobial peptides secreted by Gram-negative bacteria, known as microcins. To this purpose, Pomares et al. developed a chymotrypsin-susceptible MccJ25 variant, inactivated by digestive enzymes to be used as food preservative [5]. However, microcins are only active against Gram-negative bacteria [6]. Similar to pediocin-like bacteriocins, microcins belonging to class IIa, such as microcin V, are linear polypeptides and the removal of the leader peptide is the unique post-translational modification that they undergo before being secreted by the cells [6–8]. Most remarkable is the fact that they have a similar mechanism of action. It was found that many linear bacteriocins can disrupt the cellular membrane and kill sensitive microorganisms. The expression of an integral membrane protein that acts as a bacteriocin-specific receptor determines the sensitivity of a bacterial strain to the bacteriocin. The mannose phosphotransferase system acts as receptor for pediocin-like bacteriocins [9]. Meanwhile, three different proteins may serve as a specific receptor for linear microcins, namely the membrane component F₀ of the ATP synthase, SdaC, and the mannose permease, required by MccH47, MccV, and MccE492, respectively [10–12]. Because of the Gram-negative envelope structure, an additional step is required by class IIa microcins, i.e. an OM transporter system is used for these peptides to reach the plasma membrane receptor.

Abbreviations: AU, antimicrobial activity units; CFU, colony forming units; IM, inner membrane; LAB, lactic acid bacteria; Mcc, microcin; OM, outer membrane

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The enterocin CRL35, a pediocin-like bacteriocin isolated from Argentinean regional cheese, has a potent antilisterial activity but is inactive against Gram-negative bacteria [13]. On the other hand, microcin V previously known as colicin V is specifically active against Gram-negative bacteria [14]. In order to obtain a peptide with a broader antimicrobial spectrum the required portions of genes encoding the bacteriocins enterocin CRL35 and microcin V, namely *munA* and *cvaC*, were fused by asymmetrical PCR. The resulting *munA-cvaC* chimerical gene was cloned and expressed in *E. coli*. The hybrid bacteriocin purified from *E. coli* extracts, named Ent35–MccV, showed inhibitory activity against enterohemorrhagic *E. coli*, *Listeria monocytogenes*, and other pathogenic Gram-positive and Gram-negative bacteria.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bacteria and culture conditions used in this work are listed in Table 1. The strain *E. coli* DH5 α was used as the host for recombinant plasmids.

2.2. Genetic manipulations

In this work we fused by polymerase chain reactions (PCR) the regions of *munA* and *cvaC* encoding for the structural portion of enterocin CRL35 and microcin V, respectively. The procedure is

shown schematically in Fig. 1A. A colony of *Enterococcus mundtii* CRL35 as template and the primers munAF3 (forward-5'CATGCCATGGGTAAATACTACGGTAATGGA) and 3GentR (reverse-5'TCCTCCTCCACTTTTCCAACCAGCTGCTC) [13] were used for amplifying *munA* gene. On the other hand, the gene *cvaC* was amplified from the plasmid pHK11 using primers colVF3G (forward-5'GGAG GAGGACTTCAGGGCGTGATATTGC) and colVR (reverse-5'GGATCCTTA TAAACAAACATCACT) [15]. Recognition sites for restriction enzymes *NcoI* in munAF3 and *BamHI* in colVR and the region encoding for the hinge triglycine in 3GentR and colVF3G are underlined. The reaction was carried out under standard conditions specified by the enzyme supplier (AccuPrime™ Taq DNA Polymerase High Fidelity, Invitrogen). The PCR program included an initial denaturation step at 94 °C for 5 min, followed by 30 cycles (50 °C for 30 s, 68 °C for 40 s and 94 °C for 1 min). At the end of the PCR program, an elongation step (68 °C, 5 min) was included.

Finally, the fusion of *munA* with *cvaC* was carried out by an asymmetric PCR reaction [16]. The PCR products of *munA* and *cvaC* (1 μ l each one in a 50 μ l reaction) were used as template, munAF3 and colVR as primers at 0.4 μ M final concentration and the internal munAR3g primer at 0.4 nM final concentration was introduced to produce the fusion. Nucleotide concentrations and reaction conditions were those recommended by the supplier of the enzyme GoTaq DNA Polymerase (Promega). The program used was: 94 °C 5 min, followed by 10 cycles (94 °C 1 min, 55 °C 1 min, 72 °C 40 s), 20 cycles (94 °C 1 min, 40 °C 1 min, 72 °C 40 s) and 72 °C 10 min. The PCR product (shown in Fig. 1B) was purified from

Table 1
Inhibitory spectrum of Ent35–MccV and parental bacteriocins.

