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Heterologous Processing and Export of the Bacteriocins Pediocin PA-1 and Lactococcin A in *Lactococcus Lactis*: A Study with Leader Exchange

M. Chikindas · E. Emond · A. J. Haandrikman · J. Kok · K. Leenhouts · S. Pandian · G. Venema · K. Venema

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Abstract The bacteriocins pediocin PA-1 and lactococcin A are synthesized as precursors carrying N-terminal extensions with a conserved cleavage site preceded by two glycine residues in positions -2 and -1. Each bacteriocin is translocated through the cytoplasmic membrane by an integral membrane protein of the ABC cassette superfamily which, in the case of pediocin PA-1, has been shown to possess peptidase activity responsible for proteolytic cleavage of the pre-bacteriocin. In each case, another integral membrane protein is essential for bacteriocin production. In this study, a two-step PCR approach was used to permutate the leaders of pediocin PA-1 and lactococcin A. Wild-type and chimeric pre-bacteriocins were assayed for maturation by the processing/export machinery of pediocin PA-1 and lactococcin A. The results show that pediocin PA-1 can be efficiently exported by the lactococcin machinery whether it carries the lactococcin or the pediocin leader. It can also compete with wild-type lactococcin A for the lactococcin machinery. Pediocin PA-1 carrying the lactococcin A leader or lactococcin A carrying that of pediocin PA-1 was poorly secreted when complemented with the pediocin PA-1 machinery, showing that the pediocin machinery is more specific for its bacteriocin substrate. Wild-type pre-pediocin and chimeric pre-pediocin were shown to be processed by the lactococcin machinery at or near the double-glycine cleavage site. These results show the potential of the lactococcin LcnC/LcnD machinery as a maturation system for peptides carrying double-glycine-type amino-terminal leaders.

Keywords Bacteriocin · Lactococcin · Pediocin · Double-glycine leader

The results included herein were first presented at the NSERC-Canada Advanced Research Workshop on "Bacteriocins of Lactic Acid Bacteria-Applications and Fundamentals" held in Banff, Alberta, Canada, April17-23 (1995). These data were part of Eric Emond's Thesis "Caractérisation moléculaire de la pédiocine PA-1 et des mécanismes de la maturation et de l'exportation des bactériocines de la classe II. (1996). Université Laval, Québec, Canada.

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Preface

The history of mankind is inseparable from the history of science. However, at the end of the day, hardly anyone cares who the inventor of the steam engine, airplane, or automobile was. Yet, it is our firm belief that those who contributed to the progress of science must be known. With this in mind, we present a paper, which, to the best of our knowledge, is one of the first reports on bacteriocin expression through the processing machinery of another antimicrobial peptide. For unknown reasons, the handling manager at the time decided not to submit the manuscript for publication. The manuscript is presented here with original information and in the style it has been written…

Introduction

Bacteriocins produced by lactic acid bacteria (LAB) can be subdivided into three groups. Lantibiotics (Class I) are small peptides which are post-translationally modified and contain unusual amino acids such as dehydroalanine and dehydrobutyrine, which are dehydrated residues of serine and threonine, respectively. Other post-translational modifications produce lanthionine and β-methyllanthionine, which are thioether cross-linked amino acids (Ala-S-Ala, Aba-S-Ala). Class II bacteriocins are small heat-stable peptides with no modified amino acids. The large (>30 kDa) heat-labile bacteriocins are members of class III [18]. All bacteriocins studied so far are produced as precursor proteins containing an N-terminal extension, the so-called leader. For most bacteriocins, this leader is not of the classical type required for *sec*-dependent secretion. The leaders of most class II bacteriocins and of some of the lantibiotics share a consensus sequence [15]. The processing site is located immediately downstream of two conserved glycine residues located at position -1 and -2 of the pre-bacteriocin. The similarities between the so-called double-glycine-type leader peptides [14] suggest a common mechanism for processing and/or export of these bacteriocins.

In a number of cases, a transmembrane translocator protein of the ATP-binding cassette (ABC) superfamily [16, 17] has been implicated in the export of bacteriocins (PedD: Marugg et al., [19]; LcnC: Stoddard et al., [28]; LcnDR3: Rince et al., [23], LagD: Nes et al., [20]; SapT: Axelsson and Holck [3]; LcaD: van Belkum and Stiles [5]). Venema et al. [31] and Håvarstein et al. [14] have shown that the leader peptides of pediocin PA-1 and lactococcin G are processed by their respective ABC transporters and that cleavage could be uncoupled from transport of the bacteriocin. The proteolytic activity was associated with the cytoplasmic domain consisting of the first 150 and 190 amino acids of LagD and PedD, respectively. The proteolytic domains of these ABC translocators and that of a number of others contain common amino acid motifs thought to be part of the active site of the leader peptidase [14, 30]. A second protein has been shown necessary for the production of class II bacteriocins (LcnD: Stoddard et al., [28]; PedC: Venema et al., [31]; SapE: Axelsson and Holck [3]; LagE: Håvarstein et al., [14]). The accessory proteins are believed to be monotopic membrane proteins carrying a membrane-spanning sequence close to their N-terminal ends. The topology of one of these proteins, LcnD, has been elucidated [10]. In that study, it was shown that a short N-terminal part was located in the cytoplasm, and that the large domain downstream of the transmembrane sequence is located extracellularly. A similar topology was predicted for analogs of LcnD, suggesting that they play common roles in the maturation and/or secretion of their respective bacteriocins.

