Data mining and characterization of a novel pediocin-like bacteriocin system from the genome of *Pediococcus pentosaceus* ATCC 25745

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The genome of *Pediococcus pentosaceus* ATCC 25745 contains a gene cluster that resembles a regulated bacteriocin system. The gene cluster has an operon-like structure consisting of a putative pediocin-like bacteriocin gene (termed *penA*) and a potential immunity gene (termed *peiA*). Genetic determinants involved in bacteriocin transport and regulation are also found in proximity to *penA* and *peiA* but the so-called accessory gene involved in transport and the inducer gene involved in regulation are missing. Consequently, this bacterium is a poor bacteriocin producer. To analyse the potency of the putative bacteriocin operon, the two genes *penA-peiA* were heterologously expressed in a *Lactobacillus sakei* host that contains the complete apparatus for gene activation, maturation and externalization of bacteriocins. It was demonstrated that the heterologous host expressing *penA* and *peiA* produced a strong bacteriocin activity; in addition, the host became immune to its own bacteriocin, identifying the gene pair *penA-peiA* as a potent bacteriocin system. The novel pediocin-like bacteriocin, termed penocin A, has an isotopic mass $[M+H]^+$ of 4684.6 Da as determined by mass spectrometry; this value corresponds well to the expected size of the mature 42 aa peptide containing a disulfide bridge. The bacteriocin is heat-stable but protease-sensitive and has a calculated pI of 9.45. Penocin A has a relatively broad inhibition spectrum, including pathogenic *Listeria* and *Clostridium* species. Immediately upstream of the regulatory genes reside some features that resemble remnants of a disrupted inducer gene. This degenerate gene was restored and shown to encode a double-glycine leader-containing peptide. Furthermore, expression of the restored gene triggered high bacteriocin production in *P. pentosaceus* ATCC 25745, thus confirming its role as an inducer in the *pen* regulon.

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INTRODUCTION

A group of antimicrobial peptides, so-called bacteriocins, are produced by many Gram-positive bacteria (Jack *et al.*, 1995; Riley & Wertz, 2002). Bacteriocins are ribosomally synthesized peptides and most of them are of small size, cationic and heat-stable (Klaenhammer, 1993; Jack *et al.*, 1995; Nes *et al.*, 1996). They kill sensitive bacteria by forming pores on the cytoplasmic membrane or by inhibiting synthesis of the cell wall (Sablon *et al.*, 2000; Hechard & Sahl, 2002; Bauer & Dicks, 2005). The majority of bacteriocins have quite narrow inhibitory spectra, normally against closely related bacteria found in the same ecological niches as the producers. However, as a group, they possess a broad inhibitory spectrum including many important pathogens such as *Listeria* spp., *Clostridium* spp. and

Staphylococcus spp. (Cintas *et al.*, 1997; Eijsink *et al.*, 1998, 2002). In combination with other agents that weaken the outer membrane of Gram-negative bacteria, some bacteriocins also kill food pathogens such as *Escherichia coli* and *Salmonella* spp. (Stevens *et al.*, 1991).

Many lactic acid bacteria (LAB) are known as bacteriocin producers (Klaenhammer, 1993; Diep & Nes, 2002). This trait is believed to be an important part of the antimicrobial arsenal that prevents the growth of pathogens during food fermentation and preservation (Eijsink *et al.*, 2002; Riley & Wertz, 2002). Furthermore, some LAB are also known to have a probiotic effect on the human intestinal tract (Saxelin *et al.*, 2005). An increasing number of LAB genomes have been partly or completely sequenced during the last 5 years and, in several cases, novel bacteriocin-related genes have been identified in their genome (Bolotin *et al.*, 2001, 2004; Kleerebezem *et al.*, 2003; Pridmore *et al.*, 2004; Altermann *et al.*, 2005; Chaillou *et al.*, 2005). In some cases, bacteriocin-related genes have also been found in the genomes of non-bacteriocin producers, normally as incomplete sets of genes

Abbreviations: BU, bacteriocin unit; HPK, histidine protein kinase; RR, response regulator.

Two supplementary tables are available with the online version of this paper.

or containing mutated genes (Bolotin *et al.*, 2001; Chaillou *et al.*, 2005; Møretro *et al.*, 2005). *Pediococcus pentosaceus* ATCC 25745 is a member of the LAB. Most of its genome has been sequenced and one of the contigs published (accession no. NZ_AAEV01000009) harbours a cluster of five genes whose encoded products resemble those involved in a regulated pediocin-like bacteriocin production (Fig. 1a). However, the locus is not complete and this bacterium

is a poor bacteriocin producer. In the present study we have cloned and expressed the putative bacteriocin structural gene and its immunity gene in a heterologous host and demonstrated that high bacteriocin production was obtained when the heterologous host provides the genes necessary for regulation and transport. We have also performed genetic correction of a regulatory gene that is required for elevated bacteriocin production in the bacterial host.