Strain	Culture conditions, media/temp.	Source, reference or relevant genotype	Inhibitory activity ^a		
			Ent35–MccV	Ent35 ^b	MccV
<i>Gram-positive</i>					
<i>L. innocua</i> 7	TSB/30 °C	INRA	+++	+++	–
<i>L. monocytogenes</i> FBUNT1	TSB/37 °C	FBQF – CI	+++	+++	–
<i>L. monocytogenes</i> FBUNT2	TSB/37 °C	FBQF – CI	+++	+++	–
<i>L. monocytogenes</i> EGDe	TSB/37 °C	ATCC: BAA-679	–	–	–
<i>Enterococcus faecalis</i> FBUNT1	TSB/37 °C	FBQF – CI	–	+++	–
<i>S. aureus</i> FBUNT1	M9/37 °C	FBQF – CI	+	–	–
<i>S. aureus</i> FBUNT2	M9/37 °C	FBQF – CI	+++	–	–
<i>S. epidermidis</i> FBUNT1	M9/37 °C	FBQF	+	–	–
<i>Acinetobacter baumannii</i>	TSB/37 °C	Malbran – CI	–	–	–
<i>E. mundtii</i> CRL35	TSB/30 °C	CERELA [13]	–	–	–
<i>Gram-negative</i>					
<i>E. coli</i> MC4100	M9/37 °C	CGSC	+++	–	+++
<i>E. coli</i> O157:H7	M9/37 °C	ATCC 700728	+++	–	++
<i>E. coli</i> (UPEC215)	M9/37 °C	FBQF – CI	+++	–	++
<i>E. coli</i> (UPEC217)	M9/37 °C	FBQF – CI	+++	–	++
<i>E. coli</i> (UPEC219)	M9/37 °C	FBQF – CI	+++	–	++
<i>E. coli</i> (UPEC228)	M9/37 °C	FBQF – CI	++	–	++
<i>E. coli</i> (UPEC229)	M9/37 °C	FBQF – CI	+++	–	+++
<i>E. coli</i> (UPEC230)	M9/37 °C	FBQF – CI	–	–	–
<i>E. cloacae</i>	M9/37 °C	FBQF – CI	+	–	+
<i>S. marscecens</i>	M9/37 °C	FBQF – CI	+	–	–
<i>K. pneumoniae</i>	M9/37 °C	FBQF – CI	+	–	–
<i>Y. pestis</i>	M9/37 °C	FBQF – CI	–	–	–
<i>C. freundii</i>	M9/37 °C	FBQF – CI	–	–	–
<i>P. aeruginosa</i>	M9/37 °C	FBQF – CI	–	–	–
<i>S. Typhimurium</i> FBUNT1	M9/37 °C	FBQF – CI	–	–	–
<i>S. Typhimurium</i> SL3770	M9/37 °C	SGSC	–	–	–
<i>S. Newport</i> FBUNT1	M9/37 °C	FBQF – CI	–	–	–
<i>E. coli</i> MC4100 (pHK11)	M9/37 °C	[15]	–	–	–
<i>E. coli</i> LA1	M9/37 °C	Δ <i>sdaC::km</i> – this work	–	–	–
<i>E. coli</i> LA2	M9/37 °C	Δ <i>cirA::km</i> – this work	–	–	–
<i>E. coli</i> NC1	M9/37 °C	Δ <i>tonB::km</i> – Natalia Corbalán	–	–	–

INRA, Jouy-en-Josas, France; FBQF: Facultad de Bioquímica, Química y Farmacia; Universidad Nacional de Tucumán, Argentina; ATCC: American Type Culture Collection; CERELA: Centro de Referencias para Lactobacillos, Tucumán, Argentina; CGSC: *E. coli* Genetic Stock Center; SGSC: *Salmonella* Genetic Stock Center; Malbran: Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) Dr. Carlos Malbrán, Argentina; CI: clinical isolate.

^a Symbols represent relative activity by diffusion in agar, evaluated by measuring the average diameter (da) of inhibition zones. –: no inhibition; +: da < 5 mm; ++: 5 mm < da < 10 mm; +++: da > 10 mm.

^b Ent35: enterocin CRL35.

