Heterologous expression of the bacteriocin mesentericin Y105 using the dedicated transport system and the general secretion pathway

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Two different N-terminal extensions have been identified within class II bacteriocin precursors. The first one is a two-glycine-type leader peptide associated with a dedicated ATP-binding cassette transporter. The second is a signal peptide which directs the bacteriocin precursor to the general secretion machinery. Mesentericin Y105 is a class II anti-*Listeria* bacteriocin produced by *Leuconostoc mesenteroides* Y105 via a dedicated transport system (DTS). To investigate heterologous expression systems capable of producing mesentericin Y105 in various hosts, two different secretion vectors were constructed. One of them, containing the mesentericin Y105 structural gene fused to the segment encoding the divergicin A signal peptide, was introduced into *Escherichia coli*, *Leuconostoc* subsp. and *Lactococcus* subsp. In *E. coli*, mesentericin Y105 production was linked to a putative periplasmic toxicity. To take advantage of this secretion system, the mesentericin Y105 precursor was also produced in *E. coli*. It was demonstrated that this pre-bacteriocin exhibited some antagonistic activity against *Listeria*. To allow for a comparison between the two different transport systems, mesentericin Y105 production using the vector containing the mesentericin Y105 structural gene and its DTS transporter operon was examined. The production of mesentericin Y105 was monitored by a new fast purification method followed by MS analysis. It was shown that, in *Leuconostoc*, the production of mesentericin Y105 is enhanced via the DTS compared to the general secretion pathway.

Keywords: bacteriocin, secretion, heterologous expression, lactic acid bacteria

INTRODUCTION

Bacteriocins are proteinaceous compounds produced by many lactic acid bacteria (LAB) that typically inhibit the growth of closely related species. They were classified into four classes by Klaenhammer (1993). Class II bacteriocins produced by LAB constitute the largest group so far characterized. They consist of small heat-stable peptides possessing an N-terminal extension of

18–24 residues that contains a conserved Gly-Gly processing site (positions –2 and –1). It has been shown that most of these class II bacteriocins are exported (Stoddard *et al.*, 1992; Marugg *et al.*, 1992; Bukhtiyarova *et al.*, 1994; Axelsson & Holck, 1995; van Belkum & Stiles, 1995; van Belkum *et al.*, 1997) by at least two membrane-bound proteins that form their dedicated transport system (DTS). One of the two proteins belongs to the family of the ATP-dependent transporters (Higgins, 1992). It was demonstrated that the cleavage of the N-terminal extension of the bacteriocin occurred concomitantly to the export of the peptide through the cytoplasmic membrane (Håvarstein *et al.*, 1995). The other protein bears a still unknown function but is postulated to be an accessory protein. It

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Abbreviations: DTS, dedicated transport system; GSP, general secretion pathway; LAB, lactic acid bacteria.

is anchored to the membrane by its hydrophobic N-terminus (Franke *et al.*, 1996). Worobo *et al.* (1995) and Leer *et al.* (1995) described two new bacteriocins from LAB, divergicin A and acidocin B, respectively, which are exported by the general secretion pathway (GSP). Indeed, divergicin A and acidocin B are encoded as precursors bearing a signal peptide.

Because of the potential use of bacteriocins as food preservatives and because most industrial strains do not produce such antagonistic peptides, interest in the heterologous expression of class II bacteriocins is rapidly growing. However, most of these bacteriocins display a narrow spectrum of activity. Therefore, it may be possible to design strains that are capable of producing multiple bacteriocins targeting various species. Such an approach will require a large amount of genetic material. Indeed, the production of each bacteriocin requires the cloning of at least four genes, including the bacteriocin structural gene and its immunity gene in addition to the two genes encoding the DTS. Thus, the use of the GSP for the production of bacteriocins or the use of a single DTS for the transport of multiple bacteriocins are promising approaches. Worobo *et al.* (1995) and McCormick *et al.* (1996) showed that a protein or a peptide fused to the divergicin A signal peptide is efficiently exported and correctly matured by both Gram-positive and Gram-negative bacteria. In addition, the ability of the heterologous DTS to export class II bacteriocins has been demonstrated (Marugg *et al.*, 1992; Fremaux *et al.*, 1995; Allison *et al.*, 1995a, b; van Belkum & Stiles, 1995; van Belkum *et al.*, 1997). It appears that the level of bacteriocin production was greatly affected and was dependent on the level of homologies existing between the leader peptides of the bacteriocins.