METHODS

Bacterial strains and growth conditions. *Lactobacillus* spp., *Pediococcus* spp., *Enterococcus* spp., *Leuconostoc* spp. and *Carnobacterium* spp. were grown in MRS (Oxoid) at 30 °C; *Lactococcus* spp. in M17 medium (Difco) supplemented with 0.5% (w/v) glucose at 30 °C; *Listeria* spp. in brain heart infusion medium (Difco) at 30 °C; and *Clostridium* spp. in reinforced clostridial medium (Oxoid) at 37 °C under anaerobic conditions. For cultivation of *Lactobacillus* and *Pediococcus* transformants, erythromycin and/or chloramphenicol (each at 5 µg ml⁻¹) was added to the medium.

Cloning and transformation. All inserts were obtained by PCR using the genomic DNA of *P. pentosaceus* ATCC 25745 or *Lactobacillus sakei* 706 LMG 2334 (sakacin A producer) as template. Sequence information of all primers used in this study is listed in Supplementary Table S1, available with the online version of this paper. The primer set dbd84F and dbd104R was used to amplify the fragment containing the *sapA* promoter and the genes *sapA-saiA*, and the primer set dbd105F and dbd81R for the *penA* promoter and *penA-peaA*. To clone *penA* and *peaA* under the control of the *sapA* promoter, a two-step PCR approach was used, with the primer set dbd84F and dbd83R for the promoter sequence and the primer set dbd82F and dbd81R for the genes. Similarly, a two-step PCR approach was used to construct the fusion *penA_F* and *peaA* under the control of the *sapA* promoter, with the primer set dbd84F and dbd95R for the promoter sequence and the DNA sequence encoding the GG-leader of SapA, and the primer set dbd94F and dbd81R for the DNA sequence encoding the mature part of PenA and the entire PeaA. The resulting DNA fragments were digested with *XhoI* and ligated into the *XhoI* site of pLPV111, to obtain the plasmids pLG101, pLG111, pLG121 and pLG131 (see details in Fig. 2a and Table 1). The restoration of the inducer gene *penI_R* and the construction of the fusion gene *penI_F* (Table 1) were performed by two-step PCR, using the primer pair dbd71F and dbd68R for the sequence encoding the mature part of the inducer, and the primer pairs dbd66F and dbd72R, dbd73F and dbd74R, for the DNA sequences encoding the GG-leader of PenI and the GG-leader of PenA, respectively. The resulting PCR products were digested and cloned into pMG36e, downstream of the P32 promoter and between the *XbaI* and *XhoI* sites, resulting in plasmids pDBD298 and pDBD303 (see Table 1). The DNA insert (*peaA-penA penI_RKR*) in pDBD308 (see description in Table 1) was obtained by two-step PCR, using the internal primer pair dbd71F and dbd72R (to correct the mutation in *penI*), and the external primer pair dbd61F and dbd62R. The final PCR product was digested with *EcoRI* and ligated into pMG37e, to obtain pDBD308. Integrity of all inserts was confirmed by DNA sequencing. Transformation of *Lb. sakei* and *P. pentosaceus* was performed using protocols described by Aukrust & Nes (1988) and Caldwell *et al.* (1996).

Purification of penocin A and mass spectrometry. The bacteriocin was purified from the *Lb. sakei* clone B316 (see description of the clone in Table 2). The bacterial clone was grown in 500 ml MRS at 30 °C overnight. The bacteriocin containing cell-free

Fig. 1. (a) Genetic organization of the *pen* locus and two related regulated bacteriocin loci. Bacteriocin structural genes: *penA*, *sapA* and *sppA* (encoding penocin A, sakacin A and P, respectively). Immunity genes: *peaA*, *saiA* and *spiA*. Inducer genes: *penI_R*, *sapI* and *sppI* (*penI_R* is hypothetical, possibly being a disrupted gene with a frame shift mutation; see discussion for detail and Fig. 4). HPK and RR: *penKR*, *sapKR*, *sppKR*. ABC-transporter genes: *penT*, *sapT* and *sppT*. Accessory genes: *sapE* and *sppE* (this gene is absent in the *pen* locus). Flags indicate regulated promoters and empty arrows indicate genes commonly not involved in bacteriocin production. (b) GG-leaders with indicated conserved residues (Havarstein *et al.*, 1994) and the N-terminal part of the mature bacteriocins. *, Hydrophobic residues; □, hydrophilic residues. YGNGVXC, consensus of the pediocin-like family. (c) RT-PCR shows that the transcripts containing *penA*, *penR* and *penT* are present in cells. RNA was isolated from strain ATCC 25745, and DNase treated before first-strand synthesis of cDNA by reverse transcriptase (RT). The RT-treated (+) and non-RT-treated (–, control) RNAs were then used as template in the subsequent PCR with specific primers. Expected size of the PCR products: *penA*, 103 bp; *penR*, 231 bp; *penT*, 218 bp. Only small fragments of the 1 kb ladder (LD) are shown.

