Expression of lactococcin A and pediocin PA-1 in heterologous hosts

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M.L. CHIKINDAS, K. VENEMA, A.M. LEDEBOER, G. VENEMA AND J. KOK. 1995. Pediocin PA-1 production, immunity and secretion are specified by a cluster of four genes in Pediococcus acidilactici PAC1.0. The production by, secretion of, and immunity to lactococcin A of Lactococcus lactis are also determined by four genes. Here, expression of the pediocin operon in Lactococcus lactis is reported, which could only be achieved by placing it under control of a lactococcal promoter. Expression of the lactococcin A operon in Pediococcus is also described: recombinant clones of Pediococcus were obtained that produced and secreted both active pediocin PA-1 and lactococcin A.

INTRODUCTION

Pediocins are bacteriocins produced by Pediococcus spp. They are effective against most lactic acid bacteria and various Gram-positive pathogens (Ray et al. 1989; Klaenhammer 1993). Pediocin PA-1 is produced by several Ped. acidilactici strains (Gonzales and Kunka 1987; Motlagh et al. 1992; Nieto-Lozano et al. 1992), and is known to be an effective inhibitor of food spoilage micro-organisms, including Listeria monocytogenes (Pucci et al. 1988; Nielsen et al. 1990). The pediocin PA-1 operon of Ped. acidilactici PAC1.0 (consisting of the genes pedA, pedB, pedC and pedD) was cloned and analysed in Escherichia coli and Pediococcus (Marugg et al. 1992; Venema et al. 1995). The pedA gene encodes the pediocin PA-1 precursor, pedB determines immunity to pediocin, pedC specifies a 174-amino acid membrane-bound protein which is required for pediocin secretion, while the pedD product is the leader peptidase and belongs to the ATP-dependent translocators (Marugg et al. 1992; Venema et al. 1995). Although the transcription and translation elements of the ped operon are similar to the consensus lactococcal gene expression signals, no pediocin production was observed when the operon was cloned in Lactococcus under control of its own promoter (unpublished observation).

Pediococcus is used in meat fermentations. The activity spectrum of pediocin PA-1 makes it a useful molecule to be employed in dairy fermentations. To circumvent the need to purify pediocin PA-1 for application in dairy industry, the authors decided to express pediocin in dairy lactic acid bacteria.

Lactococcin A is a narrow specificity bacteriocin produced by several strains of Lactococcus lactis (Van Belkum et al. 1989; Holo et al. 1991; Stoddard et al. 1992). Upstream of the structural and immunity genes, lenA and lecA, two genes are located that are implicated in lactococcin A maturation and secretion (Stoddard et al. 1992).