Mesentericin Y105 is a class II anti-*Listeria* bacteriocin produced by *Leuconostoc mesenteroides* (Hécharde *et al.*, 1992) that may have application in food preservation. Its genetic determinants have been characterized and its export using the DTS has been suggested (Fremaux *et al.*, 1995). Lactacin F is another class II bacteriocin produced by *Lactobacillus johnsonii* (Muriana & Klaenhammer, 1987). The heterologous expression of mesentericin Y105 was investigated in *Lb. johnsonii* using the DTS of lactacin F; however, very poor expression of mesentericin Y105 was obtained (Fremaux *et al.*, 1995). Thus, in this study, the efficiency of the GSP for expression of mesentericin Y105 was evaluated using the signal peptide of divergicin A. In addition, by cloning the mesentericin Y105 gene clusters including their putative DTS coding genes, the expression of mesentericin Y105 was tested in various Gram-positive hosts.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 α was propagated on Luria-Bertani agar (1.5%, w/v) or in broth at 37 °C with shaking as described by Sambrook *et al.*

(1989). When appropriate, ampicillin, erythromycin, X-Gal and IPTG were used at concentrations of 100, 150, 50 and 50 $\mu\text{g ml}^{-1}$, respectively. Erythromycin-resistant transformants of *E. coli* were selected on brain heart infusion (BHI; Difco) agar plates containing erythromycin. *Leuconostoc* strains were grown in/on MRS (Difco) broth or agar (1.5%, w/v) at 30 °C. *Lactococcus* strains were propagated in M17 (Difco) containing glucose (0.5%, v/v) broth or agar (1.5%, w/v). LAB transformants were selected with 5 μg erythromycin ml^{-1} . *Listeria ivanovii* BUG 497 was grown at 37 °C in BHI media.

Bacteriocin sensitivity assays. Bacteriocin activity was assayed by spotting 5 μl cell-free heat-treated supernatant onto MRS agar (1.2%, w/v) plates and overlaying with 5 ml BHI agar (0.6%, w/v) containing a 1% (v/v) inoculum of an overnight culture of *List. ivanovii* BUG 497. Plates were incubated for 18 h before analysis.

SDS-PAGE analysis. Protein extracts obtained after ammonium sulfate precipitation were concentrated 70 times and diluted 1:1 (v/v) in sample buffer (2 $\times$) containing 5% SDS, 12% glycerol, 2% β -mercaptoethanol, 10% Coomassie brilliant blue R250 and 50 mM Tris/HCl (pH 6.8). The samples were heated for 10 min at 100 °C and loaded on a 16% (v/v) polyacrylamide gel in 0.1 M Tris/Tricine (pH 8.8) buffer (Schägger & von Jagow, 1987), which allowed suitable resolution of low-molecular-mass peptides. Electrophoresis was carried out at constant voltage (100 V) for 2 h. Gels were fixed in 50% (v/v) methanol and 10% (v/v) aqueous acetic acid for 20 min. Duplicate gels were performed. One of them was stained with Coomassie brilliant blue R250 to evaluate the molecular mass of the molecules contained in the sample. The second gel was washed with sterile water for 2 h, placed on a BHI agar plate and overlaid with 5 ml BHI agar (0.6%, w/v) inoculated with 1% (v/v) of a stationary phase culture of *List. ivanovii* BUG 497. The inhibition zones were examined after 18 h incubation at 37 °C.

Molecular cloning and transformation. Plasmids from *E. coli* and LAB were extracted and purified as previously described by Sambrook *et al.* (1989) and Muriana & Klaenhammer (1987), respectively. Plasmid DNA was digested with restriction enzymes (Gibco-BRL) according to the supplier's recommendations. Both analytical and preparative agarose gel electrophoresis in Tris/borate/EDTA (pH 8.3) were performed according to the methods described by Sambrook *et al.* (1989). DNA restriction fragments were purified from agarose gels using the Prep-a-Gene kit (Bio-Rad). Recombinant DNA was obtained using T4 DNA ligase (Gibco-BRL) following the manufacturer's recommendations. *E. coli* competent cells were prepared and transformed according to Hanahan (1983).

Leuconostoc cells were transformed by electroporation in 0.2 cm cuvettes using a Gene Pulser apparatus (Bio-Rad; settings 25 μF , 2.5 kV, 200 Ω). Electrocompetent cells were prepared according to the method of Raya *et al.* (1992). Electrocompetent *Lactococcus lactis* cells were prepared and transformed according to the method of Holo & Nes (1989).

DNA sequences were determined with an automated DNA sequencer (ALF; Pharmacia) using the Auto-read sequencing kit (Pharmacia) and appropriate primers. Sequence analyses were performed with the GCG sequence analysis software.