Fig. 2. (a) Plasmid constructs used in the heterologous expression of *penA* and *peiA*. *penA* and *peiA* encode the precursor of penocin A and the cognate immunity protein while *sapA* and *saiA* encode the precursor of sakacin A and its immunity; *penA_F* encodes a fusion precursor containing the GG-leader derived from SapA and the mature C-terminus from PenA. *Pro_{penA}* and *Pro_{sapA}* denote regulated promoters from the *pen* and *sap* systems. (b) Regulated promoters from the *sap*, *pln*, *spp* and *pen* loci. Regulated features include a pair of direct repeats (9 bp) with a spacing of 12–13 bp and poorly conserved –35 and –10 boxes. The triplet ATG at the end of each sequence signifies the start codon of the gene indicated in parentheses. TS denotes transcriptional start of the mRNA (in *plnA* and *sppA*). (c) Alignment of the regulatory repeats from the *sap* and the *pen* loci. The bottom line denotes conserved bases; R, a purine (adenine or guanine). (d) Bacteriocin plate assay. Overnight culture supernatant (5 µl) of *P. pentosaceus* ATCC 25745 (1), *E. faecium* P13 (enterocin P producer; 2), *P. acidilactici* Pac1.0 (pediocin PA-1 producer; 3), B323 (4), B314 (5), B315 (6) and B316 (7), was spotted directly on a lawn of the indicator *Lis. innocua* LMG 2785. The plate was incubated overnight at 30 °C for development of growth inhibition which is seen as clear zones. Detailed information of the clones B314, B315, B316 and B323 is given in Table 2.

supernatant (pH 4.7) was applied onto a HiPrep 16/10 SP-XL 20-m cation-exchange column (Amersham Pharmacia Biotech) pre-equilibrated with 10 mM sodium acetate buffer (pH 4.5). The column was washed with 100 ml 10 mM sodium phosphate buffer at pH 6.8 and 50 ml 0.1 M NaCl before the bacteriocin was eluted in 50 ml 1 M NaCl. The purification was completed by two rounds of reverse-phase chromatography on an Äkta purifier system (Amersham Pharmacia Biotech), using (i) the RESOURCE RPC 1 ml column and (ii) the Sephasil peptide C8, 5 µm ST 4.6/250

column (both columns from Amersham Pharmacia Biotech). In each run the peptide was eluted from the column in a water/2-propanol gradient containing 0.1% trifluoroacetic acid (TFA). The fractions showing the highest specific bacteriocin activity were pooled and used in the subsequent purification step. For mass spectra, the sample was mixed 1:1 with a saturated solution of HCCA (α -cyano-4-hydroxycinnamic acid) in TA (0.1% TFA/acetonitrile, 2:1) and deposited on a ground steel MALDI target. Mass spectra were recorded, in reflectron mode, on an Ultraflex TOF/TOF

Table 1. Plasmids and some relevant bacterial strains

Plasmid	Relevant description	Reference
pSAK20	Containing the complete set of <i>sap</i> genes except <i>sapA</i> and <i>saiA</i> ; Cam ^R	Axelsson <i>et al.</i> (1998)
pLPV111	Expression vector; compatible with pSAK20; Ery ^R	Axelsson <i>et al.</i> (1998)
pMG36e	Expression vector with the constitutive P32 promoter; Ery ^R	van de Guchte <i>et al.</i> (1989)
pMG37e	Derivative of pMG36e, without the P32 promoter	D. B. Diep, unpublished
pLG101	Derivative of pLPV111 containing <i>sapA</i> and <i>saiA</i> under control of the <i>sapA</i> promoter	This study
pLG111	Derivative of pLPV111 containing <i>penA</i> and <i>peiA</i> under control of the putative <i>penA</i> promoter	This study
pLG121	Derivative of pLPV111 containing <i>penA</i> and <i>peiA</i> under control of <i>sapA</i> promoter	This study
pLG131	Derivative of pLPV111 containing the recombinant <i>penA_F</i> (with sakacin A's leader) and <i>peiA</i> under control of <i>sapA</i> promoter	This study
pDBD298	Derivative of pMG36e containing the restored <i>penI_R</i> gene	This study
pDBD303	Derivative of pMG36e containing the fusion <i>penI_F</i> gene encoding the inducer peptide with PenA leader	This study
pDBD308	Derivative of pMG37e, containing <i>peiA-penA penI_RKR</i>	This study
Bacterial strain		
LMG 2772	<i>E. faecium</i> P13; enterocin P producer	Cintas <i>et al.</i> (1997)
LMG 2002	<i>P. acidilactici</i> Pac1.0; pediocin PA-1 producer	Marugg <i>et al.</i> (1992)
LMG 2785	<i>Lis. innocua</i> used as indicator	LMG collection*
B317	<i>Lb. sakei</i> Lb790 containing pSAK20 and pLG101	This study

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(Daltonics), using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV. The displayed spectrum is the sum of 200 laser shots, with laser power adjusted to just above threshold level.

Bacteriocin production and bioassay. *Enterococcus faecium* P13 (enterocin P), *Pediococcus acidilactici* Pac1.0 (pediocin PA-1), B317 (sakacin A) and B316 (penocin A) were used as bacteriocin producers. Bacteriocins from supernatants of overnight cultures (24 h) were concentrated by ammonium sulfate precipitation (30 %, w/v) followed by centrifugation (12 000 g for 30 min at 4 °C); the bacteriocin pellets were then suspended in distilled water prior to boiling for 5 min to inactivate bacterial growth. For inhibitory spectra,

volumes (5 µl) of three different bacteriocin concentrations (10 ×, 1 × and 0.1 × of culture supernatant) were spotted directly onto agar plates containing lawns of the indicators. After overnight incubation at appropriate conditions, inhibition was seen as clear zones on plates. Bacteriocin activity of penocin A (shown in Table 2) was determined by a microtitre plate assay as previously described (Holo *et al.*, 1991), using *Listeria innocua* LMG 2785 as indicator strain. One bacteriocin unit (BU) is defined as the minimum amount of a bacteriocin needed to obtain 50 % growth inhibition of a 200 µl indicator culture. Bacteriocin assays in the presence of 5 mM DTT were performed as previously described by Eijsink *et al.* (1998), using *Lis. innocua* LMG 2785 (whose growth is not inhibited by the presence of DTT) as indicator strain.