The homology between the leader peptides of class II bacteriocins and the structural similarities among the two-component processing/export proteins prompted us to assess the compatibility of these systems. The bacteriocins lactococcin A and pediocin PA-1 were used as models. We show that the lactococcin A processing/export proteins are able to produce both bacteriocins carrying either of the leader peptides, while the pediocin PA-1 processing/export machinery appears to be more specific for its substrate.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactococcus lactis* was grown at 30°C in M17 broth [29] supplemented with 0.5% glucose. *Escherichia coli* and *Listeria ivanovii* were grown in TY medium [24] and in tryptic soy broth supplemented with 0.6% yeast extract (TSY broth), respectively. *Pediococcus acidilactici* was grown in MRS at 30°C. Ampicillin (Amp) was added at a concentration of 100 µg/ml for *E. coli.* Erythromycin (Em) and chloramphenicol (Cm) were used at a concentration of 5 µg/ml for *L. lactis.*

Cloning, DNA Sequencing, and Primer Synthesis

Plasmid DNA was isolated from *E. coli* as described by Sambrook et al. [25]. The method of O'Sullivan and Klaenhammer [21] was used for the isolation of plasmid DNA from *L. lactis.* Restriction and modification enzymes were used according to the manufacturer's recommendations (Boehringer GmbH, Mannheim, Germany). DNA manipulation was carried out essentially as described by Sambrook et al. [25]. Competent *E. coli* cells were prepared and transformed with the gene pulser apparatus as described by the manufacturer (BioRad, Mississauga, On, Canada). The methods for preparing competent cells and electrotransformation of *L. lactis* have been described elsewhere [33]. Double-stranded plasmid DNA was sequenced with the dideoxy chain-termination method [26] using the T7 sequencing kit (Pharmacia, Upsala, Sweden). Synthetic oligodeoxyribonucleotides were synthesized using an ABI model 381A synthesizer (Foster City, CA). The sequences of the primers used are listed in Table 2.

PCR and Plasmid Constructions for Leader Peptide Exchange

The leader peptides from pre-pediocin PA-1 and pre-lactococcin A (Fig. 1) were exchanged using a two-step PCR strategy. First, the DNAs encoding the leader peptides

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant properties	Source or reference
Strains		
Escherichia coli JM101	F' *tra D36 lacI*^{q} *Δ(lacZ) M15 proA*$^{+}$*B*$^{+}$*/supE thi Δ (lac-pro AB)*	Yanisch-Perron et al. [35]
Listeria ivanovii HPB28	Indicator strain for pediocin PA-1 activity	Health Canada
Lactococcus lactis subsp. *lactis* IL1403	Plasmid free, indicator for lactococcin A, chromosomally encoded LcnC and LcnD	Chopin et al. [8]
Lactococcus lactis subsp. *cremoris* MG1363	Plasmid free	Gasson [11]
Pediococcus acidilactici PAC1.0	Plasmid-associated pediocin PA-1 production (pSRQ11) and sucrose fermentation (pSRQ10)	Gonzalez and Kunka [12]
Plasmids		
pUC18	Amp^{r}, cloning vector	Yannisch-Perron et al. [35]
pMG36ct	Cm^{r}, pMG36e-derived expression vector	C. M. Franke
pMB553	Em^{r}, containing lactococcin A operon with *lciA lcnA*	Van Belkum et al. [4]
pMC117	Em^{r}, pediocin operon under control of lactococcal promoter P32	Chikindas et al. [7]
pKV4	Em^{r}, pIL253 derivative containing *lcnC*, *lcnD*, *lcnA*, *lciA*	K. Venema (unpublished)
pCF19A	Amp^{r}, pUC18 derivative containing *lcnA*, *lciA*	C. M. Franke (unpublished)
pMC113dc	Em^{r}, *pedB* under control of lactococcal promoter P32	Venema et al. [30]
pΠ3BS	Em^{r}, low level of expression of *pedC* and *pedD* in a low-copy number plasmid	M. Chikindas (unpublished)
pPedCD	Em^{r}, pMC117 derivative expressing high level of *pedC* and *pedD*	This work
pPP233	Amp^{r}, *p-pedA*	This work
pPPI233	Amp^{r}, *p-pedA +pedB*	This work
pMG233	Cm^{r}, *p-pedA +pedB* in pMG36ct	This work
pLP242	Amp^{r}, *l-pedA*	This work
pLPI242	Amp^{r}, *l-pedA* + *pedB*	This work
pMG242	Cm^{r}, *l-pedA* + *pedB* in pMG36ct	This work
pPL267	Amp^{r}, *p-lcnA*	This work
pPLI267	Amp^{r}, *p-lcnA*	This work
pMG267	Cm^{r}, *p-lcnA* + *lciA* in pMG36ct	This work
pLL276	Amp^{r}, *l-lcnA*	This work
pLLI276	Amp^{r}, *l-lcnA* + *lciA*	This work
pMG276	Cm^{r}, *l-lcnA* + *lciA* in pMG36ct	This work

Amp: ampicillin; Em: erythromycin; Cm: chloramphenicol; ^{r}: resistance

Table 2 Primers used for exchanging the leaders of pediocin PA-1 and lactococcinA

