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Secretion of the human parathyroid hormone through a microcin type I secretion system in *Escherichia coli*

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Abstract

Background Gram negative bacteria possess different secretion systems to export proteins to the extracellular medium. The simplest one, type I secretion system (T1SS), forms a channel across the cell envelope to export proteins in a single step. Peptides secreted by the T1SSs comprise a group of antibiotics, called class II microcins, which carry an amino terminal secretion domain that is processed concomitantly with export. Mature microcins range in size from 60 to 90 amino acids and differ in their sequences. Microcin T1SSs show a high versatility in relation to the peptides they are able to secrete, being mainly limited by the length of the substrates. Different bioactive peptides unrelated to bacteriocins could be secreted by microcin V (MccV) T1SS, while retaining their biological activity.

Results In this work heterologous secretion of two variants of human parathyroid hormone (PTH) by MccV T1SS was evaluated. PTH is a bioactive peptide of 84 amino acids (PTH84), which is involved in the maintenance of bone homeostasis. Currently, a drug corresponding to the active fraction of the hormone, which resides in its first 34 amino acids (PTH34), is commercially produced as a recombinant peptide in *Escherichia coli*. However, research continues to improve this recombinant production. Here, gene fusions encoding hybrid peptides composed of the MccV secretion domain attached to each hormone variant were constructed and expressed in the presence of microcin T1SS in *E. coli* cells. Both PTH peptides (PTH34 and PTH84) were recovered from the culture supernatants and could be confirmed to lack the MccV secretion domain, i.e. microcin T1SS efficiently recognised, processed and secreted both PTH variants. Furthermore, the secreted peptides were stable in the extracellular medium unlike their unprocessed counterparts present in the intracellular space.

Conclusion The successful secretion of PTH variants using MccV T1SS could be considered as a new alternative for their production, since they would be recovered directly from the extracellular space without additional sequences. Furthermore, it would be a new example revealing the potential of microcin type I secretion systems to be conceived as a novel strategy for the production of recombinant peptides in *E. coli*.

Keywords Type I secretion system, Microcin V, Human parathyroid hormone, Recombinant peptide secretion in *Escherichia coli*

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Background

Secretion in Gram-negative bacteria requires the substrate to cross the inner and outer membranes to reach the extracellular medium. In the case of proteins, several secretion systems have been described that carry out transport across the cell envelope in one or two successive steps [1, 2]. Among those that achieve secretion in a single step, the simplest is the type I secretion system (T1SS), which is made up of three components: an inner membrane protein, a periplasmic protein, and an outer membrane protein. The first member belongs to the ATP-binding cassette transporter family ("ABC transporter"), which exports a variety of molecules and hydrolyses ATP to provide energy for the process. Those transporters dedicated to protein export contain a peptidase domain, which is involved in substrate recognition and, in some cases, in its processing. The second component, referred to as the membrane fusion protein (MFP), interacts with the ABC transporter on its inner side and with the outer membrane protein on its outer side, forming a channel that extends through the periplasm. Finally, the third component is an outer membrane protein through which the substrate reaches the extracellular space. In *Escherichia coli* cells, this function is accomplished by the trimeric porin TolC, the most characterised protein of this type, which participates in the export of a range of compounds [3–6]. Regarding the secretion mechanism, a model based on the structural and functional data of ABC transporters has been proposed. Briefly, the ABC transporter joins the substrate through the recognition of its secretion signal by the peptidase domain, and then directs the substrate through the secretion system towards the extracellular medium, while hydrolysing ATP [7–9].

Type I secretion systems are mainly found in pathogenic bacteria and their genetic determinants are located either in the chromosome or in plasmids. The export genes, particularly those encoding the ABC transporter and the MFP, usually integrate the same cluster as the substrate gene. In the case of TolC, its genetic determinant is located in the chromosome, unlinked to the other export genes. These systems direct the secretion of a variety of proteins such as digestive enzymes, adhesins, heme-binding proteins, toxins, antimicrobial peptides, among others. Their substrates range from small peptides (≤10 kDa) to large proteins (up to 1,500 kDa), which differ in the location and sequence of the secretion domain (SD) [4, 5, 7, 10]. Proteins larger than 10 kDa contain a C-terminal SD of about 50 aa, which is not processed during secretion. Consequently, protein reaches the extracellular milieu unchanged after passing through the channel formed by the MFP and the outer membrane protein. One of the best studied secretion systems of this type is that responsible for the export of the 1023/1024 aa-haemolysin A toxin produced by some uropathogenic *E. coli* strains [5]. In the case of small peptides (up to ca. 100 aa), the SD comprises the first 15 aa of the sequence, which contains a conserved double-glycine motif (Gly-Gly, Gly-Ala, Gly-Ser) [11, 12]. The ABC transporters devoted to peptide secretion have a functional peptidase domain that cleaves the SD immediately after the double-glycine motif, resulting in the concomitant export of the mature peptide [7, 12, 13]. Peptide substrates include some bacteriocins as well as peptides involved in cell-to-cell communication and biofilm formation from both Gram positive and Gram negative bacteria. In the latter case, ABC transporters form part of T1SSs devoted to export a group of ribosomally-synthesised antibiotic peptides called class II microcins, which are mainly produced by *E. coli* strains [6, 14]. These microcins contain a double-glycine secretion domain at their N-terminus so that they are synthesised as peptide precursors that mature after proteolytic cleavage during secretion. Mature microcins comprise peptides between 60 and 90 aa, with a molecular mass less than 10 kDa, high hydrophobicity and significant serine and glycine amino acid content. Their production is determined by a genetic system that includes an operon with the export genes, which share a high percentage of similarity between different microcin clusters. As for the third component, these T1SSs always employ TolC [14–18]. The microcin V (MccV) type I secretion system is the most characterised. The MccV genetic system is organized in two convergent operons: one carrying *cvi* and *cvaC* genes (*cvicvaC* operon), for the synthesis of the immunity peptide and the antibiotic peptide, respectively; and the second containing the export genes (*cvaAB* operon). The *cvaA* gene (1,275 bp) encodes the membrane fusion protein CvaA and the *cvaB* gene (2,097 bp) encodes the ABC transporter CvaB. Thus, CvaB (698 aa) and CvaA (424 aa) together with TolC (493 aa) form the MccV T1SS [15, 19, 20]. Particularly, it has been demonstrated that CvaB has a functional peptidase domain [12, 21, 22]. Therefore, MccV is synthesised as a 103 aa precursor that is released into the extracellular milieu as a mature 88 aa-peptide after CvaB cleaves its SD [11, 20, 23].

It has been shown that microcin T1SSs have a high versatility in relation to the substrates that they are able to secrete. Some of these export systems proved to be efficient for the secretion of several microcins, both naturally and heterologously [16, 18, 24]. Furthermore, other authors succeeded to secrete different antimicrobial peptides fused to the MccV secretion domain in the context of *E. coli* cells expressing the T1SS of this microcin [25–28]. Given this prior knowledge, it seems that the sequence after the SD in these peptides would not participate in export, allowing the same T1SS to secrete antibiotic peptides with different mature sequences. This

hypothesis was recently corroborated using the MccV T1SS to secrete a series of synthetic peptides with diverse sequence composition and length. They found that secretion was mainly limited by the length of the peptides, which could not greatly exceed the size of MccV. In the same work, different bioactive peptides unrelated to bacteriocins were secreted while retaining their biological activity [29].