Table 2. Bacteriocin activity in the culture supernatants from the various strains

Bacterial strain	Description	BU ml ⁻¹ *	Reference
<i>P. pentosaceus</i> ATCC 257455	Wild-type strain	5–10	ATCC
B314	<i>Lb. sakei</i> Lb790/pSAK20 + pLG111	160	This study
B315	<i>Lb. sakei</i> Lb790/pSAK20 + pLG121	1280	This study
B316	<i>Lb. sakei</i> Lb790/pSAK20 + pLG131	2560	This study
B323	<i>Lb. sakei</i> Lb790/pSAK20 + pLPV111	0	Axelsson <i>et al.</i> (1998)
B349	<i>P. pentosaceus</i> ATCC 25745/pSAK20	8	This study
B353	<i>P. pentosaceus</i> ATCC 25745/pDBD298	128 (8)	This study
B354	<i>P. pentosaceus</i> ATCC 25745/pDBD303	256 (16)	This study
B356	<i>P. pentosaceus</i> ATCC 25745/pDBD308	2560	This study
B384	<i>P. pentosaceus</i> ATCC 25745/pSAK20 + pMG36e	16	This study
B374	<i>P. pentosaceus</i> ATCC 25745/pSAK20 + p298	16	This study
B377	<i>P. pentosaceus</i> ATCC 25745/pSAK20 + p303	32	This study

*Bacteriocin activity measured in the presence of 5 mM DTT is given in parentheses.

RNA isolation and RT-PCR. RNA was isolated using the RNeasy Mini kit (Qiagen). The isolated RNA was treated with DNase I (Takara) for 30 min at 37 °C before first-strand synthesis of cDNA using Moloney murine leukaemia virus reverse transcriptase (M-MLV RT; Promega). The resulting cDNA was then used as template in PCR reactions (Dynazyme), with the primer pairs dbd131 and dbd132, dbd141 and dbd136, and dbd142 and dbd143, specific to *penA*, *penR* and *penT*, respectively. PCR conditions were: 94 °C for 4 min, 30 cycles consisting of 94 °C for 15 s, 62 °C for 15 s and 72 °C for 20 s, and the final step at 72 °C for 5 min. Sequence information of primers is listed in Supplementary Table S1, available with the online version of this paper.

RESULTS

Sequence analysis of the *pen* locus

One of the contigs published (accession no. NZ_AAEV-01000009) from the genome of *P. pentosaceus* ATCC 25745 contains a cluster of five bacteriocin-related genes, termed *penA*, *peiA*, *penK*, *penR* and *penT* (Fig. 1a). The polypeptide encoded by *penA* is 60 aa long and contains a double-glycine (GG)-leader consensus at its N-terminus (Fig. 1b), while the predicted C-terminal mature part is 42 aa long and contains the consensus of the pediocin-like bacteriocin family (KYYNGVHCGKKTCYVDWGWQATASIGKIIVNGWTQHGPWAHR; pediocin-like consensus residues are underlined) (Eijsink *et al.*, 1998). Immediately downstream of *penA* resides *peiA*, which encodes a polypeptide of 92 aa; the position and its size suggest that *PeiA* serves as an immunity protein (Klaenhammer, 1993; Nes *et al.*, 1996). The remaining three genes are divergently oriented and located upstream of *penA*. The gene pair *penKR* encode a histidine protein kinase (HPK) and response regulator (RR), respectively, proteins belonging to two-component regulatory systems that have been shown to be involved in the regulated production of a number of bacteriocins (Diep *et al.*, 1995, 1996, 2000; Nes *et al.*, 1996; Kleerebezem & Quadri, 2001). Lastly, *penT* encodes an ABC transporter with highest homology to those involved in maturation and export of bacteriocins with GG-leaders (Havarstein *et al.*, 1995). Furthermore, as for other regulated bacteriocin loci (Diep *et al.*, 1996, 2000; Brurberg *et al.*, 1997), two putative regulated promoters can be identified within the *pen* locus, one located just upstream of *penA* and the other located upstream of *penK* (see Figs 1a and 2b). The conserved features of such a regulated promoter comprise a pair of direct repeats (9 bp) separated by a spacing of 12–13 bp, and poorly conserved –35 and –10 boxes in the promoter (Diep *et al.*, 1996). We have previously demonstrated that the cognate response regulator binds specifically on the direct repeats as dimers in a cooperative manner, to activate gene expression (Risoen *et al.*, 1998, 2000, 2001).