Oligonucleotides and PCRs. Primers FBp1 (5'-AAGAAGCT-TCTGCTAAGTATTATGGGAATGGTG) and FBp2 (5'-CTCAAGCTTACTGATCGAAAACGCTG) were used for the PCR amplification of the fragment devoted to the secretion

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
<i>Escherichia coli</i>		
DH5 α	<i>recA endA1 gyrA96 thi-1 hsdR17 supE44 Δlac U169(ϕ80 <i>dlacZ</i>ΔM15) <i>deoR</i> F⁻ λ⁻</i>	Gibco-BRL
<i>Leuconostoc mesenteroides</i>		
subsp. <i>mesenteroides</i>		
Y105	Wild-type strain	Hécharde <i>et al.</i> (1992)
FR52	Wild-type strain	Mathieu <i>et al.</i> (1993)
<i>Leuconostoc mesenteroides</i>		
subsp. <i>dextranicum</i>		
DSM 20484	Wild-type strain	DSM
<i>Leuconostoc cremoris</i>		
LC	Wild-type industrial strain	Texel
<i>Lactococcus lactis</i>		
IL1403	Laboratory strain	Chopin <i>et al.</i> (1984)
RD230	Wild-type industrial strain	Texel
<i>Listeria ivanovii</i>		
BUG 497	Laboratory strain	Institut Pasteur
Plasmids		
pBSSKII+	pBluescript II SK(+) cloning vector, Ap ^r , <i>lacZ</i> , 2.96 kb	Stratagene
pGKV210	<i>E. coli</i> -LAB shuttle vector, Em ^r , 4.4 kb	van der Vossen <i>et al.</i> (1985)
pGKV259	<i>E. coli</i> -LAB shuttle expression vector, Em ^r Cm ^r , 5 kb	van der Vossen <i>et al.</i> (1987)
pTRKH2	Lactococcal cloning vector <i>lacZ</i> , Ap ^r , 6.9 kb	O'Sullivan & Klaenhammer (1993)
pHY30	<i>mesYI mesCDE</i> , 35 kb plasmid from <i>Ln. mesenteroides</i>	Fremaux <i>et al.</i> (1995)
pCD3.4	<i>dvnA dvnI</i> , 3.4 kb plasmid from <i>Carnobacterium divergens</i>	Worobo <i>et al.</i> (1995)
pRW5.6	pGKV259::514 bp <i>EcoRV</i> - <i>AccI</i> fragment from pCD3.4	Worobo <i>et al.</i> (1995)
pFBYC01	pBSSKII+::121 bp <i>EcoRV</i> - <i>HindIII</i> fragment from pCD3.4	This study
pFBYC02	pFBYC01::485 bp <i>dvnA</i> :: <i>mesY</i> , <i>mesI</i> PCR product	This study
pFBYC03	pFBYC01::557 bp <i>dvnA</i> ::pre- <i>mesY</i> , <i>mesI</i> PCR product	This study
pFBYC06	pRW5.6 Δ <i>HindIII</i>	This study
pFBYC07	pFBYC06::485 bp <i>dvnA</i> :: <i>mesY</i> , <i>mesI</i> PCR product	This study
pFBYC04	pTRKH2::7 kb <i>PstI</i> - <i>DraII</i> fragment from pHY30	This study
pCFYC5	pGKV210::1.7 kb <i>PstI</i> - <i>NsiI</i> fragment from pHY30	Fremaux <i>et al.</i> (1995)

of mesentericin Y105 (Fig. 1a). They were designed in order to generate *HindIII* restriction sites at both ends of the PCR product. The five nucleotides downstream of the *HindIII* site of FBp1 encode the C-terminus of the divergicin A signal peptide, and the remaining 19 residues are identical to the 5' end of the sequence encoding the mature mesentericin Y105. In addition to the *HindIII* site, FBp2 is complementary to the sequence encoding the C-terminus of the mesentericin Y105 immunity protein. For the PCR amplification of the DNA fragment devoted to the secretion of the pre-mesentericin Y105 (Fig. 1b), primers FBp3 (5'-AAGAAGCTTCTGCTATGACGAATATGAAGTCTGTG) and FBp2 were used. The primer FBp3 was designed similarly to FBp1; its 3' end is identical to the 5' end of the mesentericin Y105 structural gene. PCR reactions on pHY30 (Fremaux *et al.*, 1995) were performed with the *Taq* DNA polymerase from Perkin Elmer-Cetus according to the supplier's recommendations using the Gene Aaq thermocycler (Pharmacia) under the following conditions: 35 cycles of denaturation at 94 °C for 10 s, annealing at 48 °C for 10 s and elongation at 72 °C for 20 s.

Production and purification of mesentericin Y105. *Leuconostoc* strains were propagated (1%, v/v, inoculum) in 100 ml MRS broth for 24 h at 30 °C (final OD₆₀₀ $\approx$ 4) and cells were harvested by centrifugation at 6000 g for 15 min at 4 °C. After heating of the cell-free supernatant at 70 °C for 30 min, the bacteriocin was precipitated with ammonium sulfate (360 g l⁻¹) with constant stirring at 4 °C for 18 h and harvested by centrifugation (13000 g, 45 min, 4 °C). The pellet was resuspended in 10 ml water. The solution was loaded on a reverse-phase cartridge (C18 Sep-pak plus; Waters) and was eluted in steps with 5 ml 0, 10, 20, 30, 40, 60 and finally 80% (v/v) acetonitrile solutions in 20 mM ammonium acetate. Fractions were assayed for bacteriocin activity by the spot-on-lawn test. Reverse-phase HPLC was conducted on a Spectra Physics Analytical apparatus. Lyophilized active fractions were solubilized in 1 ml water before injection on a C8 column Kromasil (250 by 4.6 mm, 5 μ m particle size, 10 nm pore size, flow rate 0.8 ml min⁻¹). Separation was carried out using a water/acetonitrile/trifluoroacetic acid, 0.1% (v/v), solvent system with an elution gradient of 16–64% acetonitrile over