In this work, the heterologous secretion of two variants of human parathyroid hormone (PTH) by the MccV T1SS was assessed. PTH is a bioactive peptide which plays an important role in maintaining bone homeostasis by regulating serum calcium and phosphate. It is synthesised as a 115 aa precursor which undergoes cleavage of its 31 N-terminal residues to yield an 84 aa- mature structure, PTH84. Since the biological activity of the hormone resides in its first 34 aa, a drug that comprises this hormonal fraction (PTH34), known as Teriparatide, has been commercially produced as a recombinant peptide in *E. coli* and approved by the Food and Drug Administration and the European Medicine Agency for the clinical treatment of osteoporosis and bone destruction [30]. Nevertheless, further research is underway to improve the recombinant production of this bioactive peptide in *E. coli* [31–35]. Thus, PTH34 and PTH84 were here chosen as target peptides of MccV T1SS since their extension is in the microcins´ range, they have a simple structure without post-translation modifications, and they are bioactive peptides of clinical interest. The strategy involved the construction of gene fusions encoding the secretion domain of MccV (SD_V) attached to the N-terminus of each PTH variant, to then evaluate their secretion through MccV T1SS in *E. coli* cells. Success in the secretion of either of these two variants could be a new alternative for their production since they would be recovered directly from the extracellular space without additional sequences. Furthermore, it would also be a new example revealing the potential of class II microcin type I secretion systems as a novel strategy for recombinant protein production in *E. coli*.

Materials and methods

Bacterial strains and growth conditions

The *E. coli* K12 strains used in this study were DH5α, MC4100 and PAP222, from our laboratory collection. DH5α [*supE ΔlacU169* (*ϕ*80 *lacZ*ΔM15) *hsdR recA endA gyrA thi relA*] was employed for cloning purposes [36]. MC4100 (AC: HG738867) (*araD ΔlacU169 relA rpsL thiA*) was used as the genetic context to maintain the recombinant plasmids and as indicator in MccV production assays (see below). PAP222 is a *gyrA* derivative of W3110 (AC: AP009048), harbouring the plasmid pK270, which contains the MccV genetic system [37]. *E. coli* B BL21(DE3) (AC: CP001509) [*hsdS gal lon ompT* (*λ cIts857 ind1 Sam nin lacUV5-T7 gene 1*)] was used as the context for the analysis of the heterologous secretion of PTH variants. This strain contains the lambda DE3 prophage that carries the gene encoding T7 RNA polymerase under the control of *lacUV5* promoter, allowing its inducible expression and, consequently, the expression of genes under the control of the T7 promoter. In addition, this strain is deficient for the two main proteases of *E. coli*, Lon and OmpT, a context that reduces the degradation of heterologous proteins produced by cells [38]. LB rich medium and M63 glucose minimal medium were used for general microbiological purposes [39]. Zym 5052 medium [40] without lactose, hereafter referred to as Zym-mod, was used for the analysis of the heterologous secretion of PTH variants. Antibiotics were added to media at the following final concentrations: ampicillin (Ap), 100 μg mL^{-1}; chloramphenicol (Cm), 48 μg mL^{-1}; kanamycin (Km), 24 μg mL^{-1}. The chromogenic indicator of β-galactosidase activity X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) was added to media at the final concentration of 20 μg mL^{-1}. Isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM was used as inducer in the experiments of heterologous secretion of PTH variants. Strains harbouring recombinant plasmids were grown at 30 °C for ca. 18–20 h. Culture growth was monitored by optical density at 600 nm (OD_{600}) and by viable cell counts.

Plasmid construction

The export genes *cvaAB* of the MccV genetic system were amplified from the PAP222 cell lysate using primers pAB-F1 (5´-CAAACTAATAGTATGCAAGGAGA C-3´) and pAB-R (5´-CGCTTGTAATTCCTCTATGG TTT-3´). Specifically, these primers were designed to amplify from the Shine-Dalgarno sequence of *cvaA* to the stop codon of *cvaB*, i.e. to obtain a segment containing the two genes without the promoter of the *cvaAB* operon (Fig. 1). The 3,414 bp amplicon was cloned into the SmaI site of pUCYC5, a medium copy-number vector carrying the *cat* gene, the *ori* region from pACYC184, and the polylinker-*lacZ* region from pUC13 [41]. Transformants of DH5α were selected on LB Cm X-gal plates and Lac^- clones were purified. Among them, a plasmid harbouring *cvaAB* genes expressed under the control of the *lac* promoter was selected and named pExAB (6,324 bp). A 781 bp-amplicon carrying the *cvicvaC* operon of the MccV genetic system was obtained from the PAP222 cell lysate, employing the previously designed primers V-L (5´-CCTCCTACCCTTCACTC-3´) and V-R (5´-GAGGAATTACAAGCGTATGAGG-3´) [24]. These primers amplified a DNA segment comprising the two genes, from 75 bp upstream the start codon (attempting to include the unknown promoter of *cvicvaC*) to 141 bp downstream the stop codon of *cvaC* (to include

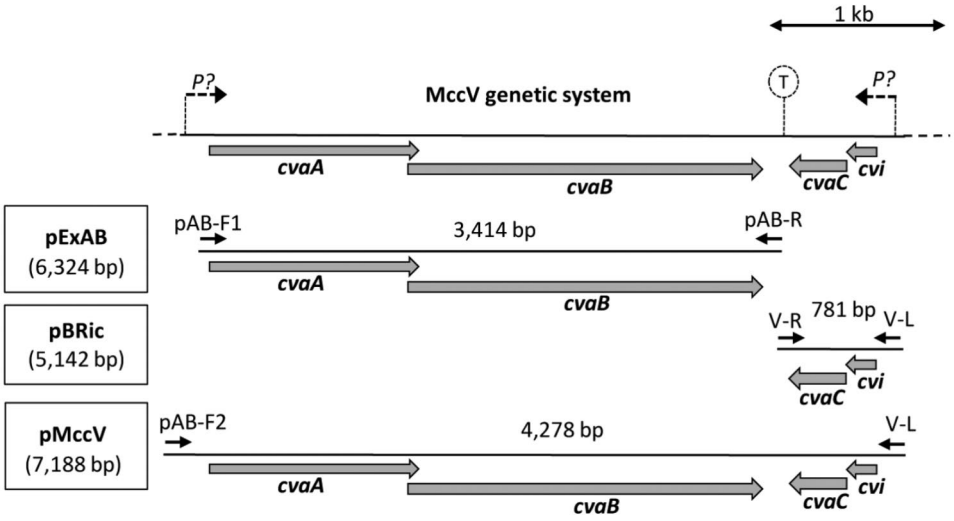

Fig. 1 MccV genetic constructions. Above, MccV genetic system. At left, name and size of the recombinant plasmids. Genes are depicted with thick grey arrows; above, the insert size is indicated; *P?*, putative location of unknown promoters; T, transcriptional terminator. Thin arrows, primers used for the constructions

the known terminator located downstream the operon) (Fig. 1). The PCR product was cloned into the EcoRV site of the pBR322 vector [39] and Ap resistant transformant DH5α clones were selected. The desired construction, containing the *cvicvaC* operon, was called pBRic (5,142 bp). The entire MccV genetic system was also cloned by amplifying a 4,278 bp segment with primers pAB-F2 (5´-TCCTGATAACTCTCCTATGTTG T-3´) and V-L, using a PAP222 cell lysate as template. Primer pAB-F2, hybridizing 104 bp upstream the *cvaA* gene, was designed to likely include the *cvaAB* promoter, which has not yet been identified (Fig. 1). The 4,278 bp amplicon was cloned into the SmaI site of pUCYC5 and transformants of DH5α were selected on LB Cm X-gal plates. Some Lac$^-$ clones were purified and assayed for the ability to produce MccV by patch test on a microcin-sensitive indicator strain. The plasmid construction carrying the entire MccV genetic system was identified and named pMccV (7,188 bp). It is important to mention that plasmid pK270, harboured by PAP222, is not sequenced. Thus, sequence of plasmid pColV-PU1 (AC: CP042245) was used for designing primers and cloning experiments. This approach was based on the fact that there are many plasmids whose sequence is deposited in data banks that share total identity at the MccV genetic system.