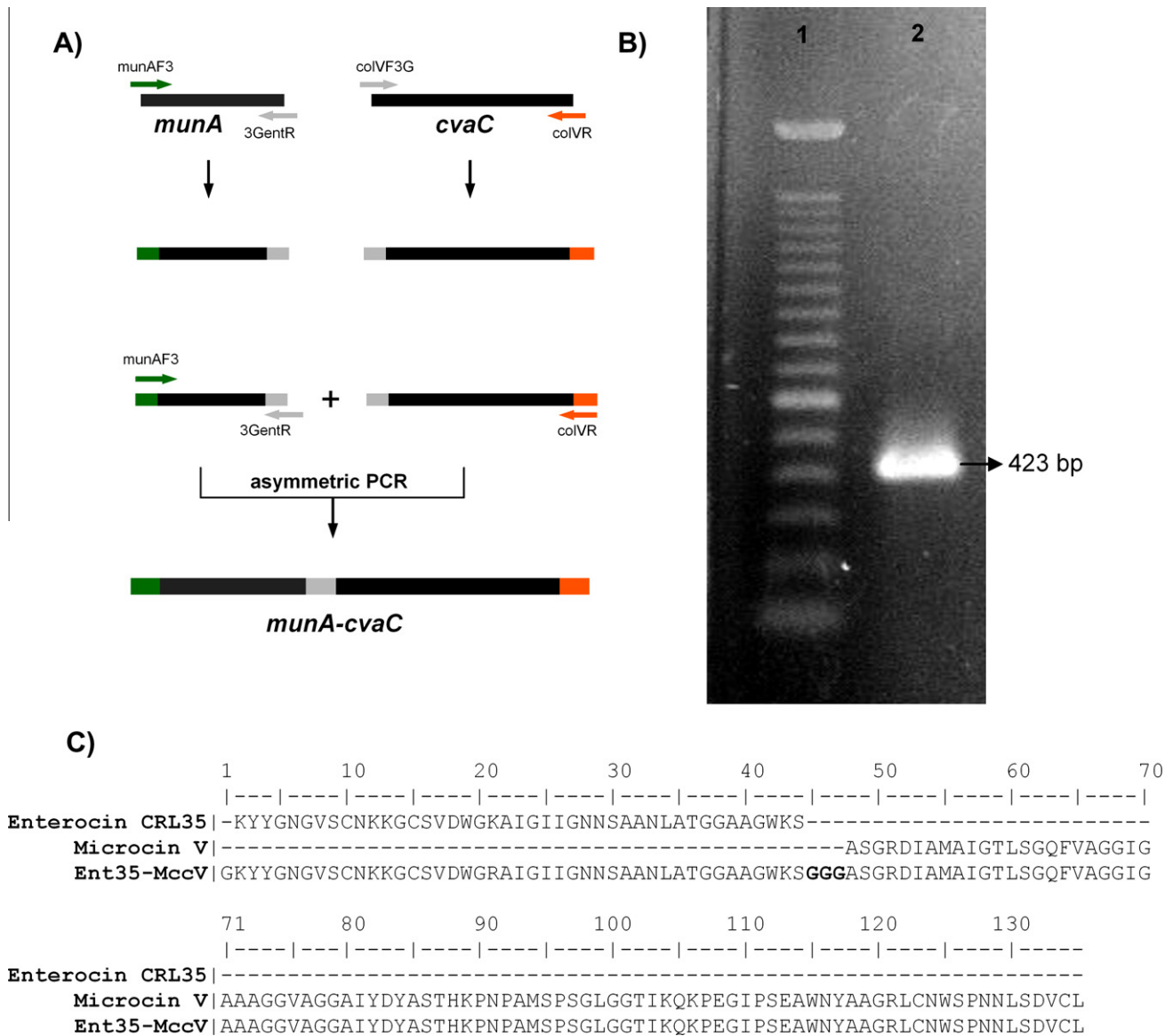


Fig. 1. *munA-cvaC* construction. (A) Schematic representation of the PCR strategy used to fuse *munA* and *cvaC*. First, each gene was amplified incorporating an overlapping fragment which encode for an interpeptide flexible region. In the second PCR, the products of *munA* and *cvaC* PCR were used as template and 3GentR (1:1000 dilution with respect to *munAF3* and *colVR* primers) was used to fuse the two DNAs. (B) Electrophoretic analysis of the product of the asymmetrical PCR in 1% agarose gel stained with (Gelred™ (Biotium). Lane 1: DNA step ladder 100 bp (Invitrogen). Lane 2: asymmetric PCR product. The arrow marks the position of migration of the hybrid product. (C) Amino acid sequences of enterocin CRL35, MccV and the hybrid Ent35-MccV. The hinge region is in bold.

agarose gel using a Gel Band Purification Kit (GE Healthcare), digested with *NcoI* and *BamHI* and cloned in linearized pET28b(+) (Novagen). The ligation mixture was used to transform *E. coli* DH5 α obtaining pMA24. The cloned gene was sequenced and analyzed for its structure.

E. coli LA1 and LA2 were obtained introducing a kanamycin resistance cassette by Phage P1 *vir* transduction from the strains *E. coli* JW2767-1 and JW2142-1, respectively [17]. The strain *E. coli* NC1 was a generous gift from Natalia Corbalán (INSIBIO, Tucumán-Argentina).

2.3. Gene expression and bacteriocins purification for activity determinations

Ent35-MccV was expressed from pMA24 in *E. coli* BL21 [DE3] (pLysS) grown in LB (Sigma) at 37 °C. When the culture reached an OD ~0.6 the Ent35-MccV synthesis was induced with isopropyl β -thiogalactoside 0.5 mM and temperature was changed to 30 °C.