Fig. 1. Schematic representation of the PCR fragments synthesized from pHY30 for the construction of the plasmids devoted to the secretion of (a) mesentericin Y105 and (b) the mesentericin Y105 precursor. Boxes represent genes that have been amplified by PCR with the primer sequences given within. The black and the dashed parts of the boxes correspond to sequences encoding the C-terminus of the divergicin A signal peptide and the mesentericin Y105 leader peptide, respectively, whereas open boxes represent genes encoding the mesentericin Y105 immunity protein (*mesI*) and the mature form of mesentericin Y105 (*mesY*). Relevant gene translation is given below the boxes; ▲, divergicin A signal peptide cleavage site; △, mesentericin Y105 leader peptide cleavage site. The *Hind*III restriction sites introduced within the primer sequences are indicated.

37 min. Elutions were monitored by measuring the A_{220} and A_{280} . All fractions corresponding to the detected peaks were assayed for activity against the indicator strains.

Protein analysis. The mass spectra of the purified mesentericin Y105 samples were obtained from positive ionspray analysis on a Perkin-Elmer Sciex API 165 mass spectrometer equipped with an ionspray source. Lyophilized active samples purified by HPLC were dissolved in a 0.25 ml solution of acetonitrile/water (1:1) containing 0.1% formic acid and infusion injected (10 μ l) at a flow rate of 5 μ l min⁻¹. Protein concentration was determined by using the bicinchoninic acid protein assay kit (Sigma) according to the manufacturer's instructions.

RESULTS

Heterologous production of mesentericin Y105 and its precursor using the GSP in *E. coli*

Preliminary experiments performed in *E. coli* strains attempted to investigate the secretion of mesentericin Y105 by the GSP. A secretion vector, pFBYC01, was constructed on the basis of the utilization of the divergicin A signal peptide placed under the control of the inducible *lac* promoter, as described in Fig. 2. To generate pFBYC01, the *Eco*RV-*Hind*III fragment from pCD3.4 (Worobo *et al.*, 1995) containing a ribosome-binding site and part of the divergicin A signal peptide gene was cloned into pBSSKII+ restricted with *Sma*I and *Hind*III enzymes.

To obtain a DNA fragment encoding a mature form of mesentericin Y105 and its immunity protein, a region of pHY30 (Fremaux *et al.*, 1995) was amplified by PCR using the primers Fbp1 and Fbp2 (Fig. 1a). The Ala-Ser-Ala cleavage site of the divergicin signal peptide, which is truncated in the *Eco*RV-*Hind*III fragment, was regenerated by the residues incorporated into the sequence of the PCR primer Fbp1. The PCR fragment was digested with *Hind*III and cloned into pFBYC01 at the unique *Hind*III site to form the mesentericin Y105

secretion plasmid pFBYC02 (Fig. 2). Sequence analysis confirmed the accuracy of the gene fusion and ensured that the correct reading frame was maintained between the sequence encoding the divergicin A signal peptide and the mesentericin Y105 structural gene, devoid of its natural leader peptide. In this construction, the transcription of the fused gene is under the control of the inducible *lac* promoter on pBSSKII+. Sequence analysis also revealed the presence of a small ORF, beginning from the *lacZ* start codon, which overlaps the fused gene and its translation initiation signals (the ribosome-binding site and the start codon of *dnaA*). To prevent any inefficient recognition of these signals, the *Sac*I restriction site located within this small ORF was opened and then blunt-ended before ligation, generating a shift in the reading frame and stop codons. Secretion of mesentericin Y105 was tested by introducing pFBYC02 into *E. coli* DH5 α cells. Upon induction with 1 mM IPTG at an OD₆₀₀ of 1 (1 cm cuvette, Hitachi U-1100 spectrophotometer), the expression of an antagonistic activity within the culture supernatant was detected by the spot-on-lawn test against a sensitive indicator strain. The activity was then analysed by an SDS-PAGE overlay assay (Fig. 3). The apparent molecular mass of the active peptide secreted by *E. coli* (Fig. 3, lane 1) was in accordance with that of the natural bacteriocin used as a control (Fig. 3, lane 4). Full induction of the antagonist in *E. coli* DH5 α caused a decline of the culture turbidity, suggesting the lysis of induced cells (data not shown).

