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.

**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; Mørætø 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-peiA. To clone penA and penA 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 and penA 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 PenI and 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 PeiA. The resulting DNA fragments were digested with Xhol and ligated into the Xhol site of pLPV111, to obtain the plasmids pLG101, pLG111, pLG121 and pLG131 (see details in Figs. 2a and Table 1). The restoration of the inducer gene penIR and the construction of the fusion gene penIR (Table 1) were performed by two-step PCR, using the primer pair dbd71F and dbd72R 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 Xhol sites, resulting in plasmids pDBD298 and pDBD303 (see Table 1). The DNA insert (penA-peiA penIR)R 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 penIR), and the external primer pair dbd61F and dbd62R. The final PCR product was digested with EcoRI and ligated into pMG36e, 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
supernatant (pH 4–7) was applied onto a HiPrep 16/10 SP-XL 20-ml 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 A¨ kta purifier system (Amersham Pharmacia Biotech), using (i) the RESOURCE RPC 1 ml column and (ii) the Sephasil peptide C8, 5 mm 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 (x-cyano-4-hydroxycinnamic acid) in TA (0·1% TFA/acetoni trile, 2:1) and deposited on a ground steel MALDI target. Mass spectra were recorded, in reflectron mode, on an Ultraflex TOF/TOF
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 ml indicator culture. Bacteriocin assays in the presence of 5 mM DTT were performed as previously described by Eijsink et al. (1998), using Listeria innocua LMG 2785 (whose growth is not inhibited by the presence of DTT) as indicator strain.

**Table 1.** Plasmids and some relevant bacterial strains

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSAK20</td>
<td>Containing the complete set of sap genes except sapA and saiA; Cam&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Axelsson et al. (1998)</td>
</tr>
<tr>
<td>pLPV111</td>
<td>Expression vector; compatible with pSAK20; Ery&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Axelsson et al. (1998)</td>
</tr>
<tr>
<td>pMG36e</td>
<td>Expression vector with the constitutive P32 promoter; Ery&lt;sup&gt;R&lt;/sup&gt;</td>
<td>van de Guchte et al. (1989)</td>
</tr>
<tr>
<td>pMG37e</td>
<td>Derivative of pMG36e, without the P32 promoter</td>
<td>D. B. Diep, unpublished</td>
</tr>
<tr>
<td>pLG101</td>
<td>Derivative of pLPV111 containing sapA and saiA under control of the sapA promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pLG111</td>
<td>Derivative of pLPV111 containing penA and peiA under control of the putative penA promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pLG121</td>
<td>Derivative of pLPV111 containing penA and peiA under control of sapA promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pLG131</td>
<td>Derivative of pLPV111 containing the recombinant penAF (with sakacin A’s leader) and peiA under control of sapA promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pDBD298</td>
<td>Derivative of pMG36e containing the restored pen&lt;sub&gt;L&lt;/sub&gt; gene</td>
<td>This study</td>
</tr>
<tr>
<td>pDBD303</td>
<td>Derivative of pMG36e containing the fusion pen&lt;sub&gt;L&lt;/sub&gt; gene encoding the inducer peptide with PenA leader</td>
<td>This study</td>
</tr>
<tr>
<td>pDBD308</td>
<td>Derivative of pMG37e, containing peiA-penA penL&lt;sub&gt;K&lt;/sub&gt;KR</td>
<td>This study</td>
</tr>
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Bacterial strain

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMG 2772</td>
<td>E. faecium P13; enterocin P producer</td>
<td>Cintas et al. (1997)</td>
</tr>
<tr>
<td>LMG 2002</td>
<td>P. acidilactici Pac1.0; pediocin PA-1 producer</td>
<td>Marugg et al. (1992)</td>
</tr>
<tr>
<td>LMG 2785</td>
<td>Lis. innocua used as indicator</td>
<td>LMG collection*</td>
</tr>
<tr>
<td>B317</td>
<td>Lb. sakei Lb790 containing pSAK20 and pLG101</td>
<td>This study</td>
</tr>
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</table>

*Authors’ laboratory.

(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.

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

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

*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 leukemia 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 a cluster of five bacteriocin-related genes, termed dbd131, 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.

*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 plu 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 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-peiA also confers immunity

In the constructs obtained above, the putative immunity gene peiA was cloned to cotranscribe with penA. To test whether the expression of peiA 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 peiA, 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 peiA 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 pediococcal-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 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 penIR; 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 penIR 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 (penIR) 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).
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. bifermentans (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.](image)
itself was found to be a poor producer, producing only residual amounts of bacteriocin as compared with other producers of the pediococcal-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, pen\textsubscript{Ig}, 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 pen\textsubscript{Ig} and the genes pen\textsubscript{KR} 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 pen\textsubscript{Ig} 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 pen\textsubscript{T} (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 (pen\textsubscript{IKR}), produced bacteriocin at high levels comparable to the \textit{Lb. sakei} clone B316, the latter displaying the highest bacteriocin activity amongst the \textit{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 \textit{Lb. sakei} 5 (Vaughan et al., 2003). The stx locus, which is composed of two bacteriocin operons (sak\textsubscript{T}, T\textsubscript{DP}, and sakXL\textsubscript{Y}), one regulatory unit (stxKRP) and the ABC-transporter gene stx\textsubscript{T} (without its cognate accessory partner), triggered sakacin X and sakacin T production when it was transferred into the recently sequenced \textit{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 \textit{P. pentosaceus} ATCC 25745, might be needed for pen\textsubscript{T} 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 \textit{P. pentosaceus} ATCC 25745 and in the pen\textsubscript{IR} or pen\textsubscript{FK}-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 pen\textsubscript{R} and sap\textsubscript{R} (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 \textit{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 \textit{Lb. sakei} contain remnants of bacteriocin-related (\textit{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 \textit{P. pentosaceus} ATCC 25745 was a consequence of a frameshift mutation in the inducer gene pen\textsubscript{I}. 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 \textit{Clostridium} and \textit{Listeria} species.
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