Recombinant plasmids encoding hybrid peptides made up of the MccV secretion domain (SD_v) plus each PTH variant (PTH34 and PTH84) were constructed in the gene synthesis service of GenScript, Hong Kong (https://www.genscript.com/). Sequences coding for the SD_v and for each PTH variant were fused in frame. PTH sequences were optimized to the *E. coli* codon usage employing the software of the company (https://www.genscript.com/gensmart-free-gene-codon-optimization.html). The gene fusions *sd$_v$-pth34* and *sd$_v$-pth84* were provided by GenScript cloned into the pET-26b(+) vector. The latter is a 5,360 bp multicopy plasmid, with the following main features: the pMB1 replication origin, a kanamycin resistant gene, a T7*lac* promoter, a ribosome binding site of high efficiency, a sequence encoding a PelB leader peptide to direct fused proteins to the periplasm, and a multi-cloning site. In the design the leader PelB coding sequence was eliminated, being substituted by a DNA segment containing the gene fusions, followed by an in frame 6xHis tag coding sequence and a double TAA stop codon. These inserts were cloned between the NdeI and BlpI restriction sites of pET-26b(+) to obtain plasmids pET-PTH34 (5,379 bp) and pET-PTH84 (5,529 bp) (Fig. 2). The synthesis service provided the plasmid constructions after corroborating their entire DNA sequence. Once received the plasmids, they were introduced into DH5α cells by transformation and the resulting clones were selected in LB plates supplemented with Km. This step was necessary to methylate the plasmid DNA for its subsequent introduction in the HsdR$^+$ genetic context of *E. coli* B BL21 (DE3).

Molecular biology techniques

Cloning experiments were carried out according to standard procedures [36]. Plasmid DNA was extracted with QIAprep Miniprep kit (Qiagen, #1018398). PCR amplifications were performed in a total volume of 30 µl, using cell lysates as templates. For cloning purposes, Phusion High-Fidelity DNA polymerase (Biolabs, #M0530) was used since this enzyme has proofreading activity and produces amplicons with blunt ends. Reaction mixes contained 1x buffer, 200 µM of each deoxynucleotide triphosphate, 500 nM of each primer, 1 U of DNA

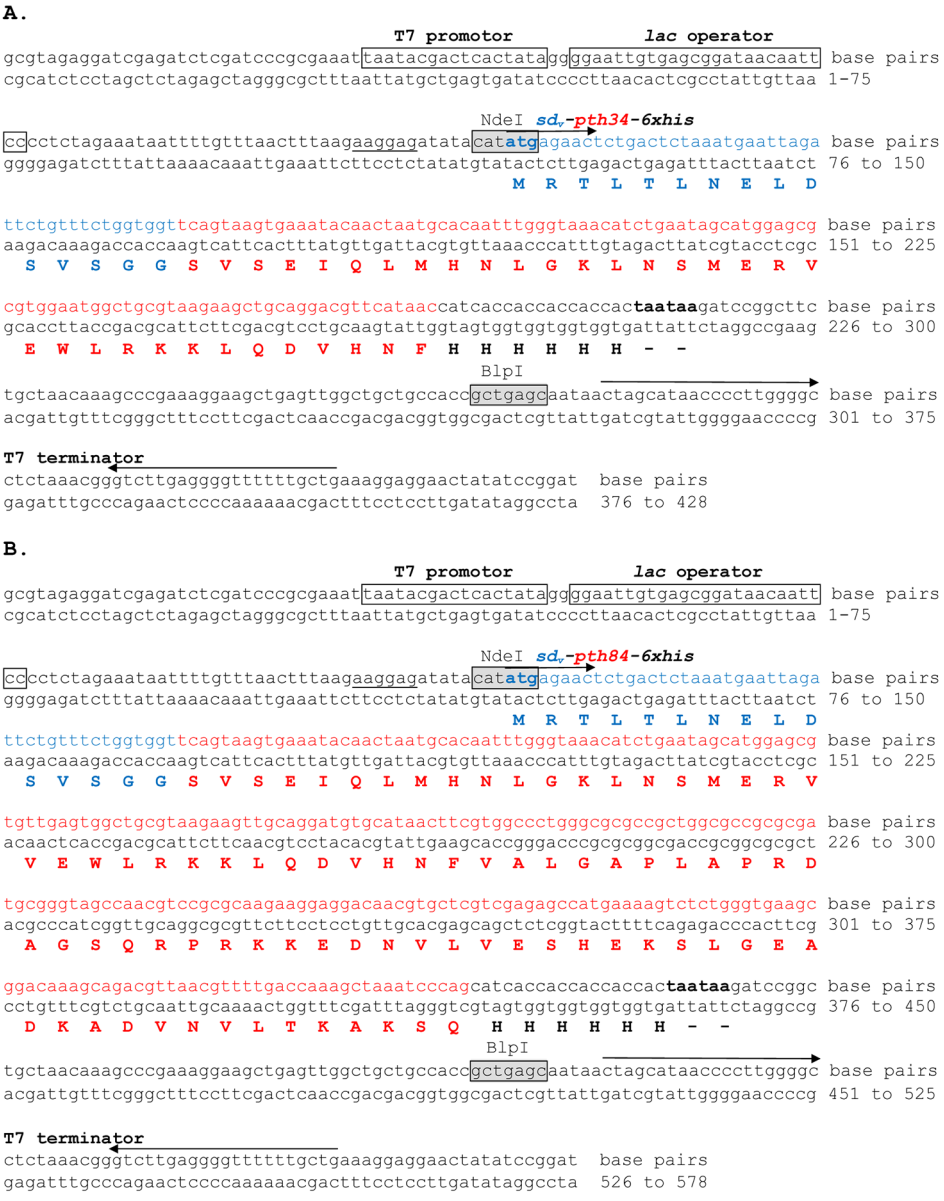

Fig. 2 Sequences containing the gene fusions cloned into pET-26b(+). Nucleotide sequences of the inserts and of the vector adjacencies are indicated. In grey and boxed, restriction sites employed for cloning the fusions. Start and stop codons are in bold and Shine-Dalgarno sequences are underlined. The amino acid sequence of the fusion products is indicated. Sequences corresponding to SD_V and PTH variants are in blue and red, respectively. (**A**) *sd$_v$-pth34-6xhis* (229 bp). (**B**) *sd$_v$-pth84-6xhis* (374 bp)

polymerase and 10 µl of cell lysate. Conditions for PCR were: 30 s at 98 °C, 30 cycles of incubation at 98 °C for 5 min, annealing temperature for 30 s (60 °C to amplify *cvaAB*, 58 °C for *cvicvaC* and 56 °C for the entire MccV genetic system), 72 °C for 30 s, and a final extension step at 72 °C for 5 min. The resulting PCR products were purified with MinElute Purification kit (Qiagen, #28006).

