Heterologous extracellular production of enterocin P from
Enterococcus faecium P13 in the methylo trophic bacterium
Methylobacterium extorquens

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Received 8 February 2005; received in revised form 15 April 2005; accepted 17 May 2005
First published online 31 May 2005
Edited by M. Moracci

Abstract

Enterocin P (EntP), a strong antilisterial pediocin-like bacteriocin from Enterococcus faecium P13, was produced by Methylobac-
terium extorquens. For heterologous expression of EntP in the methylo trophic bacterium M. extorquens, a recombinant plasmid was
constructed. The gene encoding the EntP structural gene (entP) was cloned into the plasmid vector pCM80, under control of the
methanol dehydrogenase promoter (PmxaF), to generate plasmid pS25. When M. extorquens ATCC 55366 was transformed with
pS25, EntP was detected and quantified in supernatants of the recombinant M. extorquens S25 strain by using specific anti-EntP
antibodies and a non-competitive indirect enzyme-linked immunosorbent assay (NCI-ELISA). Purification of EntP by hydrophobic
adsorption and reverse-phase (RP-FPLC) chromatographies, permitted recovery of active EntP from the supernatants of M. extor-
quens S25 grown in a synthetic defined medium.

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Keywords: Enterocin P; General secretory pathway; Heterologous gene expression; Methylobacterium extorquens

1. Introduction

Most bacteriocins produced by lactic acid bacteria (LAB) are ribosomally synthesized as biologically inactive precursors or pre-peptides containing an N-terminal extension. The mature peptides are often cationic, amphiphilic membrane permeabilizing molecules of 20–60 amino acid residues classified into three main groups:Class I consists of modified bacteriocins, the

lantibiotics, Class II is comprised of non-modified, heat-stable bacteriocins, which are divided into subclasses IIa (pediocin-like, strong antilisterial bacterio-
cins), IIb (two-peptide bacteriocins) and IIc (other peptide bacteriocins). Class III consists of the protein bacteriocins [1–3]. N-terminal extensions of lantibiotics and most non-lantibiotics are of the so-called double-glycine type (leader sequence) and are cleaved off concomitantly with export across the cytoplasmic mem-
brane by specific ATP-binding cassette transporters (ABC-transporters) and their accessory proteins [4,5]. However, some class II bacteriocins, such as acidocin
B [6], diverginic A [7], bacteriocin 31 [8], enterocin P [9], and lactococcin 972 [10] contain an N-terminal extension of the so-called sec-type (signal peptide), which could be proteolytically cleaved and externalized by the general secretory pathway (GSP) or sec-dependent pathway [11–13].

Enterocin P (EntP) is a bacteriocidal pediocin-like bacteriocin produced by Enterococcus faecium P13 isolated from Spanish dry-fermented sausages [9], and by other E. faecium strains of diverse origin [14–16]. EntP is synthesized as a 71-amino acid pre-peptide consisting of a 44-amino acid mature bacteriocin and a 27-amino acid signal peptide. The mature EntP shows a broad antimicrobial spectrum against Listeria monocyctogenes and a wide range of spoilage and food-borne gram-positive pathogenic bacteria [9], suggesting its potential application as a natural food antimicrobial agent in the food industry. However, the use of enterococci as producers of bacteriocins may be cautioned since many Enterococcus isolates code potential virulence factors [17–19]. Accordingly, the cloning and production of bacteriocins produced by enterococci in safer heterologous hosts should be evaluated. A novel expression platform using M. extorquens as a natural food antimicrobial agent in the food industry. However, the use of enterococci as producers of bacteriocins may be cautioned since many Enterococcus isolates code potential virulence factors [17–19]. Accordingly, the cloning and production of bacteriocins produced by enterococci in safer heterologous hosts should be evaluated. A novel expression platform using M. extorquens ATCC 55366 as a host capable of over-expressing large recombinant intracellular proteins in high cell density fermentations using methanol as the sole source of organic carbon and energy has been recently described [20–23]. Contributing factors to the development of the M. extorquens expression systems are: (i) the inexpensive carbon (methanol) source (http://www.methanex.com) and a synthetic defined medium [24], (ii) the development of a high cell density fermentation process [22,24]; (iii), the availability of the partial genome sequence of M. extorquens AM1 (http://www.integratedgenomics.com/genomereleases.html), and the development and availability of genetic tools and expression vectors [20,23,25]. We report in this communication the cloning and production of EntP by M. extorquens.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The EntP-producing strain E. faecium P13 [9] and the indicator strain E. faecium T136 [26] were grown in MRS broth (Oxoid Ltd., Basingstoke, UK) at 32 °C. Plasmid pCR2.1-TOPO and competent Escherichia coli TOP-10 One Shot® Electrocomp™ cells, were obtained from Invitrogen (Carlsbad, CA, USA). Plasmid pCM80 was kindly provided by Dr. M. Lidstrom (Microbiology Department, University of Washington, WA, USA). The E. coli Tuner(DE3) pLacI (pETBlue-1) and E. coli Tuner (DE3)pLacI (pJG01) cells, were obtained as previously described [27]. The E. coli cells were propagated in Luria–Bertani (LB) broth at 37 °C, while M. extorquens ATCC 55366 [24] was grown at 30 °C in the synthetic CHOI-medium containing methanol (0.5% w/v) [28]. Agar plates were made by the addition of 1.5% (w/v) agar to the broth media. The following selective agents were used: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 80 μg ml⁻¹), glucose (1% w/v), kanamycin (kan, 50 μg ml⁻¹) and/or tetracycline (tet, 20 μg ml⁻¹) all from Sigma Chemical Co., (St Louis, MO, USA). Competent M. extorquens cells were obtained and transformed as described by Figueira et al. [20].