Here the expression of the pediocin operon in L. lactis is reported. Also, the lactococcin A operon has been expressed in the wild-type, pediocin PA-1 producing Ped. acidilactici PAC1.0. The resulting Pediococcus strain produced and secreted active forms of the two bacteriocins and, thus, had a wider spectrum of inhibition.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. Pediococcus was grown in MRS broth (Difco Laboratories, Detroit, MI) at 37°C without aeration, or on MRS agar plates. Escherichia coli was grown in TY broth (Rottlander and Trautner 1970) or on TY agar. For growth of L. lactis, M17 broth (Terzaghi and Sandine 1975) or M17 agar were used. MRS broth and agar were used for determination of pediocin production by all recombinant strains. Selective antibiotic concentrations were as follows: 250 μg ml⁻¹ of ampicillin, 100 μg ml⁻¹ of erythromycin and 20 μg ml⁻¹ of kanamycin for E. coli, and 5 μg ml⁻¹ of...
Table 1 Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pediococcus acidilactici</em> PAC1.0</td>
<td>Contains 9.4-kbp plasmid pSRQ11, carrying the pediocin operon</td>
<td>Gonzales and Kunka 1987</td>
</tr>
<tr>
<td>acidilactici PAC1.14</td>
<td>Plasmid-free derivative of PAC1.0</td>
<td>Gonzales and Kunka 1987</td>
</tr>
<tr>
<td><em>pentosaceus</em> PPE1.2</td>
<td>Plasmid-free pediocin-sensitive indicator</td>
<td>Gonzales and Kunka 1983</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM101</td>
<td>supE, thi, Δ(lac-proAB), [F', irtD36, proAB, lacPZ ΔM15]</td>
<td>Yanisch-Perron et al. 1985</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> IL1403</td>
<td>Plasmid-free strain, lactococcin A indicator</td>
<td>Chopin et al. 1984</td>
</tr>
<tr>
<td>lactis LL108</td>
<td>MG1363 derivative carrying the repA gene of pWV01 on its chromosome</td>
<td>K. Leenhouts, unpublished</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pKV4</td>
<td>Em', pAMβ-1 derivative, carrying the lactococcin A operon and lcnC and lcnD</td>
<td>K. Venema, unpublished</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap', pBR322 derivative</td>
<td>Yanisch-Perron et al. 1985</td>
</tr>
<tr>
<td>pUK21</td>
<td>Km', pBR322 derivative</td>
<td>Vieira and Messing 1991</td>
</tr>
<tr>
<td>pMG36e</td>
<td>Em', pWV01 derivative, gene expression vector</td>
<td>van de Guchte et al. 1989</td>
</tr>
<tr>
<td>pSRQ220</td>
<td>Ap', pBR322-derived plasmid carrying 5.6-kbp SalI–EcoRI fragment encompassing the pediocin PA-1 operon</td>
<td>Marugg et al. 1992</td>
</tr>
<tr>
<td>pMC114</td>
<td>Ap', pUC19 derivative carrying the 5.6-kbp SalI–EcoRI fragment encompassing the pediocin PA-1 operon</td>
<td>this work</td>
</tr>
<tr>
<td>pMC115</td>
<td>Ap', pUC19 derivative carrying the promoterless pediocin operon</td>
<td>this work</td>
</tr>
<tr>
<td>pMC116</td>
<td>Km', pUK21 derivative carrying the promoterless pediocin operon</td>
<td>this work</td>
</tr>
<tr>
<td>pMC117</td>
<td>Em', pMG36e derivative carrying the pediocin operon under control of P32</td>
<td>this work</td>
</tr>
<tr>
<td>pMC121</td>
<td>Ap', pUC19 derivative containing a synthetic DNA fragment encoding start codon and leader of pediocin PA-1</td>
<td>this work</td>
</tr>
</tbody>
</table>

* Ap, Ampicillin; Em, erythromycin; Km, kanamycin; r, resistance.

Erythromycin for *Pediococcus* and *L. lactis*. All antibiotics were purchased from Sigma Chemical Co. (St Louis, MO).

**Molecular cloning**

Plasmid DNA was isolated from *E. coli* as described by Birnboim and Doly (1979). A modification of this method (Leenhouts et al. 1989) was used for isolation of plasmids from *Pediococcus* and *L. lactis*. All DNA modifying enzymes were purchased from Boehringer GmbH (Mannheim, Germany) and were used as recommended by the supplier. All DNA manipulations were carried out according to procedures described by Maniatis et al. (1982).

Electroporation was used to transform *E. coli* (Dower et al. 1988) and *L. lactis* (Holo and Nes 1989). Electroporation was used to transform *E. coli* (Dower et al. 1988) and *L. lactis* (Holo and Nes 1989). Electroporation
of *Pediococcus* was conducted by a modification of the method of Holo and Nes (1989). In short, an overnight culture of *Pediococcus* in MRS broth with 0·5 mol 1⁻¹ sucrose was diluted in fresh MRS broth containing 0·5 mol 1⁻¹ sucrose and 3·5% glycine and grown to an optical density of 0·2 at 600 nm. Cells were harvested by centrifugation, washed as described in the original protocol, and resuspended in electroporation buffer (0·5 mol 1⁻¹ sucrose, 1 mmol 1⁻¹ MgCl₂, 1 mmol 1⁻¹ KPi, pH 7·0). Electroporation was conducted according to the original procedure (Holo and Nes 1989). Cells were placed at 37°C for 2 h in recovery medium (MRS broth containing 20 mmol 1⁻¹ MgCl₂ and 2 mmol 1⁻¹ CaCl₂) and subsequently plated on MRS agar plates supplemented with a selective concentration of the appropriate antibiotic. The Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, CA) was used in all electroporation experiments.