Detection of MccV production

It was assayed by two procedures, replica-plating and patch test [39, 42]. Clones carrying plasmids pExAB and pBRic were analysed by replica-plating on a lawn of the MccV-sensitive strain MC4100 seeded on M63 minimal medium plates. After incubation, MccV production was detected as growth inhibition halos of the MC4100 indicator strain appearing around the producing clones. Similarly, in the patch test, clones were stabbed into a lawn of MC4100 seeded on minimal plates to see growth inhibition halos. MC4100 (pMccV) and MC4100 (pBRic) were used as controls of microcin producing and non-producing strains, respectively.

Preparation of samples for the analysis of heterologous PTH secretion

Experimental and control strains were grown in flasks for ca. 18 h at 30 °C with shaking at 120 rpm in Zym-mod containing the required antibiotics (chloramphenicol and/or kanamycin). Then, aliquots were subcultured (1%) in flasks with 50 mL of fresh Zym-mod supplemented with the corresponding antibiotics and incubated under the same conditions until they reached an OD_{600} of ca. 1. At this point, cultures were supplemented with 0.5 mM IPTG and further incubated for 20 h until processing [38]. Cultures were centrifuged at 6,500 rpm for 10 min at 4 °C to separately analyse cells and supernatants. Cell fractions were processed differently depending on whether they were to be analysed directly on gels or by Western blot. For analysis on Tricine SDS-PAGE, cells were harvested, resuspended in PBS 1:1, and then lysed by sonication. Aliquots were mixed with reducing sample buffer A (12% SDS, 6% mercaptoethanol, 30% glycerol, 0.05% Coomassie brilliant blue R250, 150 mM Tris/HCl pH = 7.0) and heated at 80 °C for 30 min. For Western blot, cells were resuspended in PBS 1:1, and prepared by adding reducing buffer A and heating at 42 °C for 30 min to be loaded directly onto gels. Supernatants were recovered after centrifugation, and filtered using 0.45 µm Millipore membranes to remove remaining cells. Then, samples were mixed with buffer A, heated at 42 °C for 30 min, and loaded onto gels for direct staining or for Western blotting.

Tricine SDS-PAGE electrophoresis

Proteins contained in supernatants and cell lysates were analysed on 10% Tricine SDS-PAGE, as previously described [43]. The run lasted 1,5 – 2 h as follows: 30 V (30–40 mA) for start, 60 V (40–50 mA) in the stacking and 100 V (70–90 mA) for the rest of the running. As protein controls, 1 µg of PTH34 (Abcam, #ab269813) and 1 µg of PTH84 (Abcam, #ab51234), treated as supernatant samples, were used. Pre-stained protein ladder -extra broad molecular weight (5-245 kDa) (Abcam, #ab116029) was employed as standard, loading 5 µl per gel. After the run, gels were incubated in fixing solution (95% ethanol, 10% acetic acid) for 30 min and then stained with 0.025% Coomassie R250 for 18 h.

Western blot

Samples of supernatant and cells were separately processed so that they represented equal volumes of culture. They were processed as previously described. Peptides migrated in Tricine SDS-PAGE were transferred to a 0.2 µm nitrocellulose membrane (Biorad, #162 – 0147) at ca. 280–330 mA for 2 h, using the Mini-trans Blot Electrophoretic Transfer Cell (Biorad). Then, the membrane was incubated in blocking solution [5% wt vol^{-1} low-fat milk in TTBS buffer (20 mM Tris/HCl pH = 7.5, 150 mM NaCl, 0.05% Tween20 vol vol^{-1})] for 24 h at 4 °C. Proteins were labelled by incubation with the primary Mouse Anti-His tag antibody (GenScript, #A00186), diluted 1:500 in blocking solution for 1 h at room temperature. After washing with TTBS, the membrane was incubated with a 1:20,000 dilution of the secondary Goat Anti-Mouse IgG (Fc specific)-HRP (Sigma, #A0168) in TTBS for 45 min at room temperature [36]. Clarity Max TM Western ECL Substrate kit (Biorad, #170565) was employed to visualize bands by exposing the membrane in the GBOX Chemi System XT4 (SynGene).

Mass spectrometry

Bands of interest were extracted from gels stained with Coomassie and analysed directly by mass spectrometry without further treatment. The mass spectrum of each band was determined at the "Unidad de Bioquímica y Proteómica Analíticas" of Pasteur Institute, Montevideo. Bands corresponding to supernatants were subjected to a trypsin digestion and analysed using a LTQ Velos + ETD (Thermo-Scientific) mass spectrometer associated to a nano HPLC. In the case of bands proceeding from BL21(DE3) (pExAB; pET-PTH84) lysate, trypsin digestion followed by an analysis with a MALDI TOF-TOF mass spectrometer was performed. The data base used was composed of the proteome of *E. coli* BL21(DE3) strain (https://www.uniprot.org) and of common contaminants of this type of experiments. PTH peptides of interest were included in the analysis.

Results

The aim of this work was to analyse the ability of MccV T1SS to secrete two PTH variants in *E. coli* cells. The experimental design consisted of four steps: (i) cloning the MccV export genes; (ii) construction of plasmids carrying the sd_v*-pth-6xhis* gene fusions; (iii) construction of experimental and control BL21(DE3) derivative strains, and (iv) assessment of heterologous PTH secretion.

Cloning of the MccV export genes

Considering that the type I secretion system forms a channel through the cell envelope, it seemed convenient to produce moderate levels of the constituent proteins so as not to compromise cell viability. Thus, a medium copy number vector (pUCYC5) was selected to construct plasmid pExAB, carrying the *cvaAB* genes under the *POlac* promoter. *E. coli* K12 MC4100 strain with this plasmid grew stably on LB Cm plates incubated at 30 °C. In this context the functionality of the T1SS encoded by pExAB was evaluated through its ability to secrete MccV. For this purpose, MC4100 (pExAB) was transformed with plasmid pBRic, which encodes the MccV peptide precursor CvaC and its cognate immunity Cvi, and clones were

selected on LB Cm Ap plates. Then, they were directly assayed for their ability to produce MccV by replica-plating. All transformant clones generated growth inhibition halos, indicating that pExAB encoded a functional T1SS. The same analysis was done in the context of *E. coli* B BL21 (DE3), with similar results (Fig. 3A). Subsequently, some purified clones from both contexts were analysed by patch test and proved to generate antibiosis halos of similar size to those produced by MC4100 (pMccV), which contains the entire MccV genetic system (17–18 mm in diameter) (Fig. 3B). Therefore, efficient levels of MccV secretion were achieved with plasmid pExAB in both cellular contexts *E. coli* K12 and B, the latter of which was then used to assess hormone secretion.