Primer	Sequence[a]
KOV17:	5′-*ATT ACC GTA GTA TTT* **TCC TCC GTT AGC TTC TGA AAG TTC TTC ATC**
KOV18:	5′-**GAA GCT AC GGA GGA** *AAA TAC TAC GGT AAT GGG GTT ACT TGT GGC*
KOV19:	5′-AAT GCT AGC *ATT TAT GAT TAC CTT GAT G Nhe*I
KOV21:	5′-**AAT AAA TGT TAA TTT** *ACC ACC AAT GAT ATT GGC CAT TTC TTT TTC*
KOV22:	5′-*AAT ATC ATT GGT GGT* **AAA TTA ACA TTT ATT CAA TCG ACA GCG GCT G**
KOV23:	5′-GGT CTA GAT **CAA TGG TGC AAC CCG AAA CCT CC** *Xba*I
EE7:	5′-GTC GAC GTC GAC GGG TGG CGC TGC TGG AGG TTT CGG G *Sal*I *Sal*I
EE8:	5′-CCC GGG ATC CGG AGG AAT TTT GAA **ATG AAA AAT CAA TTA AAT TTT AAT ATT G** *Bam*HI
EE9:	5′-CCC GGG ATC CGG AGG AAT TTT GAA *ATG AAA AAA ATT GAA AAA TTA ACT G Bam*HI

[a] Boldtype: *lcnA* sequence; italic: *pedA* sequence

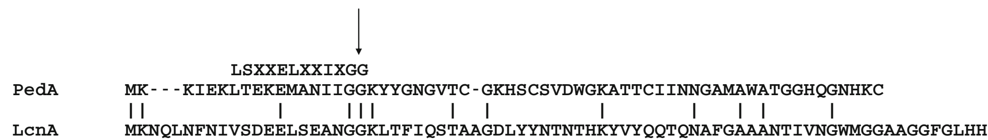


Fig. 1 Amino acid sequence alignments of P-PedA and L-LcnA. Amino acid sequences were aligned with the program alignment of the PC Gene package. The consensus sequence identified for double-glycine-type leaders [14] is shown above the alignment. The *arrow* indicates the processing sites of the two bacteriocins

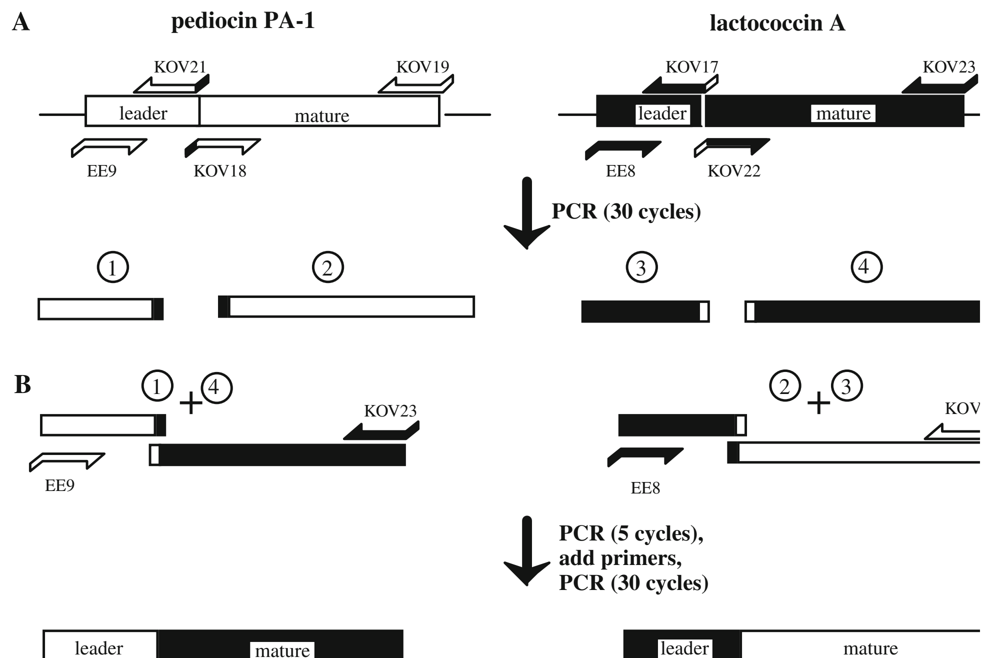


Fig. 2 Exchange of pediocin PA-1 and lactococcin A leaders using a two-step PCR strategy. Sequences specifying the leaders, and the mature bacteriocins were amplified separately using the combinations of primers depicted in panel A. Amplicons 1 and 4 and amplicons 2 and 3 were mixed together and amplified for 5 cycles without adding primer. Then, the primers illustrated in panel B were added, and the DNA was amplified in 30 cycles. PCR conditions were as follows: 94°C for 5 min; 94°C, 1 min; 50°C, 2 min; and 73°C, 1.5 min (5 cycles or 30 cycles). Sequences specifying pre-lactococcin A are in black, those encoding pre-pediocin PA-1 are in white. Numbers in circles identify the amplicons. Template DNAs used in panel A are pMC117 (*p-pedA*) and pMB553 (*l-lcnA*)

and mature portions of the bacteriocins were amplified independently. The resulting amplicons 1 to 4 (Fig. 2) either encoded leader peptide coupled to the N-terminus of the other bacteriocin (1 and 3) or the mature bacteriocin extended at its N-terminus with part of the leader peptide of the reciprocal bacteriocin (2 and 4). Second, the amplicons 1 and 4, and 2 and 3 were mixed as in Fig. 2b and amplified for 5 cycles without adding primers. Then, the proper constructs were amplified using 30 cycles of PCR, and the primers shown in Fig. 2b. The resulting amplicons encoded chimeric pre-bacteriocins in which pediocin PA-1 was fused to lactococcin leader (L-PedA), or lactococcin A was coupled to pediocin PA-1 leader (P-LcnA) (Fig. 2b). Primer EE9 was used in combination with KOV19 in order to generate wild-type pre-pediocin cassette (*p-pedA*). Similarly, the wild-type pre-lactococcin cassette (*l-lcnA*) was obtained by using primers EE8 and KOV23. The templates used in the first PCR step and for generation of the *p-pedA* and *l-lcnA* cassettes were, respectively, pMC117 and pMB553.