Pediocin assay

Screening of *Pediococcus* and *L. lactis* transformants for pediocin PA-1 production (in arbitrary units, AU) was performed as described by Henderson et al. (1992) using *Ped. pentosaceus* as the indicator strain. Lactococcin A production was tested with *L. lactis* IL1403 as indicator strain as described before (Venema et al. 1993).

RESULTS

Construction of a plasmid in which the ped operon is under control of a lactococcal promoter

To express the entire ped operon under control of a strong lactococcal promoter, the following strategy was employed (Fig. 1). The non-coding upstream region of the operon (including the putative promoter [Marugg et al. 1992]) was replaced by a synthetic DNA fragment with convenient flanking restriction enzyme sites. This DNA fragment was synthesized such that it carried the ribosome binding site of ORF32, its start codon (ATG), and the DNA region encoding the leader sequence of pediocin PA-1 until the unique B*al* site (Marugg et al. 1992) (Fig. 2). It has been demonstrated previously, that close proximity of stop and start codons can lead to an increase of expression of certain genes via translational coupling (Van de Guchte et al. 1991). This principle was employed in the work presented here, by creating a stop codon immediately upstream of the start codon by a G to T change (Fig. 2). The synthetic 82-bp DNA fragment was cloned in pUC19 using the *Pst*1- and *Eco*RI sites of fragment and vector, which resulted in plasmid pMC121. There are two recognition sites for *B*al* in pSRQ220 (one of which is in pedA). To be able to make use of the *B*al* site in pedA, the 5-6-kbp *Sal*I-*Eco*RI DNA fragment of pSRQ220 was cloned in the corresponding sites of pUC19. In this construct, pMC114, the *Sal*I site in pedA is unique. The 1109-bp *Sal*I-*B*al*I DNA fragment of pMC114 was replaced by the synthetic DNA fragment of pMC121 to obtain plasmid pMC115 (Fig. 1). In order to obtain convenient cloning sites, the *Sal*I-*Nde*I DNA fragment of pMC115 was cloned in the corresponding sites of pUK21, creating pMC116. This plasmid was cleaved with *Sma*I and *Not*I, and the DNA fragment carrying the ped operon was cloned in the *Sma*I and *Eae*I sites of pMG36e, giving pMC117 (Fig. 1). All cloning steps were carried out in *E. coli* with the exception of the latter which was done in *L. lactis*.

Expression of the pediocin operon in *Pediococcus* under control of the lactococcal promoter P32

Plasmid pMC117 (Fig. 1) was introduced into *Ped. acidilactici* PAC1.14 (a plasmid-free derivative of PAC1.0), and the pediocin-sensitive *Ped. pentosaceus* strain PPE1.2, and tested for pediocin production (Table 2). The amounts of pediocin produced by cells containing pMC117 was two- to fourfold higher than that in the wild-type pediocin producer *Ped. acidilactici* PAC1.0 (pMG36e), in which pediocin is produced from the endogenous plasmid pSRQ11.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pediocin PA-1 activity (AU 1⁻¹ x 10⁶)†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ped. acidilactici</em> PAC1.0 (pMG36e)</td>
<td>6·4</td>
</tr>
<tr>
<td><em>acidilactici</em> PAC1.14 (pMC117)</td>
<td>12·8</td>
</tr>
<tr>
<td><em>pentosaceus</em> PPE1.2 (pMC117)</td>
<td>25·6</td>
</tr>
<tr>
<td><em>L. lactis</em> LL108 (pMC117)</td>
<td>3·2</td>
</tr>
</tbody>
</table>