However, compared with the other known bacteriocin systems such as *pln* for plantaricins (Diep *et al.*, 1996), *sap* for sakacin A (Diep *et al.*, 2000) and *spp* for sakacin P (Brurberg *et al.*, 1997), two genes seem to be missing from the *pen* locus (Fig. 1a): one encoding an accessory protein that acts together with the cognate ABC transporter to

remove GG-leaders concomitant with export of the mature peptides (Stoddard *et al.*, 1992; Havarstein *et al.*, 1995) and a second gene that encodes an inducer peptide required for activation of the cognate two-component regulatory system that eventually triggers high transcription of all genes in the bacteriocin locus (Diep *et al.*, 1996, 2000). The genetic organization of these two genes is relatively conserved: the accessory gene is normally cotranscribed with and located immediately downstream of the cognate ABC-transporter gene while the inducer gene precedes the HPK gene and is also cotranscribed with the HPK and RR genes as a multicistronic transcript (Diep *et al.*, 1996; Nes *et al.*, 1996).

P. pentosaceus ATCC 25745 is a poor bacteriocin producer

Transcription analyses by RT-PCR using primers specific to *penA*, *penR* and *penT* revealed the presence of their transcripts in cells (Fig. 1c). However, very little or no growth inhibition was observed when the antimicrobial activity from its culture supernatant was assayed using an array of potential indicators, including *Listeria* species which are known to be very sensitive to pediocin-like bacteriocins (Eijsink *et al.*, 1998). For instance, the culture supernatants of the producers of enterocin P and pediocin PA-1 were 100–400 times more potent than that of *P. pentosaceus* ATCC 25745 when using the indicator strain *Lis. innocua* LMG 2785 (Table 2, Fig. 2d). These results suggest either that the bacteriocin system per se is poorly potent or that the *pen* locus is not fully functional, possibly due to the lack of the two genes described above.

Heterologous expression of *penA* and *peiA*

Next, we wanted to examine whether the gene pair *penA-peiA* represents a potent bacteriocin system when expressed in a different host that contains a complete set of genes required for regulation and bacteriocin export. We used the previously described two-plasmid system (pSAK20 + pLPV111) in *Lb. sakei* Lb790 for heterologous expression of *penA-peiA* (Axelsson *et al.*, 1998). In this system, pSAK20 contains a complete set of *sap* genes necessary for activation of the *sapA*-regulated promoter and transport of bacteriocins that apply a GG-leader to direct maturation and secretion. pLPV111, which is compatible with pSAK20, contains the bacteriocin genes to be expressed. *penA* and *peiA* were cloned into pLPV111, under the control of either its own regulated *penA* promoter or the regulated *sapA* promoter, resulting in the constructs pLG111 and pLG121, respectively (Fig. 2a). It is important to point out here that the *penA* promoter and the *sapA* promoter are quite similar with respect to their regulatory repeats (Fig. 2c). However, unlike the *pen* repeats and the repeats from the other related systems such as the *pln* and *spp*, which all have a spacing of 12 nt, the *sap* system has a 13 nt spacing between the repeats (Fig. 2b; Diep *et al.*, 1996). As the length of the spacing has previously been reported to be specifically defined for optimal gene activation (Risoen *et al.*, 2001), we anticipated

that the *sapA* promoter would be more efficient than the *penA* promoter under the *sap* regulatory regime.

After transformation of pLG111 and pLG121 into the *Lb. sakei* host, we found that both clones produced bacteriocin activity towards the indicator strain *Lis. innocua* and, as expected, the clone B315 with the *sapA* promoter produced about eightfold more bacteriocin (1280 BU ml^{-1}) than the clone B314 with the *penA* promoter (160 BU ml^{-1}).

It has previously been reported that the type of GG-leaders or subtle differences in the GG-leader sequence can significantly influence the outcome of bacteriocin production in a heterologous host (van Belkum *et al.*, 1997) and that highest production is often obtained when the leader peptide is processed by its specific ABC transporter (Horn *et al.*, 1998, 1999). As the *sap* transporter (encoded by pSAK20) is specific for the GG-leader of sakacin A, we wanted to examine whether PenA production can be further improved when it is directed by the GG-leader of SapA. Therefore, we made another construct (pLG131) that coded for a fusion polypeptide in which the predicted mature peptide of PenA was N-terminally fused with the GG-leader of SapA. As with pLG121, the same *sapA* promoter was used to drive expression of the fusion gene in pLG131 for direct comparison. When expressed under similar growth conditions, the new clone B316 (with pLG131) produced about twice as much bacteriocin as the former (Fig. 2d, Table 2), thus indicating that the GG-leader of SapA is better than that of PenA in directing heterologous bacteriocin production in this expression system.

Expression of *penA-peaA* also confers immunity

In the constructs obtained above, the putative immunity gene *peaA* was cloned to cotranscribe with *penA*. To test whether the expression of *peaA* also confers immunity to the bacteriocin (termed penocin A), this bacteriocin was added to lawns of the clones obtained above. The two clones B315 and B316, which produced the greatest amount of penocin A and presumably had a high expression of *peaA*, appeared fully immune to the bacteriocin concentration tested (Fig. 3). On the other hand, the clone B314, which produced much less bacteriocin and presumably had poor *peaA* expression, was significantly inhibited by penocin A. The immunity displayed by B315 and B316 is specific as it acts only towards penocin A but not towards any of the other pediocin-like bacteriocins such as sakacin A, enterocin P and pediocin PA-1 (Fig. 3). In contrast, the control clone B317 expressing *sapA-saiA* showed some cross-immunity to enterocin P in addition to being immune to its own bacteriocin (sakacin A). However, this clone was not immune to penocin A or pediocin PA-1.