p-pedA and *l-pedA* amplicons were cloned into *Bam*HI/*Xba*I-digested pUC18 as *Bam*HI/*Nhe*I fragments, giving pPP233 and pLP242, respectively. A *Sma*I/*Sph*I fragment from pMC113dc carrying the immunity gene for pediocin PA-1 (*pedB*; [31]) was inserted into pPP233 and pLP242 after cutting these plasmids with *Sal*I, filling in the ends with Klenow, then cutting with *Sph*I, giving pPPI233 and pPLI242, respectively. Finally, inserts specifying the pediocin or the lactococcin leader, the pediocin PA-1 mature portion and PedB were cloned into the *Eco*RI/*Sal*I site of pMG36ct. These constructs were named pMG233 and pMG242. The other two cassettes (*p-lcnA* and *l-lcnA)* were restricted and cloned into pUC18 using the *Bam*HI/*Xba*I site, and the resulting plasmids were named pPL267 and

pLL276, respectively. The lactococcin A immunity gene was obtained by PCR on pCF19A with reverse primer (Boehringer) and primer EE7. The amplicon was cloned downstream of *lcnA* in pPL267 and pLL276 using *Sal*I/*Hind*III sites, resulting in pPLI267 and pLLI276. Finally, the *Eco*RI/*Hind*III fragments from the latter plasmids were cloned into pMG36ct using the same sites. The constructs thus obtained were named pMG267 and pMG276. All of the pMG36ct derivatives were introduced into *L. lactis*, and their proper structure was confirmed by nucleotide sequencing.

To express the pediocin processing machinery (*pedC* and *pedD*), pMC117 was digested with *Acc*I and treated with Klenow polymerase. The religated product was used to transform *E. coli* and screened by restriction digestion. A plasmid, pPedCD, in which *pedA* and the 5'-end of *pedB* had been deleted was introduced into *L. lactis* and used for complementation studies.

Determination of Bacteriocin Activity

Bacteriocin activity was determined on 18-h cultures using the critical dilution method described by Daba et al. [9]. Antibiotics were not added in the overnight culture preceding the test, in order to avoid inhibition of the indicator by the antibiotic. Arbitrary units of pediocin PA-1 activity correspond to the reciprocal of the greatest dilution still completely inhibiting the growth of the indicator *L. ivanovii* HPB28 after 18 h in TSY broth at 30°C. Arbitrary unit of lactococcin A was defined similarly using *L. lactis* IL1403 grown at 30°C in glucose-M17 medium as the indicator. Specific activity was expressed as arbitrary units per mg of protein from the producing cells. A plate assay was also used for a qualitative but more sensitive detection of bacteriocin activity. An overnight culture of the bacteria containing proper antibiotics was diluted 500-fold in fresh medium (without antibiotic), and 5 µl were spotted on a plate. After 18 h of incubation at 30°C, cells were killed using chloroform. The plate was exposed to air for an hour, then overlaid with top agar seeded with the proper indicator strain (1000-fold dilution of an overnight culture). Plates were incubated overnight at 30°C and examined for halo formation.

Detection of Bacteriocin Activity on Polyacrylamide Gels

Proteins were separated on tricine–SDS–PAA gels (16.5% T, 6% C) without urea as described by Schägger and Von Jagow [27]. After electrophoresis, the gels were fixed and washed as described by Venema et al. [30]. Gels were placed on glucose M17 agar plates and overlaid using top agar seeded with the proper indicator strain (a 1000-fold dilution of an overnight culture). After incubation for 16 h, the plates were examined for zones of inhibition.

Analytical Methods

Protein Determination

The cells of one ml of culture were recovered by centrifugation, washed once with 10 mM Tris–HCl, pH 8.0, 1 mM EDTA (TE), resuspended in 1 ml of TE, and disrupted with glass beads on a multi-tube vortexer (Troemner, Model VX5000, Philadelphia, PA) at maximum setting for 5 min at 4°C. Protein concentrations were determined using the DC protein assay (Bio-Rad) according to the manufacturer's recommendations, using BSA as a standard.

Assay of Lactate Dehydrogenase

An overnight culture was inoculated (1%) in fresh medium and grown to an O.D. at 600 nm of 0.6. Cells of one ml of culture were harvested by centrifugation, and the supernatant was kept on ice. The pellet was washed once with 1 ml of the same medium and resuspended in 1 ml of 50 mM morpholinopropane sulfonic acid (MOPS), pH 7.0. Cells were disrupted with glass beads on a multi-tube vortexer (Troemner) at maximum setting for 5 min at 4°C. Lysates (5 µl) or supernatants (10 µl) was added to the reaction mixture (50 mM MOPS, pH 7.0, 20 mM pyruvate, 5 mM fructose biphosphate, 0.2 mM NADH) in a final volume of 1 ml, and activity was measured as a decrease of the O.D. at $\lambda = 340$ nm.