The biochemical and structural characterization of the mesentericin Y105 precursor will first require its purification. However, it is likely to be transiently present in the natural mesentericin Y105 producer strain and therefore difficult to purify. Consequently, we constructed the plasmid pFBYC03 to secrete the mesentericin Y105 precursor (Fig. 2). For pFBYC03 construction, the cloning strategy was identical to that described for pFBYC02, except that the PCR fragment

Fig. 2. Schematic representation of the relevant features of the plasmids (names are given on the right of the schemes) constructed for the secretion of mesentericin Y105 and of the mesentericin Y105 precursor. The boxes represent the natural or the constructed bacteriocin genes (*dvnA* for divergicin A and *mesY* for mesentericin Y105) and their corresponding immunity genes (*dvnI* and *mesI*). The black and the dashed parts of the boxes indicate the sequences encoding the divergicin A signal peptide and the mesentericin Y105 leader peptide, respectively. The promoters driving the transcription of the genes are shown on the left of the figure: *pdvn*, the natural promoter of the divergicin A operon; *p59*, a lactococcal constitutive promoter; and *plac*, the inducible promoter of the *E. coli* lactose operon. Relevant restriction sites are indicated.

Fig. 3. Detection of antagonistic activity against *List. ivanovii* BUG 497 in spent culture supernatant following Tricine-SDS-PAGE. Lanes: 1, concentrated supernatant (70×) of *E. coli* DH5α(pFBYC02); 2, concentrated supernatant (70×) of *E. coli* DH5α(pBSSKII+); 3, concentrated supernatant (70×) of *E. coli* DH5α(pFBYC03); 4, supernatant of *Ln. mesenteroides* Y105. M, Inhibition zone associated with the bactericidal activity of mesentericin Y105; P, inhibition zone associated with the putative antagonism of the mesentericin Y105 precursor.

(using primers FBp2 and FBp3) cloned into the *Hind*III site of pFBYC01 contains the whole structural gene of mesentericin Y105, including the part encoding the N-terminal extension of mesentericin Y105 (Fig. 1b). After transformation of pFBYC03 into *E. coli* DH5α and induction of the cloned gene, the presence of an active peptide against *Listeria* was determined by an SDS-PAGE overlay assay (Fig. 3). This peptide has an apparent molecular mass higher than that of the natural mesentericin Y105, and thus is likely to be the pre-mesentericin Y105. From the SDS-PAGE overlay assay, it appeared to be less active compared to mesentericin Y105, as confirmed by the absence of cell lysis upon induction by IPTG (not shown).

Heterologous production of mesentericin Y105 using the GSP in *Leuconostoc*

A secretion vector that replicates in LAB was developed using plasmid pRW5.6 constructed by Worobo *et al.* (1995). In this plasmid, the divergicin A operon is under the control of the constitutive lactococcal promoter *p59* (Fig. 2). Plasmid pRW5.6 was digested with *Hind*III, deleting part of the divergicin A structural gene and its immunity gene, then self-ligated to construct pFBYC06 (Fig. 2). Similarly to pFBYC02, a PCR-generated DNA (using primers FBp1 and FBp2) encoding a mature form of mesentericin Y105 and its immunity protein with

*Hind*III linking ends, was inserted into the *Hind*III site of pFBYC06, yielding the pFBYC07 plasmid (Fig. 2). Although the pFBYC07 replicon was functional in *E. coli* strains, no transformant was obtained with pFBYC07 in this organism probably because of the toxicity of mesentericin Y105. Alternatively, the ligation mixture of pFBYC07 was electroporated into *Ln. mesenteroides* DSM 20484. This strain, deprived of anti-*Listeria* activity, gave a high number of transformants by electroporation, and thus allowed direct transformation with ligation mixtures. Erythromycin-resistant clones of *Ln. mesenteroides* DSM 20484 obtained after electroporation were screened for antagonistic activity against *Listeria* by spot-on-lawn tests. They exhibited activity against the indicator strain (data not shown) and were found to contain pFBYC07. Similar results were obtained with pFBYC07 introduced into the industrial strain *Leuconostoc cremoris* LC.

Heterologous production of mesentericin Y105 using the DTS in *Leuconostoc*

Plasmid pCFYC5 contains the mesentericin Y105 structural gene (*mesY*) and its immunity gene (*mesI*) (Fremaux *et al.*, 1995). When introduced into *Ln. mesenteroides* DSM 20484, the resulting strain failed to produce any bactericidal activity against *Listeria* or LAB, suggesting that some essential functions are missing within the cloned fragment for the production of the bacteriocin. Previous analyses of the mesentericin Y105 genetic locus (Fremaux *et al.*, 1995) revealed the presence of an operon containing three putative genes (*mesC*, *mesD* and *mesE*) that may be involved in the specific transport of mesentericin Y105. Indeed, *mesD* and *mesE* encode the two components of a putative ATP-binding cassette transport system.