2.2. Construction of the recombinant plasmid pS25

Total genomic DNA from E. faecium P13 was isolated using the Genomic DNA Isolation Kit (Bio-Rad Laboratories, Hercules, CA, USA), and was used as template for PCR amplification of a 247-bp BamHI-KpnI fragment (S19) carrying entP and its ribosome binding site (RBS), using S1Up-Bam (5′-GGGA-TCAAAGGAGGTATTGTATTTAGAGAA-3′) and S1Dn-Kpn (5′-CGGTACCTTATGTCCCATACC-TGCCAAA-3′) as specific PCR primers. The forward primer, S1Up-Bam, contained a BamHI cleavage site (nucleotides underlined in primer above), while the reverse primer, S1Dn-Kpn, included a KpnI cleavage site (nucleotides underlined in primer above). The PCR-amplified S19 fragment was cloned into pCR2.1-TOPO to generate plasmid pS19, and competent E. coli TOP-10 cells were chemically transformed with this recombinant plasmid. Plasmid pS19 was isolated from transformed cells using a QIAprep Spin Minprep Kit (Qiagen, Hilden, Germany), and digested with BamHI and KpnI (Amersham Biosciences Corp., Piscataway, NJ, USA). The resulting 242-bp BamHI-KpnI cleaved fragment (insert S25) was recovered from agarose gels with a QIAquick Gel Extraction kit (Qiagen) and ligated (T4DNA ligase; New England BioLabs; Beverly, MA, USA) into plasmid pCM80 [25], previously digested with BamHI and KpnI, to generate plasmid pS25. Competent E. coli TOP-10 cells transformed with pS25 were assayed for its direct antimicrobial activity and purified plasmid pS25 evaluated for correct insertion of fragment S25. Competent M. extorquens ATCC 55366 cells were also transformed with pS25, and the presence of this plasmid in the M. extorquens S25 isolate was confirmed by a direct antimicrobial test and PCR-amplification using the entP specific primers S1Up-Bam and S1Dn-Kpn and the pCM80 specific primers pLAC_F (5′-GCTTTACACTTTATGCTTCCCG-3′) and lac_Z_R (5′-GCTTTACACTTTATGCTTCCCG-3′). Primers used for amplification of the inserts were designed from the published nucleotide sequence of the EntP operon [9] and pCM80 [25], and were obtained from Hukabel Scientific Ltd. (Montréal, Québec, Canada).
PCR-amplification of the inserts was performed in 100 μl reaction mixtures using 1 μl of purified DNA, 70 pmol of each primer and 5 U of Taq® DNA polymerase (Amersham Biosciences), and the PCR conditions included a hot start at 94 °C (5 min), primer annealing at 50 °C (30 s), primer elongation at 72 °C (30 s), and denaturation at 94 °C (30 s). Amplification reactions (35 cycles) and a final extension at 72 °C for 7 min were carried out in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR fragments were analyzed by electrophoresis on 2% (w/v) agarose gels at 90 V for 1 h, using the 100-bp DNA ladder (Gibco-BRL, Life Technologies Inc., Grand Island, NY, USA) as a molecular weight marker.