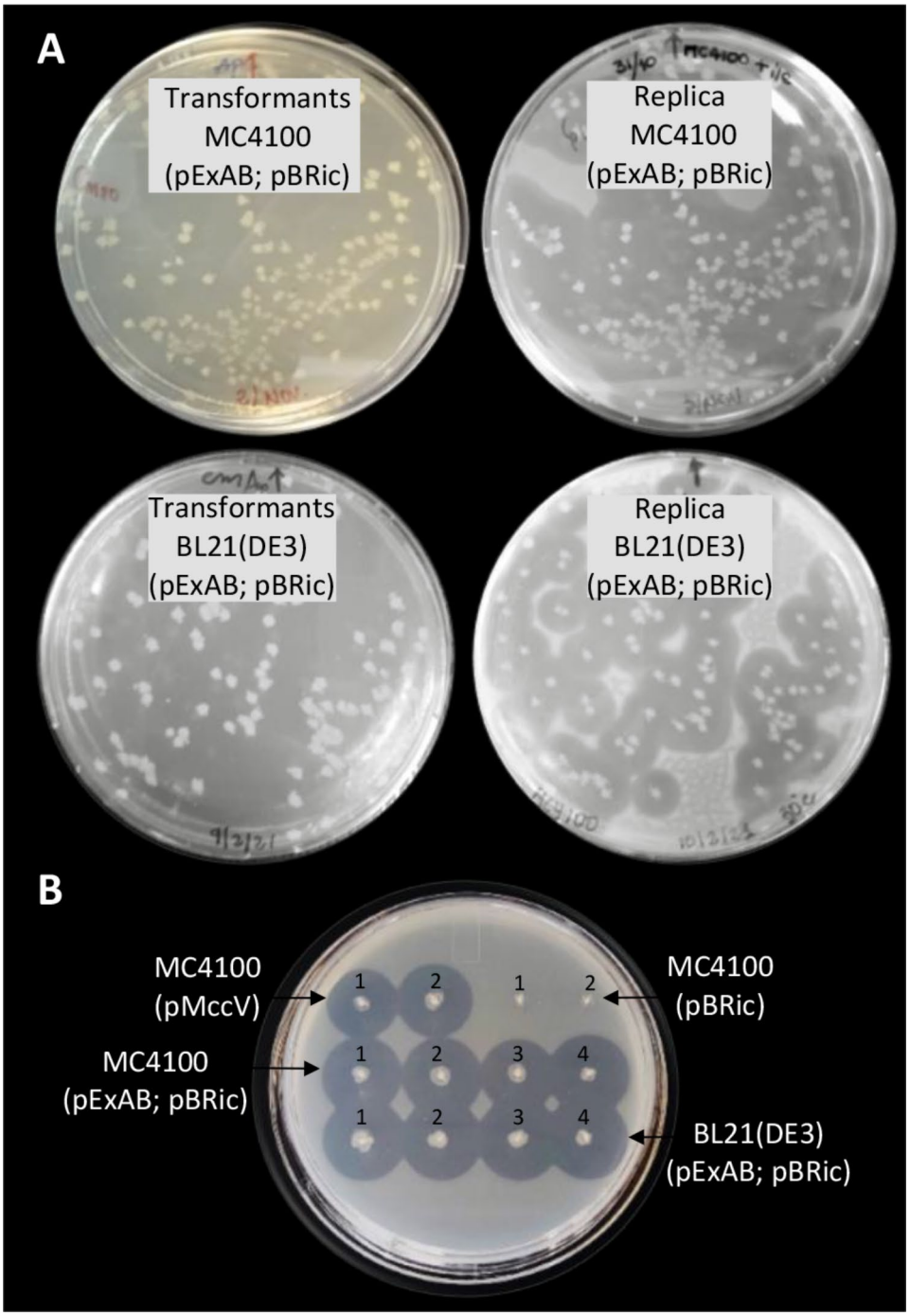

Fig. 3 Analysis of the MccV T1SS functionality. (**A**) Replica-plating assay to assess the secretion of MccV by transformant clones of the indicated strains. (**B**) Patch test performed with stabs of the indicated strains: two of each control strains and four of each experimental strains (purified transformant clones)

Construction of plasmids carrying the gene fusions *sd$_V$-pth-6xhis*

Gene fusions encoding hybrid peptides composed of the SD_V attached to each PTH variant (PTH34 and PTH84) were designed as described in Materials and methods. They were cloned into vector pET-26b(+) under the T7*lac* promoter, so that their expression would depend on the presence of the T7 RNA polymerase in the cells. The inserts containing the fusion coding sequences were introduced into the pET vector between the NdeI and BlpI sites, followed by six histidine codons and two stop codons (Fig. 2). These were of 229 bp for *sd$_v$-pth34-6xhis* and of 374 bp for *sd$_v$-pth84-6xhis*, carried by plasmids pET-PTH34 and pET-PTH84, respectively.

Construction of BL21(DE3) derivative strains

Plasmid constructions containing the gene fusions were introduced into BL21(DE3) cells in order to be expressed. This strain contains the gene for the T7 RNA polymerase which is inducible by the addition of lactose or IPTG. Under these conditions T7 RNA polymerase is produced and consequently gene fusions should be expressed [38, 44]. The secretion experiments were based on the interaction between hybrid peptides as substrates and the MccV T1SS. Each of these participants was encoded by different recombinant plasmids and these were introduced by transformation into BL21(DE3) cells. Thus, two "experimental strains", BL21(DE3) (pExAB; pET-PTH34) and BL21(DE3) (pExAB; pET-PTH84), and three "control strains", BL21(DE3) (pExAB), BL21(DE3) (pET-PTH34) and BL21(DE3) (pET-PTH84) were constructed. All strains were grown on LB medium with the corresponding antibiotics to avoid plasmid loss and incubated at 30 °C. On solid medium, the streaks tended to be rather poor, with small colonies, although with a low level of genetic instability (no evident overgrowth) (Fig. 4).

Assessment of heterologous PTH secretion

Growth of the five strains and induction with IPTG were performed as described in Materials and methods. Before sample processing, a viable cell count was carried out on plates with the corresponding antibiotics. Cultures of BL21(DE3) harbouring pET-PTH34 and pET-PTH84, alone or with pExAB, grew similarly with a viable count of ca. 2×10^9 cfu mL^{-1}, and the strain BL21(DE3) (pExAB) reached a count of 1×10^{10} cfu mL^{-1}.

As references for the analysis, the expected molecular weights of hybrid peptides (SD_V-PTH34-6xHis and SD_V-PTH84-6xHis), of their derivatives lacking the MccV secretion domain (PTH34-6xHis and PTH84-6xHis) and of pure PTH protein controls (PTH34 and PTH84) are shown in Table 1. As these peptides ranged from 4 to 12 kDa, 10% Tricine SDS-PAGE was used to analyse the protein content of supernatants and cell lysates.