† Arbitrary units (AU) of activity were determined by spot testing of supernatant fluids of cultures grown overnight in MRS at 37°C without aeration.
Fig. 1 Constructions of a plasmid expressing pediocin PA-1 from lactococcal gene expression signals. For detailed description of the strategy, see text. Only relevant restriction enzyme sites are given. SF, Synthetic fragment; Km\(^\text{r}\), kanamycin resistance marker; Em\(^\text{r}\), erythromycin resistance marker; Ap\(^\text{r}\), ampicillin resistance marker; ori, origin of replication; lacZ\(^\text{r}\), gene encoding LacZ \(\alpha\)-peptide; P32, strong lactococcal promoter (Van der Vossen et al. 1987); T, terminator; pedA, pediocin PA-1 structural gene; pedB, immunity gene; pedC and pedD, genes encoding secretion/maturation machinery; repA, gene encoding pWV01 plasmid replication protein; MCS, multiple-cloning site; ‘tet, truncated tetracycline-resistance marker
PAC1.0, the wild-type pediocin PA-1 producer. PAC1.0 was transformed with pKV4, a plasmid containing the lactococcin A structural and immunity genes and the two genes lcnC and lcnD necessary for maturation and secretion of the bacteriocin (K. Venema, unpublished results). Pediococcus acidilactici PAC1.0 (pKV4) produces normal amounts of pediocin PA-1, as was assayed using the indicator strain Ped. pentosaceus PPE1.2 (not shown). As L. lactis is insensitive to pediocin PA-1, Ped. acidilactici PAC1.0 does not inhibit the lactococcin A indicator strain L. lactis IL1403. Pediococcus acidilactici (pKV4) does inhibit strain IL1403. Table 3 shows that the lcnA operon on pKV4 is active in Ped. acidilactici under control of its own gene regulatory elements and produces an amount of lactococcin A approximately twofold lower than that of L. lactis (pKV4).

**DISCUSSION**

As reported previously, pediocin PA-1 production in Ped. acidilactici PAC1.0 is determined by an operon of four genes located on the 9.4-kbp plasmid pSRQ11 (Gonzales and Kunka 1987; Marugg et al. 1992; Venema et al. 1995). Here, it is shown that pediocin PA-1 can be produced in L. lactis. The putative promoter and the RBSs upstream of the genes in the ped operon are very similar to gene regulatory elements of lactococci (Van der Vossen et al. 1987; Marugg et al. 1992).

Nevertheless, when L. lactis carried the ped operon with its own promoter, pediocin production was not detectable. Pediocin production in L. lactis was obtained when the ped operon was cloned behind a lactococcal promoter. However, it cannot be concluded that the exchange of the original promoter for promoter P32 alone enabled L. lactis to produce pediocin. The synthetic DNA fragment encompassed the P32 promoter region and an RBS of lactococcal origin fused at a proper distance from the first gene of the ped operon, pedA. It is possible that this combination of lactococcal promoter and RBS resulted in the ability of L. lactis to produce pediocin.

Pediocin PA-1 is known as a bacteriocin with a wide spectrum of inhibition (Ray and Daeschel 1992). Pediococcus and Lactococcus strains are widely employed in the production of different foods (McKay and Baldwin 1990; Ray and Daeschel 1992). While pediococci are mainly used in meat fermentations, lactococci are primarily employed in dairy industry. The construction of a Pediococcus strain producing lactococcin A in addition to pediocin PA-1 could widen the application of this strain in food fermentations. Similarly, the production by L. lactis of pediocin should enable the production of this bacteriocin during milk fermentation processes. The availability of food grade manipulation techniques (Leenhouts 1994) opens up the

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Table 3 Lactococcin A production by Lactococcus and Pediococcus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Lactococcin A activity (AU l⁻¹) x 10⁶†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis IL1403 (pKV4)</td>
<td>6.4</td>
</tr>
<tr>
<td>Ped. acidilactici PAC1.0 (pKV4)</td>
<td>3.2</td>
</tr>
</tbody>
</table>

† Arbitrary units (AU) of activity were determined by spot testing of supernatant fluids of cultures grown to early stationary phase in MRS at 37°C (for Pediococcus) or in GM17 at 30°C (for L. lactis).

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possibility of making *L. lactis* and *Pediococcus* strains that produce lactococcin A and pediocin PA-1 in a food grade way for use in dairy or meat fermentations.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


lactococcin B, a thiol-activated bacteriocin from Lactococcus lactis. Applied and Environmental Microbiology 59, 1041–1048.