After 3 h, cells were collected by centrifugation in 10 mM HEPES, pH 7, PMSF 0.5 mM (buffer A), lysed using a French press and centrifuged at 47,000xg, 4 °C for 1 h. The supernatant was heated (10 min, 100 °C) and centrifuged (10 min, 10,000xg). The resultant supernatant was then purified through a C18 cartridge (Supelco) and eluted with a stepwise buffer A:acetonitrile gradient (0%, 5%, 10%, 20%, 30% and 50% v/v). Ent35-MccV eluted with 50% acetonitrile. This fraction was used to test the antimicrobial activity after solvent removal (Savant SpeedVac® System). Protein concentration was determined using Quant-iT™ Protein Assay Kit (Invitrogen). The parental bacteriocins, enterocin CRL35 and MccV, were purified as previously described [13,18].

2.4. RP-HPLC purification of Ent35-MccV and MALDI-TOF MS analysis

The supernatant obtained from the centrifugation of the heated extract (Section 2.3) was precipitated with ammonium sulfate to a final concentration of 70% (w/v). Proteins were harvested by

centrifugation, re-suspended with 0.1% (v/v) aqueous trifluoroacetic acid (TFA) and fractionated by Reversed Phase (RP)-HPLC using a modular system HP 1100 Agilent Technology (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C₄ column (214TP52, 5 µm, 250 × 2.1 mm i.d.). After 5 min of isocratic elution at 10% solvent B (0.1% TFA in HPLC-grade acetonitrile, v/v), a 10–70% linear gradient of B over 60 min was applied at a flow rate of 0.2 ml/min. Solvent A was 0.1% TFA in HPLC-grade water (v/v). For each analysis, aliquots of approximately 100 µl of heated supernatant were fractionated. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected and concentrated in a speed-vac for mass spectrometry (MS) analysis. Prior to MS analysis HPLC fractions were further desalted by C₄ Zip Tip[®] micro-columns (Millipore, Bedford, CA, USA), washing with 0.1% TFA and eluting with 70% acetonitrile (v/v) containing 0.1% TFA.

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS experiments were carried out on a Voyager DE-Pro spectrometer (PerSeptive BioSystems, Framingham, MA, USA) equipped with a N₂ laser (λ = 337 nm). Sinapinic acid (Sigma), dissolved in 50% acetonitrile containing 0.1% TFA at a concentration of 10 mg/ml, was used as the matrix. Purified HPLC fractions (1 µl) were mixed with matrix (1 µl) directly on the MALDI target and air dried. The mass spectra were acquired in the positive linear ion mode using the Delayed Extraction (DE) technology. The instrument operated at an accelerating voltage of 20 kV. External mass calibration was performed with a commercial standard protein mixture (Sigma), including bovine insulin (average MH⁺ 5734.5), cytochrome c (average MH⁺ 12362.0) and horse myoglobin (average MH⁺ 16952.3). Raw data were analyzed using the software program Data Explorer 4.0 purchased with the spectrometer.

2.5. Antimicrobial activity and bacterial viability assays

Drops of each purified peptide (20 µl, 350 µg of protein/ml) were spotted in a TSB (Difco) or M9 (Sigma) plate. Once the droplets were adsorbed, the plate was covered with 4 ml of 0.6% agar previously inoculated with 10⁶ cells of the test strain. Incubation was carried out overnight at 30 °C or 37 °C. Antimicrobial activity was evaluated by measuring the diameter of the inhibition halos. The peptide solutions were successively diluted before the drops were spotted. The antimicrobial activity units (AU/ml) were calculated as the reciprocal of the highest two-fold dilution showing growth inhibition halo against *Listeria innocua* 7 or *E. coli* MC4100.

For viability determination *E. coli* MC4100 and *L. innocua* 7 were grown to mid-log phase, harvested by centrifugation (10 min, 10000xg), and re-suspended at approximately 1.10⁷ colony forming units per milliliter (CFU/ml) in 50 mM HEPES, pH 7.4, 12.5 mM glucose. The suspensions were treated with the bacteriocin (50 AU/ml corresponding to each assayed bacterial strain) and the CFUs versus time were determined.

2.6. Tricine SDS-PAGE

Electrophoresis gels were prepared as previously described [19] and the samples were treated with loading buffer without β-mercaptoethanol. After fixing, the gels were washed successively with ethanol 70%, 30% and sterile bidistilled water. The gels were placed on TSB or LB (Sigma) agar plates, covered with 10 ml of 0.6% (w/v) agar inoculated with 10⁶ cells of *L. monocytogenes* FBUNT1 or *E. coli* MC4100. The plates were incubated at 37 °C for 16 h and the inhibition zones were examined.

2.7. Nucleotide sequence accession number

The DNA sequence of *munA-cvaC* was deposited at the EMBL Nucleotide Sequence Database, EMBL-Bank ID: FR865441.