First, an assay focusing on culture supernatants was performed, in order to analyse whether the MccV T1SS was indeed capable of secreting the hormone peptides. It was expected that CvaB recognized and processed the SD_V bound to the N-terminus of each PTH variant, and that the PTH-6xHis peptides would be released into the extracellular medium as a consequence of the secretion process. Thus, supernatants of the five strains were recovered after 20 h of induction with IPTG, then filtered, and aliquots were analysed on a Tricine SDS-PAGE gel. After staining with Coomassie R250, there was a faint band profile in all lanes. However, clear bands appeared in the supernatants of the experimental strains with slightly less migration than that of the pure PTH proteins (Fig. 5A). Taking into account that these bands did not have counterparts in the supernatants of any control strain, they were extracted from the gel and analysed by mass spectrometry. Indeed, it was determined that they corresponded to the processed PTH peptides (Fig. 5B). In both cases, the N-terminal peptide resulting from the T1SS specific cleavage was detected, i.e. sequences started in

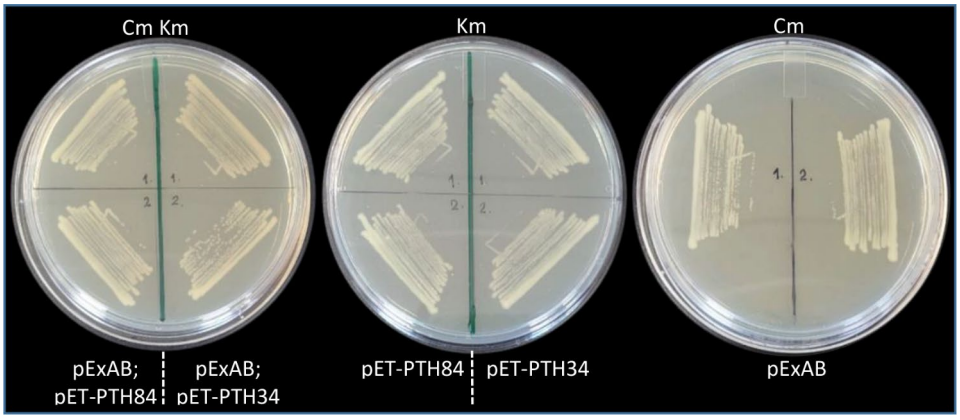

Fig. 4 Growth of experimental and control BL21(DE3) derivative strains on LB solid medium. 20 h incubation. Above, antibiotics added to plates; below, plasmids carried by strains. Two independent transformant clones of each strain are shown

Table 1 Features of PTH peptides

Peptide	Sequence[a]	Extension [aa]	MW [kDa][b]
SD_V-PTH34-6xHis	MRTLTLNELDSVSGGSVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF*HHHHHH*	55	6.52
PTH34-6xHis	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF*HHHHHH*	40	4.94
SD_V-PTH84-6xHis	MRTLTLNELDSVSGGSVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALGAP LAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ*HHHHHH*	105	11.82
PTH84-6xHis	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALGAP LAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ*HHHHHH*	90	10.25
PTH34	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF	34	4.12
PTH84	SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNFVALGAP LAPRDAGSQRPRKKEDNVLVESHEKSLGEADKADVNVLTKAKSQ	84	9.43

[a] In red, SD_V; regular letter, PTH sequence; italic, 6xHis tag
[b] Calculated molecular weight (https://www.bioinformatics.org/sms/prot_mw.html)

the residue immediately after the double-glycine motif of the SD_V. Thus, this result demonstrated that the hybrid peptides SD_V-PTH34-6xHis and SD_V-PTH84-6xHis were specifically recognized, processed and secreted by the MccV T1SS. Considering the amount of pure PTH proteins loaded into the gel, it could be visually estimated that each experimental PTH band contained ca. 100 ng, which would correspond to ca. 10 mg L^{-1} in the culture (Fig. 5A).

It should be noted that the migration of the protein ladder did not agree with that of the commercial pure PTH proteins and thus, with that of bands recovered from the experimental supernatants. This could be due to the fact that migration of this molecular weight marker has never been analysed in Tricine SDS-PAGE gels (personal communication with Abcam).

To search for the PTH peptides in the intracellular milieu of the experimental strains, cell lysates were run on Tricine SDS-PAGE gels and stained with Coomassie (Fig. 6A). A strong band of the expected size for SD_V-PTH84-6xHis peptide was seen in the lane of BL21(DE3) (pExAB; pET-PTH84), i.e. this band migrated above but close to the pure PTH84 protein. Below, faint bands appeared, which were suspected to be degradation products of the SD_V-PTH84-6xHis peptide, although a PTH84-6xHis peptide, processed by the MccV T1SS, could not be discarded. Thus, the strong band, called band 1, as well as those migrating immediately below, jointly named as band 2, were extracted from the gel and analysed by mass spectrometry. The analysis confirmed that band 1 corresponded to the unprocessed hybrid peptide SD_V-PTH84-6xHis: an N-terminal trypsin peptide encompassing the junction between the secretion domain (SD_V) and the PTH84 variant was identified (Fig. 6B). Regarding band 2, the presence of PTH84-related peptides was confirmed through the detection of their C-terminal residues while no information could be obtained of their N-terminus (Fig. 6B). In the case of BL21(DE3) (pExAB; pET-PTH34) lysate, no bands appeared in the zone of migration expected for SD_V-PTH34-6xHis peptide or for its possible derivatives (data not shown). It is important to note that in this experiment, as well as in subsequent ones, both PTH34-derived peptides and pure PTH34 were not visible on the gels.

To get further insight into the analysis of PTH peptides secretion, Western blot experiments were performed to detect them with more sensitivity, using an anti-His tag antibody, as explained in Materials and methods. Once again, positive results were obtained only with samples from the BL21(DE3) (pExAB; pET-PTH84) culture. In this case, there were clear bands from both types of sample supernatant and cells. Their migration revealed the expected size difference between the SD_V-PTH84-6xHis and PTH84-6xHis peptides. The first appeared in the cell extract while the second only in the supernatant (Fig. 7). It should be noted that in the cell fraction faint secondary bands appeared below the main one; in any case, all of them were higher than that observed in the supernatant. Therefore, it is most likely that they corresponded to degradation derivatives of the original SD_V-PTH84-6xHis peptide. On the contrary, no traces of degradation could be seen below the main band in the supernatant.