2.3. Antimicrobial activity assays

The antimicrobial activity of E. coli and M. extorquens cells was assayed by a stab-on-agar test, as previously described [15]. Briefly, the E. coli isolates were stabbed onto LB plates containing glucose (1% w/v) and incubated at 37 °C for 6 h to initiate bacterial growth and bacteriocin production, and the M. extorquens isolates were developed on CHOI plates at 30 °C for 36 h. Then, 15 ml of MRS soft agar (0.8% agar) containing about 10^7 cfu ml⁻¹ of the indicator E. faecium T136 were poured over the plates. Following incubation at 32 °C for 18 h, the plates were examined for growth inhibition zones. Cell-free culture supernatants from E. faecium, E. coli and M. extorquens cells were obtained by centrifugation of cultures at 12,000g for 10 min at 4 °C, adjusted to pH 6.2 with 1 M NaOH, filtered through 0.22 μm-pore-size-filters (Millipore). Then, 50 g (5% [w/v]) of Amberlite XAD-16 (Sigma) was added to the supernatant, and the sample kept at 4 °C with stirring for 2 h. The sample was loaded into an econo-column chromatography support (Bio-Rad) and, when the supernatant drained off, the matrix was washed with 100 ml distilled water in the first step and with 75 ml of 40% ethanol in water (v/v) later, to remove weak or non-hydrophobic compounds. The adsorbed bacteriocin was eluted with 200 ml of 85% 2-propanol pH 2.0 in water (v/v) and the remaining 2-propanol was eliminated by mild heating (35–40 °C) of the sample in a rotavapor (Büchi, Switzerland). After addition of 0.1% trifluoroacetic acid (TFA) to the sample, the antimicrobial activity was further subjected to reverse-phase chromatography in a C2 to C18 column (PepRPC HR 5/5) integrated in a FPLC system (RP-FPLC). The bacteriocin was eluted from the column with a 55 min linear gradient of 20–35% 2-propanol in aqueous 0.1% TFA at a flow rate of 0.5 ml min⁻¹. Purified EntP was stored in 60% 2-propanol with 0.1% TFA at −20 °C. Pure enterocin Q, pediocin PA-1 and EntP from E. faecium P13 and M. extorquens S25 were subjected to Tricine-SDS-PAGE, as described by Shägger and Von Jagow [30]. Protein electrophoresis was performed on Novex 16% Tricine gels in a XCell SureLock Mini-Cell at 80 V constant current. Gels were blotted onto a PVDF membrane, and the Western blotting completed as previously described [29].

2.4. Enzyme-linked immunosorbent assay (ELISA)

Polyclonal antibodies with specificity for EntP and a non-competitive indirect ELISA (NCI-ELISA), used as previously described [29], were employed to detect and quantify production of EntP by M. extorquens S25. Briefly, wells of flat-bottom polystyrene microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight (4 °C) with pure EntP or supernatants of M. extorquens S25. After coating, wells were blocked and then washed. Next, diluted anti-P3-KLH (C-terminal epitope EntP) serum was added to each well, unbound antibodies were removed by washing, and goat anti-rabbit IgG peroxidase conjugate (Cappel Laboratories, West Chester, PA, USA) was added. Bound peroxidase was determined with ABTS (2,2’-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid], Sigma) as the substrate by measuring the absorbance of the wells at 405 nm with a Labsystems iEMS reader (Helsinki, Finland). Known concentrations of EntP in MRS and CHOI were used to construct standard curves.

2.5. Purification of enterocin P, protein electrophoresis and Western blotting

Ent P was purified from E. faecium P13 as previously described [29]. Briefly, supernatants from 1-l cultures were subjected to precipitation with ammonium sulfate, desalting by gel filtration and further subjected to cation-exchange and hydrophobic-interaction chromatography, followed by reverse-phase chromatography in a FPLC system (RP-FPLC). The purification of the EntP produced by M. extorquens S25 was achieved from a 1-l culture grown in CHOI at 30 °C with shaking for 48 h. Cells were removed by centrifugation at 12,000g for 10 min at 4 °C, and the supernatant filtered through 0.22 μm-pore-size-filters (Millipore). Then, 50 g (5% [w/v]) of Amberlite XAD-16 (Sigma) was added to the supernatant, and the sample kept at 4 °C with stirring for 2 h. The sample was loaded into an econo-column chromatography support (Bio-Rad) and, when the supernatant drained off, the matrix was washed with 100 ml distilled water in the first step and with 75 ml of 40% ethanol in water (v/v) later, to remove weak or non-hydrophobic compounds. The adsorbed bacteriocin was eluted with 200 ml of 85% 2-propanol pH 2.0 in water (v/v) and the remaining 2-propanol was eliminated by mild heating (35–40 °C) of the sample in a rotavapor (Büchi, Switzerland). After addition of 0.1% trifluoroacetic acid (TFA) to the sample, the antimicrobial activity was further subjected to reverse-phase chromatography in a C2 to C18 column (PepRPC HR 5/5) integrated in a FPLC system (RP-FPLC). The bacteriocin was eluted from the column with a 55 min linear gradient of 20–35% 2-propanol in aqueous 0.1% TFA at a flow rate of 0.5 ml min⁻¹. Purified EntP was stored in 60% 2-propanol with 0.1% TFA at −20 °C. Pure enterocin Q, pediocin PA-1 and EntP from E. faecium P13 and M. extorquens S25 were subjected to Tricine-SDS-PAGE, as described by Shägger and Von Jagow [30]. Protein electrophoresis was performed on Novex 16% Tricine gels in a XCell SureLock Mini-Cell at 80 V constant current. Gels were blotted onto a PVDF membrane, and the Western blotting completed as previously described [29].