3. Results

3.1. Construction of *munA-cvaC*

As described in Section 2.2, the hybrid *munA-cvaC* was obtained by asymmetrical PCR (see Fig. 1A). First, each gene was amplified using the previously described primers. The DNA sequence encoding the hinge region was incorporated with 3GentR (reverse primer for *munA*) and colVF3G (forward primer for *cvaC*) and overlapping fragments were generated. After that, the two fragments were fused by an asymmetrical PCR (Fig. 1A) [20]. The hybrid gene was cloned, expressed in *E. coli* BL21 [DE3] and purified by reverse phase chromatography. The hinge region between two antimicrobial peptides was reported to be critical for obtaining active antimicrobial hybrid peptides [21]; thus, a Gly–Gly–Gly flexible region was introduced between enterocin CRL35 and MccV. The amino acid sequence of parental and the hybrid Ent35–MccV are shown in Fig. 1C.

3.2. Spectrum of action

As shown in Table 1, the antimicrobial spectrum of Ent35–MccV was assayed against a number of Gram-positive and Gram-negative bacteria. In agreement with well-established data, none of the Gram-positive strains tested were inhibited by MccV and enterocin CRL35 was not active against Gram-negative bacteria. Similar to the parental bacteriocins, the hybrid peptide displayed potent antimicrobial activity against *Listeria* spp. and *E. coli*. Ent35–MccV was active against *L. innocua* 7, two clinical isolated strains of *L. monocytogenes*, namely FBUNT1 and FBUNT2, and was inactive against the enterocin CRL35 natural resistant strain *L. monocytogenes* EGDe. All the Gram-negative bacteria inhibited by MccV were also inhibited by Ent35–MccV. In particular, the peptide displayed antimicrobial activity against *Enterobacter cloacae* and *E. coli*; including the laboratory strain MC4100, the enterohemorrhagic *E. coli* O157:H7 and 5 out of 6 clinical isolates of uropathogenic strains (UPEC). In fact, only the strain UPEC230 was not affected by Ent35–MccV. However, UPEC230 is a microcinogenic strain that produces an antimicrobial compound which was characterized by cross-streaking as MccV [22]. It was previously suggested that a relationship between higher-molecular-mass microcins, such as MccV and urovirulence may occur [23]. Moreover, Ent35–MccV displayed antimicrobial activity against other clinical isolates Gram-positive and Gram-negative strains on which enterocin CRL35 or MccV are inactive, such as *Staphylococcus aureus* FBUNT1 and FBUNT2, *Staphylococcus epidermidis*, *Serratia marcescens* FBUNT1 and *Klebsiella pneumoniae* FBUNT1. The hybrid peptide was inactive against *Enterococcus faecalis* FBUNT1, on which enterocin CRL35 is active. Some bacteria tested (*Acinetobacter baumannii*, *Yersinia pestis*, *Citrobacter freundii*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *S. enterica* serovar Newport) were resistant.

3.3. Mechanism of action

To explore the mechanism of action of Ent35–MccV, its antimicrobial activity was assayed against wild type *E. coli* MC4100 and isogenic mutant strains, bearing mutations in the *cirA*, *tonB* or *sdaC* genes. All the mutant strains are resistant to MccV because CirA and TonB are necessary to transport the peptide across the OM [24], and SdaC is the IM receptor [11]. The results showed that the mutant strains (*E. coli* LA1, LA2 and NC1) were also resistant to Ent35–MccV, indicating that the same proteins are required by the hybrid bacteriocin in order to exert antimicrobial action on *E. coli*.

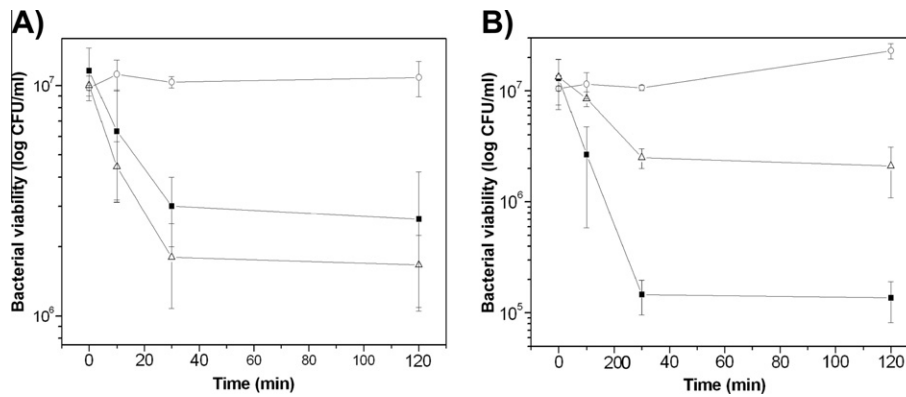


Fig. 2. Effect of Ent35-MccV and parental bacteriocins on the viability of *L. innocua* 7 and *E. coli* MC4100 cells. The bacterial suspensions were treated with 50 AU/ml of each bacteriocin (see Section 2.4). Cell viability is expressed as CFU/ml at different times. (A) *L. innocua* 7 cells without treatment (○), Ent35-MccV (■), and enterocin CRL35 (Δ). (B) *E. coli* MC4100 control without treatment (○), Ent35-MccV (■), and MccV (Δ). The results represent the average of three independent experiments.