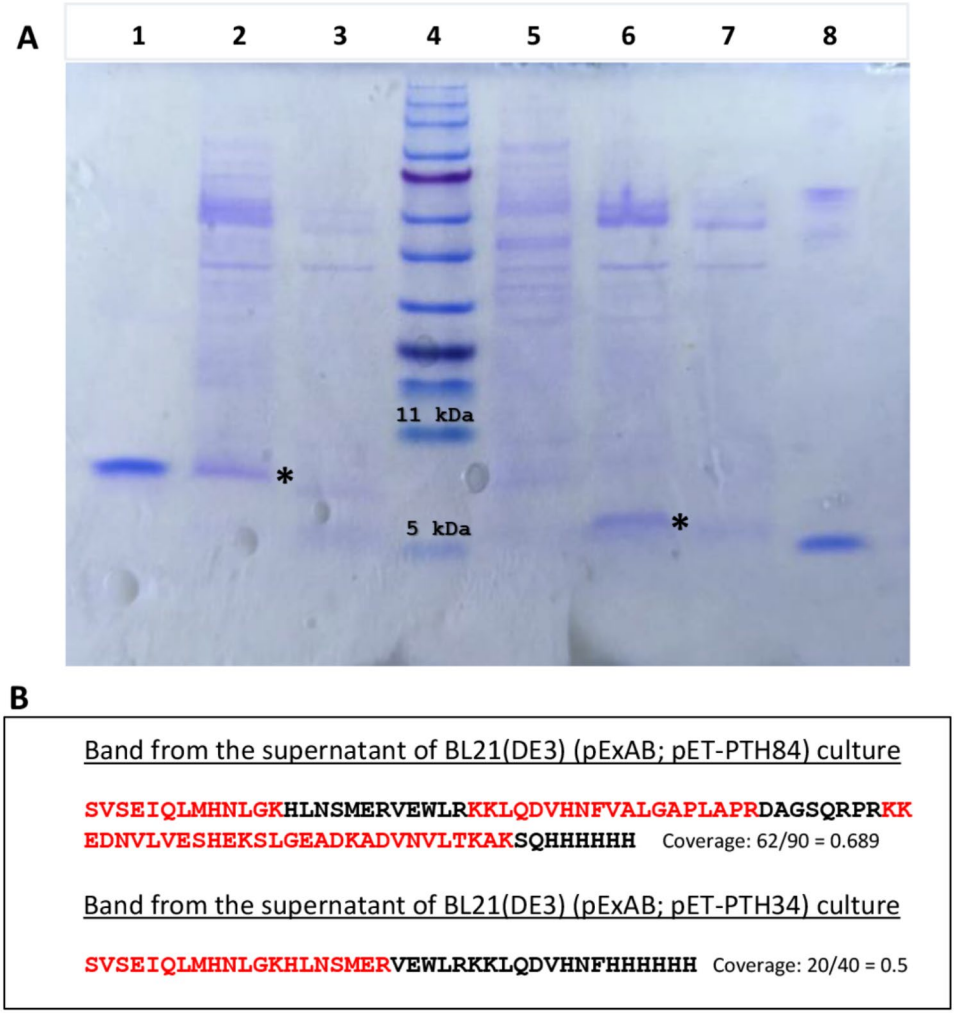

Fig. 5 Secretion of PTH peptides: supernatants. (**A**) Tricine SDS-PAGE of supernatants from cultures of control and experimental strains. Aliquots of 10 µl of each supernatant were loaded. (1): 1 µl of PTH84 (1 µg); (2): BL21(DE3) (pExAB; pET-PTH84); (3): BL21(DE3) (pET-PTH84); (4): protein ladder (5 µl); (5): BL21(DE3) (pExAB); (6): BL21(DE3) (pExAB; pET-PTH34); (7): BL21(DE3) (pET-PTH34); (8): 1 µl of PTH34 (1 µg). Asterisks, bands analysed by mass spectrometry. (**B**) Mass spectrometry analyses from bands marked in A. PTH84-6xHis and PTH34-6xHis sequences were included in the data base of the analysis. Red, peptides detected by mass spectrometry

Discussion

Export of recombinant proteins in *E. coli* is a relevant research topic for the biotechnology industry. Crossing the cell envelope and obtaining a protein in the extracellular space has many advantages: facilitates purification, reduces toxicity for the producing bacteria, minimizes the action of intracellular proteases, among others. Basically, two strategies have been developed for recombinant protein secretion in *E. coli*: the so-called two-step secretion mechanism, depending on the Tat or Sec pathways plus some strategy to cross the outer membrane, and the one-step secretion mechanism, which includes the T1SS. In general terms, they all present some disadvantages, that could be low yields, narrow substrate range, recovering the target protein in a mix of periplasmic and some cytoplasmic proteins, among others [45–47]. Therefore, further efforts to set up an efficient secretion mechanism to obtain recombinant proteins in the extracellular milieu continue being of interest. In the context of biotechnological interest in recombinant peptide secretion, a few articles using a T1SS have been published in recent years. For example, secretion of nanobodies fused to the HlyA secretion domain has been reported in *E. coli* cells [48]. Recently, four natural peptides, including the human epidermal growth factor, were secreted heterologously by the MccV T1SS [29]. This latter is precisely the closest antecedent to this work.

Here, PTH peptides were produced as fusion proteins containing the MccV secretion domain at their N-terminus and a 6xHis tag at their C-terminus. In the context of *E. coli* B cells, the MccV T1SS secreted both PTH variants through the recognition and processing of the

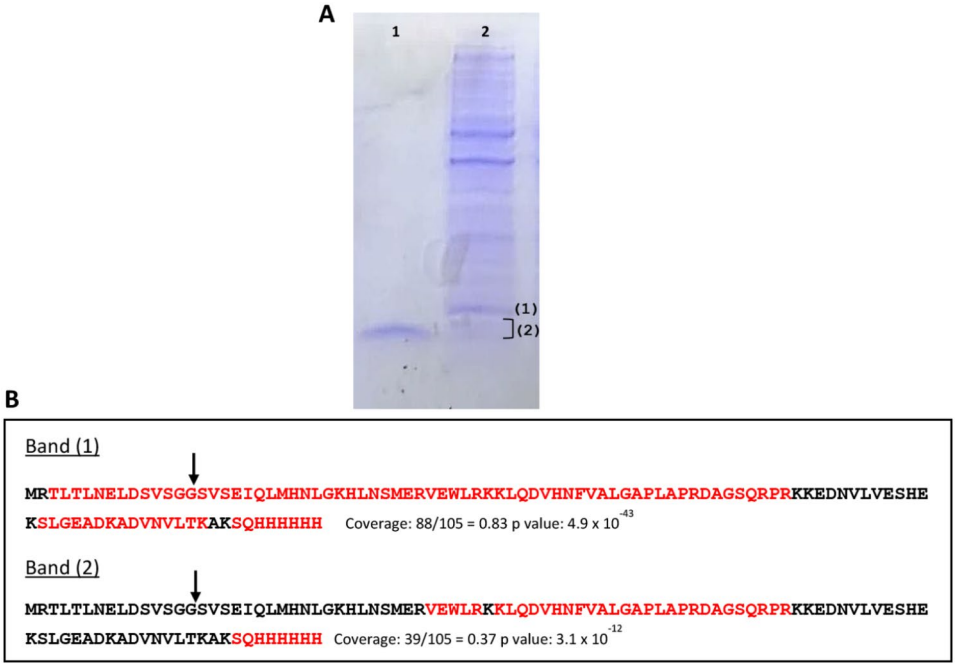

Fig. 6 Intracellular PTH peptides: cell lysates. (**A**) Tricine SDS-PAGE of BL21(DE3) (pExAB; pET-PTH84) lysate. Lines: 1, 1 µl of PTH84 (1 µg); 2, 10 µl of cell lysate. (1) and (2), bands analysed by mass spectrometry. (**B**) Mass spectrometry analyses. Data base for band (1) included the unprocessed SD_V-PTH84-6xHis sequence, and for band (2), the processed PTH84-6xHis sequence. Red, peptides detected by mass spectrometry. Arrow, specific cleavage site performed by the MccV T1SS

SD_V. Several experimental evidences confirm this result. Assays comparing filtered culture supernatants of experimental and control strains revealed that only the experimental strains secreted the PTH peptides (Fig. 5). Their identity was confirmed by mass spectrometry and the analysis revealed that they corresponded to PTH-6xHis peptides lacking the MccV secretion domain. Therefore, these peptides had been specifically secreted by the MccV T1SS. Moreover, no trace of them could be seen in the supernatant of the control strains only encoding the hybrid peptides. Another point that supports the result was the poor protein background exhibited in gels by all the supernatants, in agreement with the described scarce secretome of *E. coli* B cells [49]. This evidence, together with the fact that cells were completely removed by filtration, discards cell lysis as the reason for the presence of PTH peptides in the supernatant. It should also be stressed that small amounts (10 µL) of raw supernatants analysed on gels revealed a clear contrast between the PTH bands and the faint background in the lanes (Fig. 5). Regarding the estimated amount of the secreted peptides (ca. 10 mg L^{-1}), their secretion level would be comparable to that of other nonbacterial bioactive peptides exported by the MccV T1SS [29]. In addition, it is noteworthy that the secreted PTH peptides were seen in the crude supernatant fractions, without any processing - such as purification steps - that could affect the detection of their true amount in the culture supernatant.