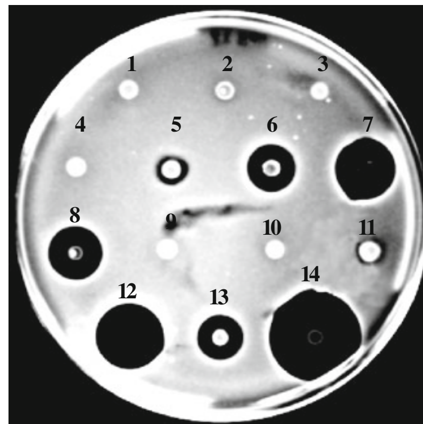
Results

The Limiting Factor in Pediocin PA-1 Production is the Processing/Export Machinery

Plasmid pMG233 encodes wild-type pediocin (P-PedA) and the pediocin immunity protein PedB. When this plasmid was introduced into *L. lactis* MG1363, no pediocin activity could be detected by either the plate assay or by the critical dilution method (Fig. 3, row 4, spot 4). As strain MG1363 does not express a bacteriocin processing/export machinery, pre-pediocin, most probably, remained intracellular. Cell-free extracts of MG1363 (pMG233) did not show any bacteriocin activity, which can be explained by the fact that pre-pediocin has only very low bacteriocin activity [31]. When MG1363 carried, in addition to pMG233, a plasmid expressing the pediocin PA-1 processing/export machinery PedC/PedD (either from pII3BS or pPedCD), bacteriocin activity was seen by halos around colonies of such strains (Fig. 3, spots 5

Fig. 3 Assays on pediocin PA-1 production by the critical dilution method (*upper panel*) and the halo assay (*lower panel*) of lactococcal cells producing wild-type or chimeric pre-pediocin PA-1 in the presence or absence of the pediocin PA-1 or the lactococcin A processing/export machinery. The *upper panel* gives an explanation of the plasmids and relevant proteins present in lactococcal cells, and the *lower panel* shows the antagonistic activity of colonies of cells carrying these constructs on the indicator strain *L. ivanovii* HPB28. N.D.: activity not detected. The numbers in the *lower panel* correspond to the rows in the *upper panel*. P: leader sequence of pediocin PA-1; L: leader sequence of lactococcin A

	Strain (plasmid)	Bacteriocin(s)	Processing/export system	Halo	Pediocin PA-1 Spec. Act. (A.U./mg prot.)
1	MG1363	—	—	-	N.D.
2	IL1403	—	—	-	N.D.
3	MG1363 (pKV4)	L-LcnA	LcnC/D	-	N.D.
4	MG1363 (pMG233)	P-PedA	—	-	N.D.
5	MG1363 (pMG233, pII3BS)	P-PedA	PedC/D	+	N.D.
6	MG1363 (pMG233, pPedCD)	P-PedA	PedC/D	+	N.D.
7	MG1363 (pMG233, pKV4)	{ P-PedA L-LcnA	LcnC/D	+	545
8	IL1403 (pMG233)	P-PedA	LcnC/D	+	40
9	MG1363 (pMG242)	L-PedA	—	-	N.D.
10	MG1363 (pMG242, pII3BS)	L-PedA	PedC/D	-	N.D.
11	MG1363 (pMG242, pPedCD)	L-PedA	PedC/D	+/-	N.D.
12	MG1363 (pMG242, pKV4)	{ L-PedA L-LcnA	LcnC/D	+	1100
13	IL1403 (pMG242)	L-PedA	LcnC/D	+	20
14	MG1363 (pMC117)	P-PedA	PedC/D	+	4550



indicator: *L. ivanovii* HPB28

and 6, respectively). MG1363 (pMG233) carrying pPedCD produced more pediocin activity than that carrying pII3BS. Since the amount of pre-pediocin expressed by pMG233 is expected to be the same in each strain, the difference in pediocin activity can be attributed to the different copy numbers of pPedCD and pII3BS, indicating that the limiting factor in pediocin PA-1 production is the processing/export machinery. Because pMG233, pPedCD, and pMC117 carry the same origin of replication of the lactococcal plasmid pWV01 and the same lactococcal promoter P32 to drive gene expression [13], strains MG1363 (pMG233, pPedCD) and MG1363 (pMC177) (Fig. 3, spots 6 and 14, respectively) were expected to express levels of pediocin PA-1 of the same order of magnitude. The difference in specific activity reported in Fig. 3 is due to the instability of pPedCD. Indeed, several colonies of MG1363 carrying pPedCD were subjected to restriction enzyme analysis, and all of them had deletions within the region encoding PedC and PedD (data not shown).

The Lactococcin Secretion Machinery can Export Pediocin PA-1 Carrying its own Leader Sequence

L. lactis IL1403 carries the genes *lcnC* and *lcnD* encoding the lactococcin processing/export machinery on its chromosome [32]. When pMG233 (*p-pedA*) was introduced

into that strain, active pediocin PA-1 was found by the plate assay (Fig. 3, spot 8). Enough pediocin PA-1 was produced to allow determination of the specific activity by the critical dilution method (Fig. 3, row 8). Apparently, pediocin PA-1 can be activated and exported by the lactococcin LcnC/LcnD system. MG1363 carrying a plasmid encoding the LcnC/LcnD system (pKV4) together with pMG233 (*p-pedA*) expressed about 14-fold more pediocin PA-1 activity than IL1403 (pMG233) (compare Fig. 3, rows 7 and 8). MG1363 (pMG233, pKV4) produced, in addition to pediocin PA-1 encoded by pMG233 (Fig. 3, spot 7), lactococcin A which is encoded by pKV4 (Fig. 5, spot 1). These results show that pediocin PA-1 can compete with lactococcin A for secretion by the lactococcin LcnC/LcnD system.