A 7 kb *Pst*I–*Dra*II restriction fragment containing *mesYICDE* was introduced into pTRKH2, a high-copy-number *E. coli*–LAB shuttle vector (O'Sullivan & Klaenhammer, 1993). The resulting plasmid, pFBYC04 (Table 1), was able to direct the production in *Ln. mesenteroides* DSM 20484 of a bactericidal compound active against *Listeria*. Similarly, the industrial *Ln. cremoris* LC strain produced an antagonistic activity against *Listeria* when electroporated with pFBYC04. This demonstrates that *mesC*, *mesD* and/or *mesE* are required for the expression of mesentericin Y105. It has been shown in a highly homologous bacteriocin, leuconcin A (van Belkum & Stiles, 1995), by gene inactivation that similar dedicated transport genes are required for export.

Analysis of mesentericin Y105 expressed in *Ln. mesenteroides*

The antagonistic compound, produced by *Ln. mesenteroides* DSM 20484 transformed with pFBYC04 and pFBYC07, was purified from cell-free culture supernatants by a three-step protocol involving ammonium sulfate precipitation, C18 solid-phase extraction and

Fig. 4. Reverse-phase HPLC elution profiles (C8 Kromasil AIT) of active fractions obtained after cartridge chromatography of concentrated spent culture supernatants from: *Ln. mesenteroides* Y105 (lane 1); *Ln. mesenteroides* DSM 20484(pFBYC04) (lane 2); *Ln. mesenteroides* DSM 20484(pFBYC07) (lane 3); control, homologous fraction from *Ln. mesenteroides* DSM 20484 (lane 4).

reverse-phase HPLC. Almost all of the antagonistic activity was found in the ammonium sulfate pellet. After resuspending this pellet in water, proteins were fractionated on C18 cartridges using an increasing acetonitrile gradient. The active fraction eluted at 40% acetonitrile was further purified by reverse-phase HPLC. The elution profiles showed a single protein peak at a retention time of 22 min (peak A; Fig. 4). The biological activity of the HPLC fractions corresponding to the peaks was assayed against the indicator strain, revealing an antagonistic activity. The purified protein was subjected to MS analysis using an ionspray ionization source instrument. As shown in Fig. 5(a), the final estimated mass was 3868.27 ± 0.25 Da for the purified peptide from *Ln. mesenteroides* DSM 20484 containing either pFBYC04 or pFBYC07. This value corresponds to the molecular mass of mesentericin Y105 purified from the supernatant of the natural producer strain *Ln. mesenteroides* Y105 (lane 1, peak A; Fig. 4), and is in agreement with the theoretical molecular mass for an oxidized form of mesentericin Y105 calculated from its amino acid sequence (Fremaux *et al.*, 1995). The amount of mesentericin Y105 purified from a culture of each of the transformants and of the natural producer was estimated. A similar amount of mesentericin Y105 was obtained from a 100 ml culture of *Ln. mesenteroides*

Fig. 5. Ion spray mass spectra of the purified fractions from *Ln. mesenteroides* Y105 of (a) peak A and (b) peak B obtained after reverse-phase HPLC (see Fig. 4, lane 1).

DSM 20484(pFBYC04) and *Ln. mesenteroides* Y105 (142 and 129 µg, respectively), whereas 34 µg mesentericin Y105 was obtained from *Ln. mesenteroides* DSM 20484(pFBYC07), under identical culture conditions and using the same purification procedure. In addition to peak A corresponding to mesentericin Y105, a second protein peak (lane 1, peak B; Fig. 4) was revealed on a HPLC chromatogram from a culture of the natural mesentericin Y105 producer. Peak B, with a 27.7 min retention time, showed an antagonistic activity against *Leuconostoc* strains (data not shown) but not *Listeria*, suggesting the production of a second bacteriocin. Indeed, it is not likely to correspond to a degradation product from mesentericin Y105, since it was not detected in *Ln. mesenteroides* DSM 20484(pFBYC04) culture supernatant. Mass spectrum analysis of peak B gave a molecular mass of 3445.95 ± 0.35 Da (Fig. 5b). With respect to the molecular mass, this compound resembles mesenterocin 52B, previously described by Revol-Junelles *et al.* (1996), a class II bacteriocin known for its activity against *Leuconostoc* strains but not *Listeria*. Our results demonstrate the production of a second bacteriocin by *Ln. mesenteroides* Y105 that was not previously detected because mesentericin Y105 masked its activity in spot-on-lawn tests.

Heterologous expression of mesentericin Y105 in *Lc. lactis*

To further study the heterologous expression of mesentericin Y105, plasmids pFBYC04 and pFBYC07 were also introduced into *Lc. lactis* strains IL1403 and RD230. The ability of the transformants to produce mesentericin Y105 was examined. None of the supernatants of *Lc. lactis*/pFBYC04 or *Lc. lactis*/pFBYC07 transformants revealed any detectable antagonistic activity. These data cannot be correlated to a transcriptional deficiency, since Northern blot hybridization revealed the presence of the expected transcript (data not shown). To test if, in *Lactococcus*, mesentericin Y105 is expressed in an inactive form or alternatively is not present at all in the extracellular medium, we looked for its presence in the supernatant as described before. The HPLC chromatogram did not show any peak at a retention time of 22 min. This seems to confirm the inability to produce mesentericin Y105 from pFBYC07 or pFBYC04 in *Lactococcus*.