Restoration of the bacteriocin production in *P. pentosaceus* ATCC 25745

Interestingly, there are some features resembling remnants of a disrupted inducer gene located between one of the

Fig. 3. Immunity test on clones expressing *peaA*. The bacteriocins penocin A (PenA), sakacin A (SakA), enterocin P (EntP) and pediocin A (PedA) were spotted directly onto lawns of the following indicator clones: B314 with low penocin A production; B315 and B316, both with high penocin A production; and the control B317 with sakacin A production. A $5 \mu\text{l}$ volume of $10\times$ concentrated bacteriocin-containing culture supernatant was added onto each spot. Growth inhibition is seen as clear zones after overnight incubation at 30°C . Detailed description of bacterial clones is given in Tables 1 and 2.

putative regulated promoters and the HPK gene *penK*, a region where an inducer gene normally resides in other systems (Fig. 1a). These features include (i) a short ORF that is preceded by a conserved ribosome-binding site (AAGAAG) and encodes a polypeptide containing part of a GG-leader-like sequence and (ii) a sequence on an overlapping reading frame that encodes a small cationic inducer-like peptide that starts with two consecutive glycine residues (Fig. 4a). This glycine-duplet, together with the preceding amino acid sequence encoded by the other reading frame, seems to constitute a complete GG-leader (Figs 1b and 4b). To test our hypothesis, we corrected the putative disrupted inducer gene by removing a nucleotide (adenine) from the joining region between the two halves (Fig. 4c and d). The resulting restored ORF (termed *penI_R*; R for being restored) encodes a polypeptide of 39 aa, of which the first 16 residues contain a GG-leader consensus and the remaining C-terminal sequence is of 23 aa and has a relatively high pI (10.4), features typical of the small inducer peptides involved in regulated bacteriocin systems (Nes *et al.*, 1996). The restored *penI_R* was cloned into the expression vector pMG36e where its expression was driven by the constitutive promoter P32, and the resulting plasmid (pDBD298) was transformed into the pediococcal host. As shown in Table 2 and Fig. 4(e), the clone B353 expressing pDBD298 has a marked increase in bacteriocin production, about 15-fold more than the wild-type strain. Increased bacteriocin production was also obtained when the pediococcal clone B354 expressed the other plasmid pDBD303, which harbours a fusion gene (*penI_F*) encoding a polypeptide in which the mature part of the inducer peptide was N-terminally fused with the GG-leader of PenA (Table 2, Fig. 4e).

Fig. 4. Restoration of the disrupted inducer gene (*penI*) from the *pen* locus. (a) DNA sequence with two translated reading frames in the region containing the disrupted *penI*. Part of the GG-leader sequence is located on the first half of the first reading frame while the entire mature part of the inducer peptide is located on the second frame (bold letters). (b) Removal of the indicated nucleotide (arrow in a) restores the integrity of *penI*. The continuously translated N-terminal GG-glycine leader is underlined. (c, d) Chromatograms show the DNA sequence before (c) and after (d) restoration of *penI*. (e) Bacteriocin plate assay with the culture supernatant derived from *P. pentosaceus* ATCC 25745 (1), B349 expressing pSAK20 (2), B353 expressing the restored *penIR* (3), B354 expressing the fusion gene *penIF* (with PenA GG-leader) (4), B384 expressing pSAK20 and pMG36e (control, 5), B374 expressing pSAK20 and *penIR* (6) and B377 expressing pSAK20 and *penIF* (7). The amount of bacteriocin used in the assay was 5 μ l of 10 $\times$ concentrated supernatant of overnight-grown cultures and the indicator was *Lis. innocua* LMG 2785.

For some regulated bacteriocin systems such as *nis* and *pln* for nisin and plantaricin production, the inducer peptide by itself has bacteriocin activity (Hauge *et al.*, 1998; Kleerebezem *et al.*, 1999). To exclude the possibility that the increased bacteriocin activity observed above was directly due to the products of *penIR* and *penIF*, we performed the bacteriocin assay in the presence of 5 mM DTT, a reducing agent that destroys the disulfide bridge in penocin A (see below), thereby weakening its antimicrobial activity (Eijsink *et al.*, 1998). The sequence of the inducer peptide (Fig. 4b) has no cysteine residues to form a disulfide bridge and consequently should not be affected by the presence of DTT. As expected, a drastic reduction in bacteriocin activity was observed when the bacteriocin assay was performed under the reducing conditions, amounting to less than one-tenth of the activity that was assayed without DTT (Table 2). Based on these results, we conclude that *penIR* is an inducer gene required to activate the production of penocin A in *P. pentosaceus* ATCC 25745.