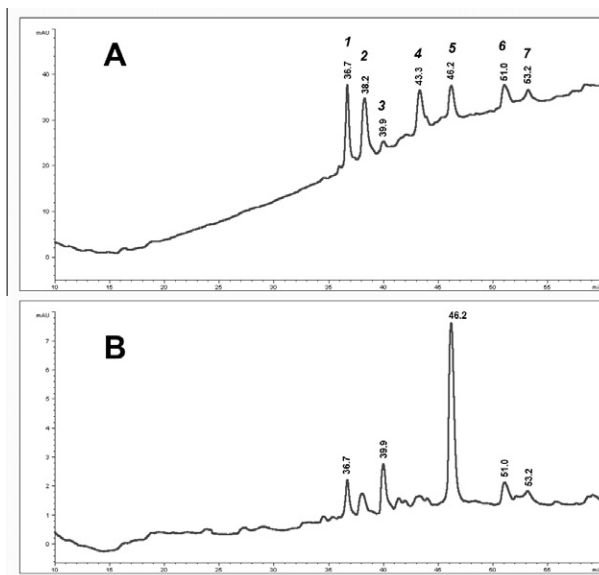


Fig. 3. RP-HPLC separation of the heated cellular extract. Cellular extract of *E. coli* BL21 [DE3] (pMA24) were treated as described in Section 2.4. Chromatogram was monitored at (A) $\lambda = 220$ nm and (B) $\lambda = 280$ nm. Peaks were collected and individually analyzed by MALDI-TOF MS.

The enterocin CRL35 producer and immune bacterial strain, *E. mundtii* CRL35, was immune to the hybrid peptide, indicating that the immunity protein for enterocin CRL35 protects against Ent35-MccV [13]. Similarly, *E. coli* MC4100 cells harboring pHK11, which encodes the MccV immunity protein Cvi [15], were also immune to Ent35-MccV.

Both, Enterocin CRL35 and MccV are bactericidal [11,25]. To investigate whether Ent35-MccV is able to kill the target cells, we studied the time course killing effect on *E. coli* MC4100 and *L. innocua* 7 using the same AU/ml (Fig. 2). Ent35-MccV was able to decrease the viability of both bacteria. After two hours of treatment with Ent35-MccV the *L. innocua* 7 viable cells decreased one order of magnitude compared to the control curve in the absence of the peptide. A similar result was obtained with enterocin CRL35 (Fig. 2A). When the experiments were carried out with *E. coli* as sensitive strain, cell viability decreased two orders of magnitude by treatment with Ent35-MccV, meanwhile the same antimicrobial activity units of MccV decreased only one log, compared to the control without antimicrobial peptide (Fig. 2B). These results clearly demonstrate the bactericidal ability of Ent35-MccV against both bacteria.

3.4. Ent35-MccV characterization

The MALDI-TOF MS analysis of Ent35-MccV samples prepared as described in Sections 2.3 and 2.4 showed a single signal at m/z 7271.6. According to previously published MS data [26,27] this component was tentatively identified as to the *E. coli* cold-shock-C secreted protein.

The missed detection of any further protein component has to be most likely ascribed to a high ionization efficiency of the CSP-C and to subsequent suppression ion effects. Therefore, the sample was subjected to preliminary RP-HPLC separation. In Fig. 3A and B are shown the UV chromatograms at $\lambda = 220$ nm and $\lambda = 280$, respectively. Peaks were manually collected and analyzed by MALDI-TOF MS. The dominant component with m/z 7271.6 was contained in the peak no. 1 at retention time (RT) 36.7 min. The MALDI-TOF peptide mass mapping carried out after tryptic hydrolysis (not shown) confirmed, through the use of the Protein Prospector search engine (<http://prospector.ucsf.edu>), that it is the CSP-C of *E. coli* (Uniprot accession: P0A9Y6). Though at a low degree, the CSP-C also contaminated the later eluting HPLC fractions.

The MALDI-TOF spectrum of the peak no. 5 at RT 46.2 min (Fig. 4) displayed a signal at m/z 13179.8. The measured MW is compatible with that expected for Ent35-MccV in which a proteolytic cleavage at the Gly¹ has occurred. Interestingly, the corresponding chromatographic peak resulted clearly increased by the $\lambda = 280$ detection (Fig. 3B), if compared to the other peaks. This was attributed to the presence of as many as 4 Trp residues in the sequence of Ent35-MccV, that strongly enhanced $\lambda = 280$ absorbance.

The remaining main signals in the MALDI spectrum can be assigned to other co-eluting fragments that arise from proteolysis of Ent35-MccV as schematized in Table 2, thereby supporting the identification of Ent35-MccV. The proteolytic cleavages preferentially involved the carboxy-terminal of specific Gly residues. No further polypeptide chains ascribable to fragments of Ent35-MccV were detected by the MS analysis of the remaining HPLC fractions. It has to be underlined that the attempt of characterizing Ent35-MccV by MALDI peptide mass mapping was unsuccessful, most likely because of both a low degree of trypsinolysis of the chimeric protein and the occurrence of interfering peptides generated from *E. coli* CSP-C.