DISCUSSION

Large quantities of a bacteriocin are required for structural analysis. In attempts to obtain a sufficient amount of mesentericin Y105 for elucidating its spatial conformation, we investigated its heterologous expression in various bacterial species using two export systems: (i) the GSP and (ii) the putative mesentericin Y105 DTS.

First, we took advantage of the divergicin A signal peptide (Worobo *et al.*, 1995) for the construction of two plasmids (pFBYC03 and pFBYC02) capable of secreting the mesentericin Y105 precursor and the mature mesentericin Y105, respectively. In these plasmids, the structural gene of mesentericin Y105 or the part of the gene encoding the mature peptide is fused to the signal peptide of the divergicin A encoding region. This synthetic gene was placed under the control of the β -galactosidase inducible promoter. Cloned in *E. coli* DH5 α , the two plasmids allowed the production of both peptides. Interestingly, the bacteriocin precursor (that possessed a 24 N-terminal amino acid extension) exhibited a weak antagonistic activity against *Listeria*. This is in agreement with the observation by Quadri *et al.* (1997) for the carnobacteriocin B2 precursor. Previous work demonstrated the ability of *E. coli* to produce heterologous bacteriocins originated from LAB. Pediocin PA-1 was produced via its own DTS (Marugg *et al.*, 1992) and divergicin A was exported using the leucocin A or colicin V export machinery (van Belkum *et al.*, 1997). On the contrary, divergicin A failed to be produced by *E. coli* using the lactococcin A DTS (van Belkum *et al.*, 1997). In our case, very poor antagonistic activity (about 100 times less than the natural producer) was detected in the supernatant of *E. coli* DH5 α (pFBYC02) cultures. Nevertheless, the divergicin A signal peptide was shown to efficiently direct the production of alkaline phosphatase to the culture supernatant in *E. coli* (Worobo *et al.*, 1995). However,

these authors reported that one-third of the secreted alkaline phosphatase was located within the periplasm. In addition, although colicin V is efficiently secreted across the cytoplasmic membrane via the GSP, secretion through the outer membrane is not achieved and the molecule is trapped within the periplasm (Zhang *et al.*, 1995). Despite previous analyses demonstrating that mesentericin Y105 was inactive on whole cells of *E. coli*, upon IPTG induction, cell lysis was observed in *E. coli* DH5 α (pFBYC02) cultures. This result, together with the low level of production, suggested that at least a part of mesentericin Y105 is trapped within the periplasm and that the toxicity of mesentericin Y105 may lead to cell lysis. Interestingly, a lytic activity of mesentericin Y105 on mitochondria (Maftah *et al.*, 1993) and erythrocytes (A. Maftah, personal communication) was previously described. These three lots of data suggest that mesentericin Y105 may act without the intermediate of a specific receptor when the cytoplasmic membrane is directly accessible. Thus, the cell wall is likely to give its specificity to the bacteriocin. The immunity protein may ensure the cytoplasmic membrane integrity and may not be expressed enough in the *E. coli* DH5 α (pFBYC02) strain. Indeed, we were unable to construct an *E. coli* strain expressing mesentericin Y105 in which the immunity gene was disrupted, suggesting that pFBYC02 encodes a functional immunity protein in *E. coli*.

The toxicity of mesentericin Y105 produced by a Gram-negative host led us to investigate the secretion of the bacteriocin in *Lactococcus* and *Leuconostoc*. Heterologous expression in *Lc. lactis* of bacteriocins fused to the divergicin A signal peptide has been investigated previously. Divergicin A and carnobacteriocin B2 were found to be secreted by *Lc. lactis* (Worobo *et al.*, 1995; McCormick *et al.*, 1996), proving the efficiency of the GSP for the expression of bacteriocins in Gram-positive species. A plasmid (pFBYC07), based on the cloning vector pGKV259, was constructed in which the gene encoding the secreted mesentericin Y105 is under the control of the constitutive lactococcal promoter p59. Three lactococcal strains (laboratory strains IL1403 and MG1363 and one industrial strain RD230) transformed with pFBYC07 failed to produce any detectable amount of bacteriocin. Plasmid pFBYC07 was then isolated from one of the lactococcal transformants and used to transform *Leuconostoc* strains. In contrast to *Lc. lactis* strains, all the *Leuconostoc* transformants were found to produce small amounts of mesentericin Y105 in the culture supernatant. Low level secretion of mesentericin Y105 is consistent with the weak activity detected for the secreted divergicin A and carnobacteriocin B2 in heterologous hosts (Worobo *et al.*, 1995; McCormick *et al.*, 1996).