Pediocin PA-1 Carrying the Lactococcin A Leader is Poorly Recognized by PedC/PedD but Efficiently by LcnC/LcnD

No halo was visible around colonies of MG1363 cells carrying pMG242 (*l-pedA*) and pII3BS (*pedC/pedD*) (Fig. 3, spot 10). However, the faint inhibition zone around MG1363 (pMG242 (*l-pedA*), pPedCD) suggests some recognition of the chimeric molecule by the PedC/PedD system (Fig. 3, spot 11). Chimeric pre-pediocin PA-1 (L-PedA) was recognized by both the chromosomal and plasmid-borne lactococcin LcnC/LcnD system (Fig. 3 spots 12, 13). The specific activity of cells carrying pMG242 (*l-pedA*) and pKV4 (*lcnC/lcnD*) was twofold higher than of cells producing pediocin with its own leader peptide (P-PedA) (Fig. 3, compare spots 7 and 12). The reverse was observed when complementing chimeric pre-pediocin with the chromosomally encoded LcnC/LcnD system in *L. lactis* IL1403 (Fig. 3, spots 8 and 13).

Lactococcin A Carrying the Pediocin PA-1 Leader is Poorly Recognized by the PedC/PedD Machinery

As expected, MG1363 cells expressing chimeric (P-LcnA) or wild-type (L-LcnA) pre-lactococcin A did not showed inhibitory activity as these strains do not express a bacteriocin export machinery (Fig. 4, spot 4 and spot 9). MG1363 (pMC117, pMG267) expressed high amounts of pediocin activity from the *ped* operon present on pMC117 (Fig. 5, spot 3). However, despite the presence of *p-lcnA* on pMG267, no detectable extracellular production of lactococcin A was observed (Fig. 4, spot 5). Lactococcin A activity could be detected as a slight zone of inhibition on the indicator strain *L. lactis* IL1403 when pMG267 (*p-lcnA*) was present in a cell together with pPedCD (Fig. 4, spot 7). These results suggest that lactococcin A carrying the pediocin leader can be processed by PedC/PedD at low efficiency.

The Lactococcin Processing/Export System LcnC/LcnD Recognizes Chimeric Pre-Lactococcin A

In the presence of the chromosomally encoded lactococcin export machinery LcnC/LcnD in *L. lactis* IL1403, comparable amounts of extracellular lactococcin A are produced from pMG267 (*p-lcnA*) and pMG276 (*l-lcnA*) (Fig. 4, spot 8 and spots 12). MG1363 cells carrying pMG276 (*l-lcnA*) together with either pII3BS (PedC/PedD) or pMC117 (complete pediocin operon) did not produce detectable lactococcin A activity, showing that either wild-type pre-lactococcin A is not recognized by the pediocin PA-1 machinery or that it can not compete with pre-pediocin PA-1. We were not able, in several attempts, to introduce pPedCD in MG1363 (pMG276).

Wild-Type and Chimeric Pediocin PA-1 are Processed by the LcnC/LcnD System

Supernatants of cultures of strains carrying either *p-pedA* or *l-pedA* in combination with genes encoding either the LcnC/LcnD or PedC/PedD processing/export systems were examined for the presence of pediocin PA-1 by tricine–SDS–PAGE. Figure 6 shows that pediocin PA-1 could only be detected in those cases in which *lcnC* and *lcnD* were present in multiple copies (on pKV4). Pediocin PA-1 produced by these two strains runs to a similar position as pediocin PA-1 produced by the original producer organism, *P. acidilactici* PAC1.0. Activity of strains expressing the pediocin processing/export machinery on pPedCD or pII3BS was not high enough to be detected by the gel overlay method. Similarly, none of the strain producing lactococcin A produced enough bacteriocin activity to be detected on acrylamide gel except for the positive control: MG1363 containing pKV4 (data not shown).

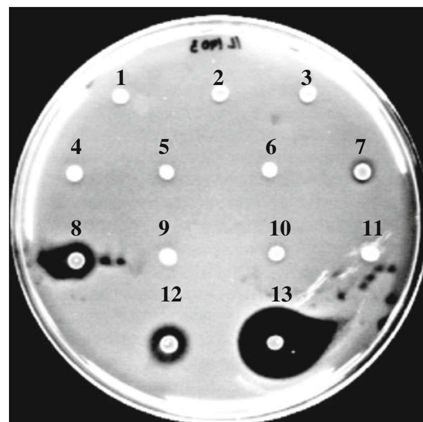
Discussion

Pediocin PA-1 and lactococcin A depend on a two-component processing/export system for their maturation and secretion [28, 31]. This seems to be the case for all bacteriocins with double-glycine-type leaders studied so far. The sequences of the leaders of pediocin PA-1 and lactococcin A conform to the consensus identified for class II bacteriocin leaders: $L_{-12}S_{-11}XXE_{-8}L_{-7}XXI_{-4}XG_{-2}G_{-1}$ [14]. All members of this class of bacteriocins, some lantibiotics, the *Enterococcus faecalis* hemolysin/bacteriocin (CylL1, CylL2), and the microcin colicin V from *E. coli* have homologous leaders which have been shown (or are likely) to be processed after the conserved glycine doublet [14, 30].