3. Results

3.1. Heterologous production of EntP by M. extorquens

A 242-bp BamHI-KpnI cleaved fragment (S25), carrying the structural EntP gene (entP) and its ribosome
binding site (RBS) from \( E. \) \( \text{faecium} \) P13, was cloned into plasmid pCM80 resulting in the recombinant plasmid pS25 (Fig. 1). \( E. \) \( \text{coli} \) TOP-10 colonies transformed with pS25 produced biologically active EntP, as evidenced by halos of inhibition against \( E. \) \( \text{faecium} \) T136 by the stab-on-agar test (Fig. 2). However, when competent \( M. \) \( \text{extorquens} \) ATCC 55366 were transformed with pS25, colonies of the recombinant \( M. \) \( \text{extorquens} \) S25 host did not show antimicrobial activity against \( E. \) \( \text{faecium} \) T136. To evaluate production of EntP under control of the \( P \) \( \text{maxF} \) promoter, \( M. \) \( \text{extorquens} \) S25 cells were grown in the CHOI medium at 30°C for 72 h, and production of EntP in the culture supernatants was measured by specific anti-EntP antibodies and a NCI-ELISA. As shown in Fig. 3 production of EntP by \( M. \) \( \text{extorquens} \) S25 was apparent after 36 h of growth, reached its maximum after 48 h and remained stable after 72 h of growth. Moreover, growth kinetic parameters were not affected by production of EntP in \( M. \) \( \text{extorquens} \) S25 as compared to \( M. \) \( \text{extorquens} \) (pCM80) (results not shown). Strikingly, production of EntP by \( M. \) \( \text{extorquens} \) S25 could not be detected in supernatants of \( M. \) \( \text{extorquens} \) S25 by using an antimicrobial activity assay (ADT) with \( E. \) \( \text{faecium} \) T136 as the indicator microorganism.

3.2. Purification of enterocin P and Western blotting

The recombinant EntP produced by \( M. \) \( \text{extorquens} \) S25 was further purified by hydrophobic adsorption and reverse-phase (RP-FPLC) chromatographies. The purification process permitted recovery of an 0.8 ml fraction containing, as determined by NCI-ELISA, 160 ng of EntP in 40% 2-propanol, which represents a...
0.1% of the bacteriocin initially determined in the cell-free culture supernatant. Most importantly, as shown in Fig. 4, the heterologously EntP produced by M. extorquens S25 showed antimicrobial activity against E. faecium T136. Western blotting results of purified EntQ, pediocin PA-1 and EntP produced by E. faecium P13 and M. extorquens S25 shows (Fig. 5), that the anti-EntP antibodies only recognizes reactive antigenic bands in lanes corresponding to purified EntP from both microorganisms, and that such bacteriocin shows a great tendency to form aggregates.

4. Discussion

Bacteriocins produced by LAB have received increased scrutiny over the past few years due to the their potential use as food preservatives [31,32]. In order for the application of bacteriocins to be economically viable, certain concerns must be addressed, namely product yield, production costs and ease of scale up. Attempts to increase product yield through the development of expression systems, mostly for food-grade LAB have been investigated [33]. However, low production yields of recombinant bacteriocins by LAB have been the norm rather than the exception. The pink facultative methylotrophic bacteria, M. extorquens, has been developed for its potential as an expression system of recombinant proteins in high cell density fermentation. Recombinant green fluorescent protein (GFP), the Lactobacillus casei esterase, the Bifidobacterium infantis β-galactosidase, and the Bacillus thuringiensis Cry1Aa toxin have been produced by M. extorquens at yields ranging from milligrams to grams per litre [22,23].