3.5. Tris-tricine SDS-PAGE and Ent35-MccV assay

Purified preparations containing Ent35-MccV and the parental bacteriocins were analyzed by tricine SDS-PAGE. Gels revealed by

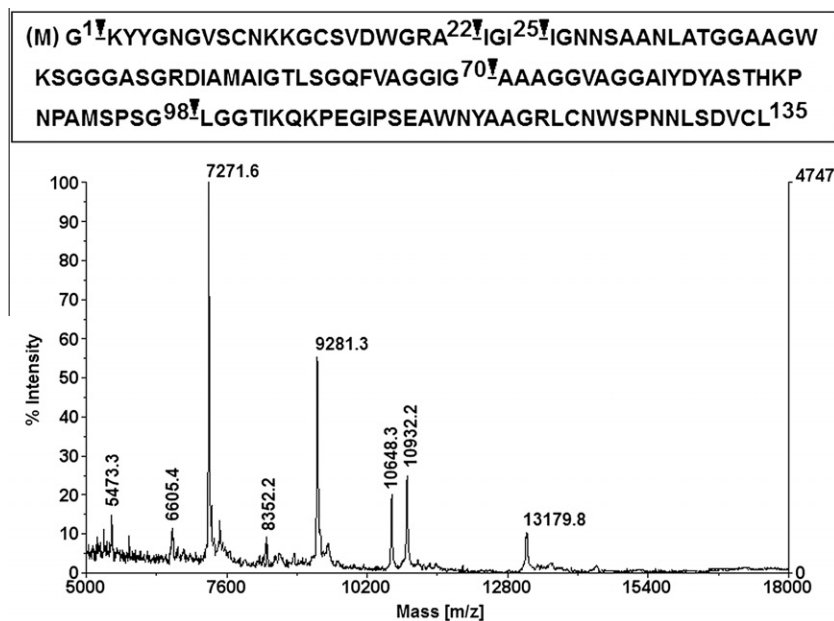


Fig. 4. MALDI-TOF MS analysis of the HPLC fraction no. 5. The mass signal 13179.8 is compatible with the expected molecular weight of Ent35–MccV (2–135 chain). The other signals are assigned in Table 2. The upper panel shows the Ent35–MccV sequence in which the proteolytic cleavage sites are indicated.

Table 2
MALDI-TOF MS assignment of Ent35–MccV proteolytic fragments in the HPLC peak no. 5.

Measured m/z	Expected m/z^a	Assignment	Cleavage
13179.8	13202.8	Ent35–MccV (2–135)	G ¹
10932.2	10930.2	Ent35–MccV (23–135)	A ²²
10648.3	10646.8	Ent35–MccV (26–135)	I ²⁵
9281.3	9276.4	Ent35–MccV (1–98)	G ⁹⁸
6605.4	6602.4	Ent35–MccV (71–135)	G ⁷⁰

^a The expected m/z of the Ent35–MccV fragments were calculated considering Cys engaged in disulfide bonds.

biological activity against *L. monocytogenes* FBUNT1 and *E. coli* MC4100 are shown in Fig. 5A and B, respectively. Ent35–MccV inhibition zones were located at \sim 13.3 kDa (lane 2, band (a) for both gels), close to the migration band expected for unhydrolyzed Ent35–MccV (theoretical MW 13259.8 Da). These results demonstrated that the hybrid peptide is actually responsible for the antimicrobial activity, different from any small-sized proteolytic fragment produced during the purification. There is an additional inhibition zone (c), which could be attributed to Ent35–MccV oligomers. It was only observed when antimicrobial activity was tested against *E. coli* MC4100 SDS-PAGE detection (Fig. 5B).

4. Discussion

In this paper, a new hybrid bacteriocin between the pediocin-like bacteriocin, enterocin CRL35 and the linear microcin, MccV is reported. Johnsen et al. reported the fusion of N- and C-terminal portions of various “pediocin-like” bacteriocins and found that the C-terminal half of the peptide determines the spectrum of action against species and strains of Gram-positive bacteria [28,29]. Moreover, a 15-mer peptide that spans pediocin PA-1 from the center toward the C-terminal portion inhibited its biological activity. Thus, the receptor binding region could be localized within the C-terminal portion of these bacteriocins [30]. The N-terminal region must be involved in the membrane perturbation induced by the peptide, since a 15-mer peptide derived from enterocin

CRL35 produced an enhancement of enterocin CRL35 antimicrobial activity and showed antimicrobial activity [31,32]. On the other hand, Azpiroz and Laviña constructed hybrid microcins fusing the N- and C-terminal domains of MccV and MccH47. They arrived to the conclusion that the C-terminal domains of these class II microcins are involved in the recognition of the OM receptor for the target cell uptake and the N-terminal domain is the toxic region of the molecule [33]. Both, class IIa of LAB bacteriocin and class II of microcins must have an IM receptor binding region.