The results also indicate that a functional machinery exists for exporting penocin A in *P. pentosaceus* ATCC 25745 although the *pen* locus in its genome seems to lack an

accessory gene. To examine whether the presence of a cloned accessory gene can further increase the production of penocin A, we transformed the plasmid pSAK20, which contains the accessory gene *sapE*, into *P. pentosaceus* ATCC 25745 (with or without the co-expression of pDBD298 or pDBD303). Surprisingly, we did not observe any increase in bacteriocin production; rather, activity was reduced in comparison with those (B353 and B354) expressing only pDBD298 or pDBD303. On the other hand, when we transformed the pediococcal host with a plasmid (pDBD308) which harbours the bacteriocin operon (*penA-peIA*) and the restored regulatory unit (*penIRKR*), to increase the dose of these genes, the bacteriocin production was increased significantly, reaching the high level (2560 BU ml⁻¹) observed for the *Lb. sakei* host B316 (see Table 2 and further discussion below). These results strongly suggest the presence of an efficient transport apparatus for GG-leader-containing peptides, in *P. pentosaceus* ATCC 25745.

Physical properties and purification of penocin A

The antimicrobial activity of penocin A was found to be heat-stable (resistant to boiling for at least 10 min) but

protease K-sensitive (data not shown), properties typical of small bacteriocins (Jack *et al.*, 1995). The bacteriocin peptide was purified to homogeneity from the culture supernatant of the *Lb. sakei* clone B316 and its isotopic mass $[M+H]^+$ was 4684.6 Da as determined by mass spectrometry (Fig. 5), which is about 2 Da less than the calculated $[M+H]^+$ mass of the expected mature peptide (42 aa and 4686.29 Da). This discrepancy corresponds well to a removal of two hydrogen atoms upon the formation of a disulfide bridge between two cysteine residues (positions 9 and 13), a maturation process which is common for bacteriocins within the pediocin-like family (Eijsink *et al.*, 1998).

Comparison of the inhibitory spectra of penocin A and three related bacteriocins

A collection of 71 strains from eight different genera of Gram-positive genera were used as indicators in the bacteriocin assay. The inhibitory spectrum of penocin A was compared with that of enterocin P, sakacin A and pediocin PA-1, in Supplementary Table S2, available with the online version of this paper. The results show that pediocin PA-1 and penocin A have a broader inhibitory spectrum than that of sakacin A and enterocin P, i.e. inhibition rates are 58 % and 48 % for pediocin PA-1 and penocin A, versus 39 % and 35 % for sakacin A and enterocin P, respectively. Like the other pediocin-like bacteriocins, penocin A was not very antagonistic to lactococcal species; only two of 18 tested lactococcal indicators were sensitive. Variation in sensitivity between species within a genus was observed with *Clostridium*. Only *C. butyricum* (two of two strains) was found sensitive to penocin A and the other bacteriocins, while *C. bif fermentans* (two strains), *C. sporogenes* (three strains) and *C. tyrobutyricum* (two strains) were not sensitive to any of these bacteriocins at the concentrations applied. Importantly, although the four bacteriocins differ from each other in inhibitory spectra, they all display a consistent potency towards listerial species: nine of nine

strains from three different species were found sensitive to all four bacteriocins.

Cross-resistance between penocin A and the other pediocin-like bacteriocins

Recently, a number of reports have indicated that pediocin-like bacteriocins exploit the mannose PTS sugar permease as a receptor when attacking *Listeria* and *Enterococcus* species (Hechard *et al.*, 2001; Gravesen *et al.*, 2002), and that many spontaneous mutants of *Listeria* resistant to this group of bacteriocins were found to exhibit a reduced synthesis of one of the subunits of the permease complex (Gravesen *et al.*, 2002). Spontaneous mutants of the indicator strain *E. faecium* P13 resistant to penocin A or pediocin PA-1 were found to occur at high frequencies and also displayed cross-resistance towards each other (data not shown). The cross-resistance phenomenon also occurs with penocin A-resistant mutants of *Listeria*. Thus, mutants of *Listeria monocytogenes* LMG 2653, which had acquired resistance to any of the four bacteriocins, penocin A, pediocin PA-1, enterocin P and sakacin A, appeared resistant to the other three bacteriocins as well. Taken together, the results strongly suggest that penocin A exploits the same receptor or mechanism as the other pediocin-like bacteriocins, when killing species of *Listeria* and *Enterococcus*.

DISCUSSION

Compared with other known regulated bacteriocin loci (Diep *et al.*, 1995, 1996; Nes *et al.*, 1996; Eijsink *et al.*, 2002), two genes seem to be missing from the *pen* locus (see Fig. 1a): an inducer gene which is part of the regulatory system required for gene activation, and an accessory gene involved in export of GG-leader-containing peptides (Stoddard *et al.*, 1992; Havarstein *et al.*, 1995). Such genes have previously been shown to be necessary for bacteriocin production (Kuipers *et al.*, 1993; van Belkum & Stiles 1995; Diep *et al.*, 2001). Consistently, *P. pentosaceus* ATCC 25745

Fig. 5. Mass spectrometry indicates an isotopic mass $[M+H]^+$ of 4684.6 for penocin A containing a disulfide bridge. Shown above is the suggested structure of penocin A.

itself was found to be a poor producer, producing only residual amounts of bacteriocin as compared with other producers of the pediocin-like family (Fig. 2d).