Allison et al. [1, 2], Piard et al. [22], and van Belkum and Stiles [5] have demonstrated that extracellular production of

Fig. 4 Assays on lactococcin A production by the critical dilution method (*upper panel*) and the halo assay (*lower panel*) of lactococcal cells producing wild-type or chimeric pre-lactococcin A in the presence or the absence of the pediocin PA-1 or the lactococcin A processing/export machinery. The *upper panel* gives an explanation of the plasmids and relevant proteins present in lactococcal cells, and the *lower panel* shows the antagonistic activity of colonies of cells carrying these constructs on the indicator strain *L. lactis* IL1403. N.D.: activity not detected. The numbers in the *lower panel* correspond to the rows in the *upper panel*. P: leader sequence of pediocin PA-1; L: leader sequence of lactococcin A

	Strain (plasmid)	Bacteriocin(s)	Processing/export system	Halo	Lactococcin A Spec. Act. (A.U./mg prot.)
1	MG1363	—	—	-	N.D.
2	IL1403	—	—	-	N.D.
3	MG1363 (pMC117)	P-PedA	PedC/D	-	N.D.
4	MG1363 (pMG267)	P-LcnA	—	-	N.D.
5	MG1363 (pMG267, pMC117)	{ P-LcnA P-PedA	PedC/D	-	N.D.
6	MG1363 (pMG267, pII3BS)	P-LcnA	PedC/D	-	N.D.
7	MG1363 (pMG267, pPedCD)	P-LcnA	PedC/D	+/-	N.D.
8	IL1403 (pMG267)	P-LcnA	LcnC/D	+	N.D.
9	MG1363 (pMG276)	L-LcnA	—	-	N.D.
10	MG1363 (pMG276, pMC117)	{ L-LcnA P-PedA	PedC/D	-	N.D.
11	MG1363 (pMG276, pII3BS)	L-LcnA	PedC/D	-	N.D.
12	IL1403 (pMG276)	L-LcnA	LcnC/D	+	N.D.
13	IL1403 (pKV4)	L-LcnA	LcnC/D	+	10774



indicator: *L. lactis* IL1403

bacteriocin is possible using heterologous secretion systems. In a study by van Belkum et al. [6], divergicin A, a bacteriocin normally secreted via the general *sec*-dependent pathway [34], was used as a reporter to investigate whether double-glycine-type leader peptides of the precursor bacteriocin are the target for the processing/export system. These authors showed that divergicin A fused to the leader peptides of leucocin A, lactococcin A, or colicin V could be secreted by their cognate secretion systems and, with the exception lactococcin (LcnC/LcnD) and colicin (CvaA/CvaB) secretion systems which could not recognize each other's leader peptides, by the heterologous processing/export systems. Here, we show that pediocin PA-1 can be processed and exported by the lactococcin LcnC/LcnD system, irrespective of whether its precursor carries the pediocin or lactococcin leader. Moreover, wild-type and chimeric pre-pediocin PA-1 could compete with pre-lactococcin A for secretion by the LcnC/LcnD system, as some of the strains presented here produced both bacteriocins simultaneously. Results obtained with wild-type and chimeric pre-lactococcin A confirmed that the lactococcin A processing/export system recognizes the leader peptides of both bacteriocins.

van Belkum et al. [6] also showed that chimeric pre-divergicin A was properly processed by the leucocin processing/export system, whether it carried the leucocin A leader or that of lactococcin A. Similarly, the lactococcin A LcnC/LcnD system appears to be able to process both

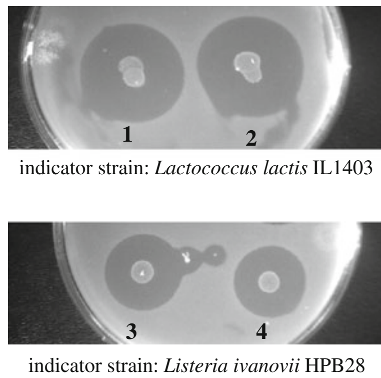


Fig. 5 Detection by the plate halo assay of wild-type lactoccin A production by its cognate processing/export system (LcnC/LcnD) in the presence of wild-type or chimeric pre-pediocin (*upper panel*) and of wild-type pediocin PA-1 production by its cognate processing/export system (PedC/PedD) in the presence of wild-type or chimeric pre-lactococcin (*lower panel*). 1: MG1363 (pMG233 [P-PedA$^+$], pKV4 [L-LcnA$^+$]); 2: MG1363 (pMG242 [L-PedA$^+$], pKV4 [L-LcnA$^+$]); 3: MG1363 (pMG267 [P-LcnA$^+$], pMC117 [P-PedA$^+$]); and 4: MG1363 (pMG276 [L-LcnA$^+$], pMC117 [P-PedA$^+$]). Indicator strains used in the assays are indicated