Leucocin A is an anti-*Listeria* peptide closely related to mesentericin Y105 (Hastings *et al.*, 1991). These two peptides only differ by two residues, alanine 22 (mesentericin Y105) versus phenylalanine (leucocin A) and isoleucine 26 versus valine. These differences are conservative changes, and thus one can expect leucocin A to behave similarly to mesentericin Y105. Leucocin A has

been produced previously in *Lc. lactis*, using its own DTS (van Belkum *et al.*, 1997). To understand the problems encountered for the secretion in *Lc. lactis*, similar constructs were made with the mesentericin Y105 genetic determinants. Firstly, plasmid pCFYC5, which contains the mesentericin Y105 structural gene and its corresponding immunity gene, was constructed and introduced in *Ln. mesenteroides* DSM 20484, a strain devoid of antagonistic activity. Transformants failed to produce any detectable bacteriocin activity. Secondly, another construct, pFBYC04, which contains the entire mesentericin Y105 gene cluster described by Fremaux *et al.* (1995), was introduced in this strain. The resulting clones displayed bactericidal activity against *Listeria*, demonstrating the presence within the cloned fragment of the genes encoding the DTS for mesentericin Y105, as proposed previously by Fremaux *et al.* (1995). However, similarly to pFBYC07, plasmid pFBYC04 cloned in *Lc. lactis* failed to produce mesentericin Y105. Northern blot analysis proved that the mesentericin Y105 gene cluster in pFBYC04 and the *dvnA::mesY* gene fusion in pFBYC07 were well transcribed in *Lactococcus* (data not shown). In addition, bacteriocin genes originating from LAB are usually efficiently translated in *Lactococcus* and this species was shown to be capable of exporting and processing a few bacteriocins from other LAB species (Worobo *et al.*, 1995; van Belkum & Stiles, 1995; van Belkum *et al.*, 1997; Horn *et al.*, 1998). Thus, we suspect that difficulties encountered in producing mesentericin Y105, but not leucocin A, in *Lactococcus* may be associated with either peptide degradation or trapping specified by the bacteriocin sequence itself. Indeed, lactococcal strains are known to produce many peptidases (Kunji *et al.*, 1996) and the binding properties of bacteriocins have been previously reported (Coventry *et al.*, 1996). It is, however, interesting to note that failure to produce a bacteriocin using its own DTS in a heterologous host has already been described (van Belkum *et al.*, 1997).

The level of mesentericin Y105 expression by recombinant and wild-type *Leuconostoc* strains was evaluated through a new three-step purification method. This mesentericin Y105 purification strategy appeared to be highly reproducible and more efficient than previously described methods (Hécharde *et al.*, 1992; Revol-Junelles *et al.*, 1996). Comparison of the yield of purified bacteriocin showed that mesentericin Y105 is produced about three times more when exported by its own DTS, i.e. $>1 \text{ mg l}^{-1}$ for cultures of *Ln. mesenteroides* Y105 and *Ln. mesenteroides* DSM 20484-(pFBYC04), than by the GSP, i.e. $\sim 300 \mu\text{g l}^{-1}$ for a culture of *Ln. mesenteroides* DSM 20484(pFBYC07). However, this may be not only related to the efficiency of the export system itself (DTS versus GSP), but also to gene copy numbers (pTRKH2 versus pGKV259) and/or promoter efficiencies (mesentericin Y105 promoter versus p59 promoter).

Very few industrial LAB are known to produce bacteriocins. To take advantage of these GRAS (Generally Recognized As Safe) antagonistic peptides in the control

of spoilage and pathogenic bacteria, various strategies have been proposed previously to produce bacteriocins in heterologous hosts, especially in industrial strains. The secretion pathway appeared to be a 'premium' choice because of its ubiquity among bacteria. The feasibility of bacteriocin secretion has been shown (Worobo *et al.*, 1995; McCormick *et al.*, 1996). However, we demonstrated that bacteriocin yield was poor and may not fit industrial requirements. Some authors also investigated the possibility of producing multiple bacteriocins using the DTS of other bacteriocins (Allison *et al.*, 1995a, b; Fremaux *et al.*, 1995; van Belkum & Stiles, 1995). They demonstrated the poor efficiency of such heterologous association, suggesting that DTSs are somehow specific for a bacteriocin. Thus, the leader peptide associated with its own DTS fused to a mature peptide seemed to be an attractive alternative. However, at least for mesentericin Y105, the primary sequence of the bacteriocin affects the efficiency of its expression. Therefore, it appears that the best compromise consists in the production of bacteriocins by strains of 'homologous genera' using their own DTS. For example, we were able to express the mesentericin Y105 in an industrial strain, namely *Ln. cremoris* LC, at a similar level to that of the natural producer. In addition, such approaches are more likely to be in accordance with legal issues concerning the release of genetically modified micro-organisms.

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