The heterologous extracellular production of EntP by M. extorquens S25, a transformed derivative of M. extorquens ATCC 55366 with the recombinant plasmid pS25, was determined by using specific anti-EntP antibodies and a NCI-ELISA. The maximum amount of EntP in the supernatants of M. extorquens S25 was 155 ng ml⁻¹ (Fig. 3), which represents, approximately, a 2% of the EntP produced by E. faecium P13 in MRS (7.5–8.0 µg ml⁻¹). Despite this, the production of EntP by M. extorquens S25 constituted a 25-fold increase respect to the EntP produced by E. coli Tuner(DE3)placI (pJG01), under the control of the inducible T7 promoter [27]. Moreover, in contrast to the toxicity produced by synthesis of EntP in the recombinant E. coli cells [27], the growth profile of the M. extorquens S25 remained unchanged as compared to the wild type (untransformed) culture. Biomass yield (dry weight/methanol substrate consumed) and maximum specific growth rates of recombinant M. extorquens remained essentially unaffected by production of EntP (results not shown). It is tempting to speculate that M. extorquens S25 cells are less prone to toxicity by EntP although it could occur that a higher production of EntP by such cells would show a larger toxic effect. The EntP was also produced by M. extorquens S25 in absence of its putative immunity protein. Consequently, M. extorquens S25 may be employed as alternative host for production and secretion of EntP. Nevertheless, contrary to the situation with the EntP produced by E. coli [27], the EntP produced by M. extorquens S25 was not biologically active in the supernatant of the producing cells.

Purification of EntP from the supernatant of M. extorquens S25 permitted recovery of 160 ng of EntP, which represents a 0.1% of the bacteriocin initially present in the culture supernatants. This purification protocol permits a higher recovery of the EntP produced by M. extorquens S25 than from E. faecium P13 (0.01–0.02%) [27], although lower than the 13.3% recovery of the EntP produced by E. coli Tuner(DE3)placI (pJG01) [29]. Most importantly, the EntP purified from M. extorquens S25 was biologically active (Fig. 4). The absence of EntP active in the supernatants of M. extorquens S25 may be due to the formation of inactive aggregates or to the existence of compounds in the growth media interfering with bacteriocin activity. Salt inhibition has been already reported to reduce the inhibitory activity of antimicrobial peptides, including nisin [34]. Components of the CHOI medium may also activate extracellular proteinases with inhibitory effect against EntP. It may also occur that processing of the prebacte-riocin does not happen in the right site lowering the activity of produced EntP in the supernatant. Western blotting of pure pediocin PA-1, enterocin Q, and EntP with anti-EntP antibodies (Fig. 5), suggests that EntP is a hydrophobic bacteriocin with a strong tendency to form aggregates and, although the molecular structure
of such aggregates is not yet known, their formation would reduce the biological activity of EntP.

The heterologous expression system developed in this work has permitted, as far as we know, the first extracellular production of an small antimicrobial peptide EntP by the Gram-negative methylotrophic bacterium M. extorquens, presumably through the involvement of the heterologous host sec-dependent pathway. This work merits also recognition for evaluation of M. extorquens for heterologous production of a prepeptide with a signal peptide for processing and transport of the mature peptide out of the M. extorquens recombinant cells. Moreover, EntP purified from M. extorquens S25 was biologically active. However, the low production of EntP in Gram-negative bacteria, such as E. coli and M. extorquens, as compared to the Gram-positive bacterium E. faecium may respond to several factors: (i) N-terminal signal peptides and other components of the General Secretory Pathway (GSP) in Gram-negative bacteria may differ from that of the Gram-positive bacteria; (ii) differences in codon usage, stability and translational efficiency of the mRNA; (iii) in contrast to Gram-negative bacteria, secreted proteins of Gram-positive bacteria only need to traverse a single membrane to enter the extracellular environment; and (4), rapid degradation of secreted recombinant proteins by cell proteases. Nevertheless, the ability of M. extorquens S25 to grow and produce EntP in an economical and simple medium facilitates purification of this bacteriocin for its use as a natural preservative in the food industry. Moreover, the production of EntP by M. extorquens S25 might permit the heterologous production in this host of other bacteriocins, peptides and proteins of interest, by fusing their structural genes to the nucleotide fragment encoding the EntP signal peptide.

Acknowledgements

This work was partially supported by Grants 07G/0026/2000 from the Comunidad de Madrid, and Grants AGL2000-0707 and AGL2003-01508 from the Ministerio de Educación y Cultura, Spain. J. Gutiérrez is recipient of a fellowship from the Ministerio de Ciencia y Tecnologia (MCYT), and R. Criado holds a fellowship from the Ministerio de Educación, Cultura y Deporte (MECD), Spain. We thank L. Beaulieu for helpful discussions.

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