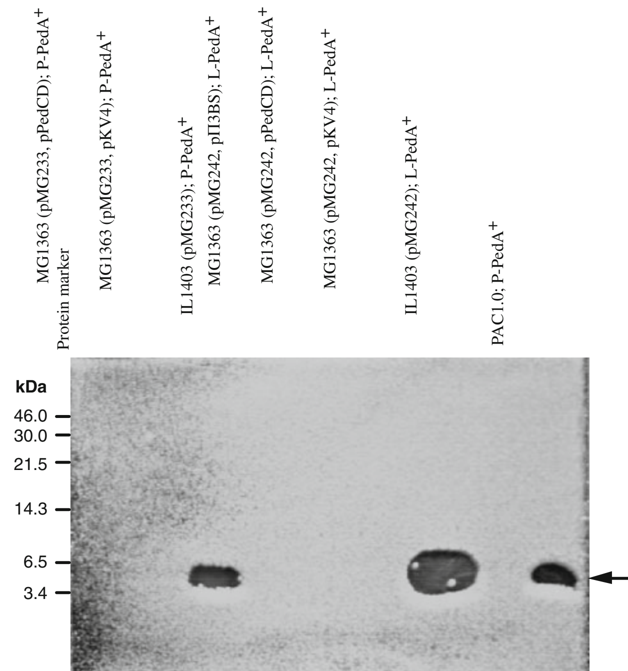


Fig. 6 Antimicrobial activity of wild-type (P-PedA) and chimeric (L-PedA) pre-pediocin PA-1 produced in the presence of PedC/PedD or LcnC/LcnD. Culture supernatants (20 µl, except for PAC1.0 (1 µl)) of various plasmid containing strains indicated in the top margin were subjected to tricine–SDS–PAA (16% without urea) gel electrophoresis. The gel was washed and placed on a petri dish containing TSY agar, overlaid with TSY soft agar seeded with the indicator strain *L. ivanovii* HPB28 and incubated for 18 h at 30°C. The rainbow low-molecular size marker was used as a standard (Amersham, Oakville, On, Canada). The *arrow* indicates the inhibition zone of pediocin PA-1 produced by *P. acidilactici* PAC1.0

wild-type and chimeric pre-pediocin near to or at the original processing site, since the secreted peptides migrated at the same position on an SDS–polyacrylamide gel as mature pediocin PA-1 produced by the wild-type strain *P. acidilactici* PAC1.0.

The pediocin PA-1 processing/export system, on the other hand, seems to be more specific than that of lactococcin A. Apparently, PedC/PedD does not recognize chimeric pre-pediocin (L-PedA), chimeric pre-lactococcin (P-LcnA), and wild-type pre-lactococcin (L-LcnA). These results suggest that PedC/PedD require not only the proper leader peptide for efficient secretion to take place, but also some sequence or structure provided by the mature part of the bacteriocin. van Belkum et al. [6] suggested that, in addition to the leader peptide, the hydrophobic nature of the passenger protein fused to the leader may be important for efficient secretion. We observed very slight zones of inhibition when chimeric pre-pediocin (L-PedA) and chimeric pre-lactococcin A (P-LcnA) were produced in the presence of the pediocin processing/export system (in MG1363 [pMG242, pPedCD] and strain MG1363 [pMG267, pPedCD]). It is possible that P-LcnA and L-PedA are recognized and cleaved by PedD, but that subsequent steps are blocked. The observed activity would correspond to intracellularly located mature lactococcin A or pediocin PA-1 leaking from lysing cells within a colony. In agreement with this hypothesis is the observation by Venema et al. [31] that *E. coli* over-expressing pediocin PA-1 produced intracellular (but not extracellular) active pediocin PA-1 if complemented with (part of) PedD alone. Activation was correlated with cleavage of the leader of P-PedA by PedD. In their case, pediocin PA-1 was over-expressed using the *E.* coli T7 over-expression system and even under those conditions, very little pre-pediocin was actually processed. Here, an assay on cell-free extracts from MG1363 (pMG267, pPedCD) and MG1363 (pMG267, pMC117) did not reveal inhibitory activity (data not shown). It is highly likely that the low level of production of intracellularly processed pediocin PA-1 caused this.

A reason for the specificity of bacteriocin secretion observed with the PedC/PedD couple may lie with the rather peculiar accessory protein PedC. This protein shows no homology to the other accessory proteins involved in the secretion of double-glycine-type bacteriocins and may, therefore, be specific for its wild-type substrate (P-PedA). To test this hypothesis, we attempted to combine pedD with the lactococcin accessory protein LcnD. Unfortunately, we were unable to clone *pedD* alone in *L. lactis*, due to toxicity of its product (data not shown). Venema et al. [31] have shown that two transcripts are produced from the *ped* operon. The most abundant transcript of 1.2 kb encompasses *pedA*, *pedB*, and *pedC*, while the minor

transcript (3.5 kb) included all of the genes, including *pedD*. Possibly, this is a way for *P. acidilactici* to limit the amount of PedD protein and avoid any toxic effects. Toxicity of PedD would also explain the instability of pPedCD in *L. lactis* which resulted in rapid loss of the plasmid from the cells during successive rounds of propagation. The integrity of the cells appeared to be maintained even in the presence of pPedCD, because we did not detect the activity of the intracellular enzyme lactate dehydrogenase in the supernatant of cultures at the end of the exponential phase of growth (data not shown).

Several studies have shown that bacteriocin secretion systems of lactic acid bacteria can be used to direct the externalization of heterologous bacteriocins [1, 2, 5, 6, 22], and thus, they could function as general maturation and secretion systems for the construction of multiple-bacteriocin-producing strains with potential applications in food and feed preservation.

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