The incompleteness of the *pen* locus was confirmed when we complemented the pediococcal host with the expression of the restored gene, *penI_R*, which was required for elevated bacteriocin production. This restored gene encodes a small cationic inducer-like peptide containing a GG-leader and has a conserved genetic organization, namely being located immediately upstream of the HPK gene (Fig. 1a). Based on our results, we believe that *penI_R* and the genes *penKR* together make up a complete signal transduction system required to activate the regulated promoters in the *pen* locus. Such regulatory systems are known to modulate gene expression in a 'quorum-sensing' manner (Diep *et al.*, 1996; Kleerebezem & Quadri, 2001; Eijsink *et al.*, 2002). In such a system, a low or basal level of the inducer is believed to be constantly secreted and used as a means to monitor cell density in the environment. Only when the inducer reaches a certain threshold concentration (or a defined 'quorum' of cells) in the environment does this signal trigger high expression of a defined set of genes through the action of the cognate HPK and RR. By analogy, it is therefore reasonable to think that the presence of the *pen* transcripts in the pediococcal cells as detected by RT-PCR (Fig. 1c) was probably a result of basal transcription taking place prior to gene activation and, as the inducer gene in the *pen* regulon was disrupted in the natural strain, bacteriocin production was therefore repressed at a low level throughout the bacterial cycle.

The elevated bacteriocin production in the pediococcal clone expressing the restored *penI_R* indicates that a functional transport apparatus exists in cells to mediate export of GG-leader-containing peptides, despite the fact that only the ABC-transporter gene *penT* (without a cognate accessory gene) is found in the *pen* locus. Furthermore, this export apparatus seems to be relatively efficient; this is based on the fact that the pediococcal clone B356, which contains a higher gene dose of the bacteriocin operon (*penA-peIA*) and the regulatory unit (*penI_RKR*), produced bacteriocin at high levels comparable to the *Lb. sakei* clone B316, the latter displaying the highest bacteriocin activity amongst the *Lb. sakei* clones (Table 2). A search in the sequenced part (1 762 192 nt and 1653 genes) of the pediococcal genome did not reveal any orphan bacteriocin-related accessory gene (data not shown), suggesting that the ABC transporter PenT might, by itself, constitute a functional apparatus for bacteriocin export. To our knowledge, only one bacteriocin system has previously been reported to be functional without an accessory protein, namely the *stx* system from the malt isolate *Lb. sakei* 5 (Vaughan *et al.*, 2003). The *stx* locus, which is composed of two bacteriocin operons (*sakT_αT_βI_T* and *sakXI_X*), one regulatory unit (*stxKRP*) and the ABC-transporter gene *stxT* (without its cognate accessory partner), triggered sakacin X and sakacin T production when it was transferred into the recently

sequenced *Lb. sakei* strain 23K, which does not have a bacteriocin-related accessory gene in its genome. By analogy it is tempting to speculate that the *pen* export apparatus might function without an accessory protein in a similar manner as observed for the *stx* system. However, we can not exclude the possibility that an orphan accessory gene, in a yet unsequenced region in the genome of *P. pentosaceus* ATCC 25745, might be needed for *penT* to constitute a complete export apparatus. The conclusive answer awaits a complete genome sequence from this bacterium.

In an attempt to further improve the bacteriocin production in *P. pentosaceus* ATCC 25745 and in the *penI_R*- or *penI_F*-expressing pediococcal clones B353 and B354, pSAK20 was transformed into these clones. This plasmid contains the genes required for bacteriocin export and gene activation, and was expected to have a positive effect on penocin A production. Surprisingly, a significant reduction (about 10-fold or more) in bacteriocin production was found (Table 2). We do not know what caused the reduced production. Possibly, the presence of two highly similar response regulators *penR* and *sapR* (sharing about 50 % identity at amino acid level) within the same cell might somehow interfere with each other's function, thereby causing an inefficient gene activation. Supporting this notion is our previous study on the regulation of plantaricin production in *Lactobacillus plantarum* C11, which possesses two very similar RR proteins, PlnC and PlnD (Diep *et al.*, 1996). These two proteins, sharing about 60 % identity and whose genes are located within the same operon, individually act as gene activators but they antagonize each other when they are overexpressed within the same host, with PlnD being converted from a weak activator to a strong negative regulator (Diep *et al.*, 2001, 2003).

Bacteriocin production can be considered as an accessory pathway because this trait is commonly not critical for the normal growth of the bacterial host. However, as the responsible loci are often located on mobile elements such as transposons or transferable plasmids, these loci are therefore frequently transferred to other bacterial lineages, thereby being prone to genetic rearrangement or mutation. For instance, a recent survey by Møretrø *et al.* (2005) shows that a collection of 15 different sakacin P-sensitive and non-producing strains of *Lb. sakei* contain remnants of bacteriocin-related (*spp*) genes/loci in their genome. The degeneracy of these loci varies among strains, with some containing a point mutation in a critical gene whilst others had one or several genes deleted. In the present study, we have demonstrated that the poor bacteriocin production in *P. pentosaceus* ATCC 25745 was a consequence of a frameshift mutation in the inducer gene *penI*. Once restored, this gene activated bacteriocin production and the encoded bacteriocin was found to be a potent peptide, with a relatively wide inhibitory spectrum including important food-borne pathogens such as *Clostridium* and *Listeria* species.

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