The antimicrobial spectrum displayed by Ent35–MccV demonstrates that both bacteriocins are active in the chimerical peptide. Ent35–MccV must have two receptor binding regions, two toxic regions and the OM receptor binding domain in the MccV portion. Although the hybrid bacteriocin appears to have a similar mechanism of action than the parental bacteriocins and is recognized by the two immunity proteins, i.e. MunC and Cvi, the antimicrobial activity of the hybrid does not correspond exactly to the sum of the antimicrobial activities of the parental peptides. The toxic domains belonging to both peptides might be active simultaneously once Ent35–MccV binds to the plasma membrane receptor in *E. coli* cells. This fact could explain the better capacity to kill *E. coli* cells than MccV and the better performance killing *E. coli* than *L. innocua* cells. There is a persistence of *L. innocua* 7 and *E. coli* MC4100 populations larger than 1.10^6 CFU/ml and 1.10^5 CFU/ml after treatment with the hybrid antimicrobial peptide. Vignolo et al. observed that population of *L. monocytogenes* decreased initially after enterocin CRL35 treatment and increased upon further incubation [34]. Another microcins and bacteriocins have similar kinetics of cell destruction [35,36]. Although more experiments testing the antimicrobial activity of Ent35–MccV in foods or complex fluid matrices (whole blood, plasma or serum) must be performed, in order to find the right conditions in which the spontaneous emergence and outgrowth of a bacteriocin-resistant bacterial population can be avoided. In fact, we strongly believe that Ent35–MccV could be employed in combination with other bacteriocins or other chemical or physical food treatments [4,34,37].

The antimicrobial activity displayed by the hybrid against bacterial strains which are not inhibited by the parental bacteriocins

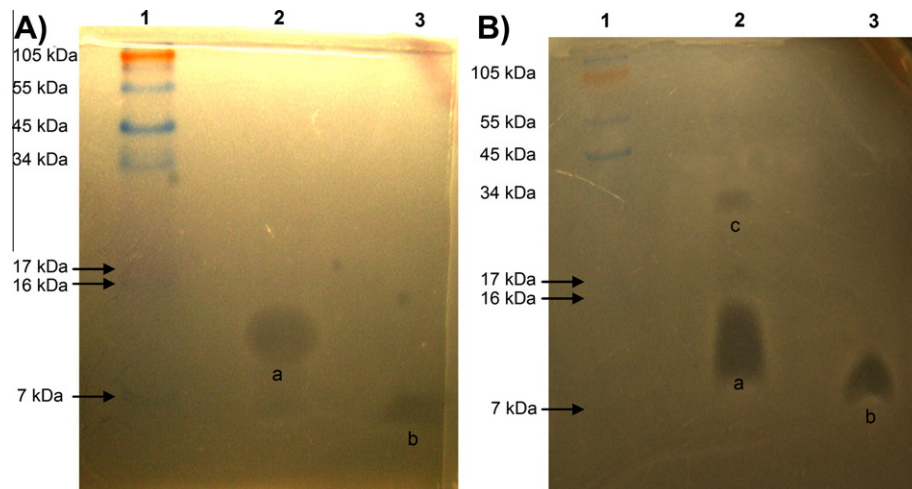


Fig. 5. Bacteriocins analyzed by Tris-Tricine SDS-PAGE and revealed by biological activity. Lane 1 contained the prestained molecular mass marker – see Blue Plus2, Invitrogen (both gels). (A) Gel revealed by biological activity against *L. monocytogenes* FBUNT1 showing the zones of growth inhibition by the bacteriocin bands (a) and (b). Lanes: 2, Ent35–MccV; 3, enterocin CRL35 (MM: 4289.8 Da). (B) Gel revealed using *E. coli* MC4100 as indicator strain. Lanes: 2, Ent35–MccV (band a); 3, MccV, MM: 8735.7 Da (band b).

could be explained by the presence of multiple domains or modules in Ent35–MccV. It is possible that a membrane protein of the target cells serves as a receptor for one of the parental bacteriocins or Ent35–MccV. However, a steric barrier or an inappropriate bacteriocin position could impede the interaction between the toxic domain and the cellular membrane. Therefore, the parental bacteriocins could not act causing the disruption of the cell membrane. After receptor binding, the hybrid peptide could have increased possibilities for a favorable toxic domain-membrane interaction. These results must be thoroughly investigated in future research in order to gain more insight into the mechanism of action of membrane active bacteriocins.

Ent35–MccV is active against the emergent foodborne pathogens, the Gram-negative enterohemorrhagic *E. coli* O157:H7 and the Gram-positive *L. monocytogenes*. More broad spectra hybrid bacteriocins could be obtained based on the idea proposed in this work. Furthermore, chimerical bacteriocin–microcin peptides must be useful to study the mechanism of action of bacteriocins and for applications in medicine, veterinary or food industry.

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