Additional evidence supporting the specific secretion of PTH peptides by the MccV T1SS was provided by the analysis of the cell fractions. These experiments could only be performed with the PTH84 peptide because the small PTH version proved to be extremely unstable. The unprocessed peptide SD_V-PTH84-6xHis could be identified by mass spectrometry in cell fractions (Fig. 6). As far as we know, this would be the first characterization at the sequence level of an intracellular non-bacterial peptide intended to be processed and secreted by a T1SS. Coherently, in a Western blot assay the difference in migration of the unprocessed intracellular (SD_V-PTH84-6xHis) and processed extracellular (PTH84-6xHis) forms was evident (Fig. 7). It is noteworthy that unprocessed peptide did not appear in the supernatant nor did processed peptide appear in the cellular fraction, indicating that processing and secretion would be concomitant. This provides physiological support to the hypothesis of a coupled mechanism of substrate processing and secretion, which has been proposed for the ABC transporters involved in peptide export [7, 12, 13, 50]. Moreover, in this work, results indicate that the directionality of the process would not be restricted to the ABC protein but to the entire three-component T1SS.

Considering that in the experiment shown in Fig. 7 equal volumes of cells and supernatant proceeding from the same culture were assessed, and judging by the intensity of the main bands in both fractions, it could

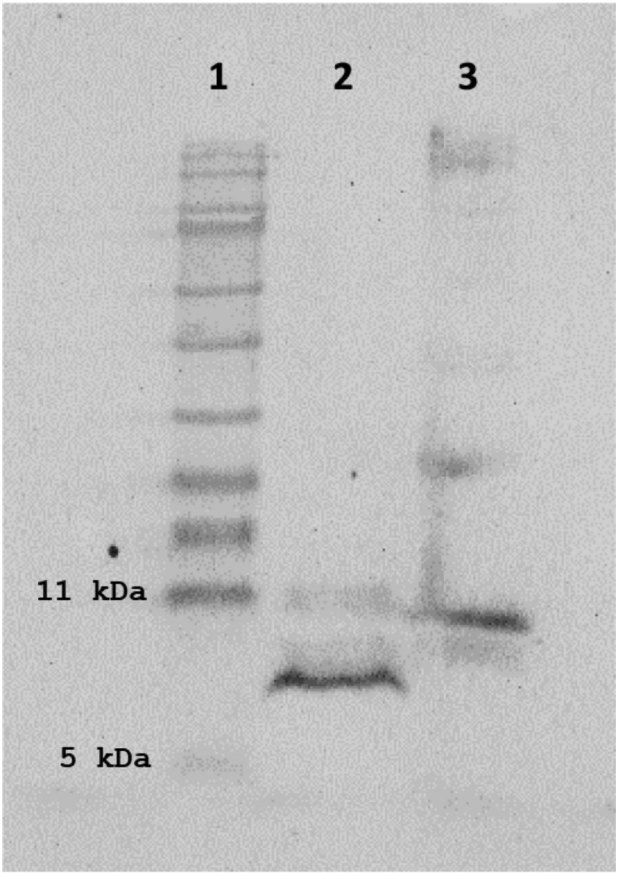

Fig. 7 Western blot assay for the identification of PTH84-6xHis peptide forms. Membrane transferred from Tricine SDS-PAGE loaded with samples from BL21(DE3) (pExAB; pET-PTH84) culture. Detection with Anti-His tag antibody. (1): protein ladder (5 µl); (2): 15 µl of supernatant; (3): 15 µl of cell lysate

be estimated that about half of the synthesised peptide was secreted into the extracellular medium. It should be stressed that in the supernatant no bands smaller than that corresponding to the secreted PTH could be detected, a fact that indicates that no important degradation would occur in the extracellular milieu. Therefore, this location seems to favour the hormone´s stability. On the contrary, the intracellular extract showed not only the band of the unprocessed PTH peptide but also one or more faint bands below, which could correspond to degradation products (Figs. 6A and 7). This interpretation agrees with the mass spectrometry analysis of band 2 from an intracellular sample, in which the N-terminal sequence was not recovered (Fig. 6B).

In general terms, a clear trend towards hybrid peptides degradation was observed, being most pronounced in the case of PTH34. In fact, the analysis with this small peptide was finally abandoned in view of the low reproducibility of the results. It is known that degradation by cellular proteases is one of the reasons for poor production of recombinant proteins in *E. coli*. This phenomenon is particularly relevant in the case of peptides, whose degradation is faster the shorter they are [51]. This could be the reason for the extreme instability of PTH34 compared to that of PTH84 chimeras. Nevertheless, other investigators have concentrated in the production of the PTH34 variant, which has been commercially launched by Eli Lilly as a recombinant peptide produced in *E. coli* [52]. This drug, called Teriparatide, is employed for the treatment of osteoporosis [30, 53, 54]. Several authors have reported problems with the recombinant production of the two hormone variants (PTH34 and PTH84) in *E. coli* cells, which are mainly related to their intracellular degradation and formation of inclusion bodies. In this sense, improvements were achieved by combining the hormone with different protein partners. However, these strategies did not include any specific secretion step so the hybrid proteins had to be purified from cellular components and their partners removed [31–35, 55–60]. It is worth mentioning that some non-specific procedures have been tested to obtain hormones in the extracellular space, which consist of their leakage together with periplasmic proteins or their export using a Gram-positive bacterial model [31, 35, 56, 61]. In this work, the specific secretion of the two PTH peptides in *E. coli* cells is demonstrated. More complete information was reached with the PTH84 variant: while its peptide precursor underwent a partial degree of degradation in the intracellular milieu, the secreted mature peptide remained stable in the extracellular space.

Conclusion

In this work a recombinant system designed to synthesize and secrete two variants of the human parathyroid hormone in *E. coli* cells is presented. The type I secretion system naturally devoted to export microcin V proved to be proficient for specifically secreting the native hormone peptides with the sole addition of a 6xHis tag at their C-terminus. The peptides could be recovered directly from the extracellular milieu where they appeared to be stable, i.e. no degradation was detected. These are preliminary results that should be optimized to overcome intracellular peptide instability and to increase extracellular production yields. This would settle the basis for further determinations on the hormone´ s functionality and sample contaminants. In sum, these results could be seen as a new example of the potential of microcin type I secretion systems to be conceived as a new biotechnological tool for the secretion of recombinant peptides in bacteria.

Abbreviations

T1SS	Type I Secretion System
ABC transporter	ATP-Binding Cassette transporter
MFP	Membrane Fusion Protein
SD	Secretion Domain

SD_V	Secretion Domain of MccV
MccV	Microcin V
PTH	Human Parathyroid Hormone
Ap	Ampicillin
Cm	Chloramphenicol
Km	Kanamycin
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
IPTG	Isopropyl β-D-1-thiogalactopyranoside
OD_{600}	Optical Density at 600 nm
6xHis	Six Histidine Codons

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Author contributions
V.F. designed and performed the experiments, and analysed the data. J.M. designed the experiments and analysed the data. M.L. analysed the data, review and editing the manuscript. M.F.A. designed and performed the experiments, analysed the data, wrote the original draft and review and editing the manuscript. All the authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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