CHAPTER VIII

Functional analysis of the pediocin operon of *Pediococcus acidilactici* PAC1.0:
PedB is the immunity protein and PedD is the precursor processing enzyme.

Koen Venema, Jan Kok, Joey D. Marugg, Marjolein Y. Toonen,
Aat M. Ledeboer, Gerard Venema, and Michael L. Chikindas.

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CHAPTER VIII

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**SUMMARY**

The pediocin PA-1 bacteriocin operon of *Pediococcus acidilactici* PAC1.0 encompasses four genes, *pedA*, *pedB*, *pedC* and *pedD*. Transcription of the operon results in the formation of two overlapping transcripts, probably originating from a single promoter upstream of *pedA*. The major transcript comprises *pedA*, *pedB*, and *pedC*, while a minor transcript encompasses all of these genes and *pedD*. By deletion analysis and overexpression of *pedB* in *P. pentosaceus* we demonstrate that this gene encodes the pediocin PA-1 immunity protein. Prepediocin is active in *E. coli* and when *pedA* was expressed concomitantly with *pedD* both the precursor and the mature form of pediocin were observed intracellularly. Extracellular pediocin was only detected if both *pedC* and *pedD* were present. The N-terminal domains of PedD and a subgroup of bacteriocin ABC-transporters is conserved. Expression of only this domain of PedD in cells producing prepediocin was sufficient for prepediocin processing. From these results we conclude that both PedC and PedD are essential for pediocin transport, and that PedD is capable of processing prepediocin.

**INTRODUCTION**

*Pediococcus* belongs to the homofermentative Gram-positive lactic acid bacteria of which many strains produce bacteriocins (Klaenhammer, 1993). Bacteriocins produced by *Pediococcus* spp., designated pediocins, are effective against most lactic acid bacteria and various Gram-positive pathogens and are known to be inhibitors of food spoilage microorganisms, including *Listeria monocytogenes* (Pucci et al., 1988; Nielsen et al., 1990; Klaenhammer, 1993). Pediocin PA-1 is produced by several *P. acidilactici* strains and is identical to pediocin AcH (Henderson et al., 1992; Motlagh et al., 1994).

Recently, genes involved in the production of pediocin PA-1 and AcH were cloned and sequenced (Marugg et al., 1992; Motlagh et al., 1994). The genes required for pediocin PA-1 production are organized in an operon on the 9.4-kbp plasmid pSRQ11 in *P. acidilactici* PAC1.0 (Marugg et al., 1992). The *ped* operon, comprising the genes *pedA*, *pedB*, *pedC*, and *pedD*, was cloned in pBR322. Analyses in *E. coli* showed that *pedA* and *pedD* are essential for pediocin PA-1 production (Marugg et al., 1992). *pedA* encodes the precursor of pediocin PA-1, while the *pedD* gene product belongs to the group of ATP-dependent translocators (Fath and Kolter, 1993) and, thus, was implicated in transport of pediocin across the cytoplasmic membrane. The *pedB* product, a small positively charged protein of 112 amino acids, was not essential for pediocin production. Its role as well as that of *pedC* was not further analyzed.

Recently, Bukhtiyarova et al. (1994) analyzed the pediocin AcH gene cluster which is identical to the pediocin PA-1 operon. They concluded that only *papA* (the pediocin structural gene) and *papD* (the ABC-homologue) are required for production of active extracellular pediocin in *E. coli*. The function of the gene products could not be established in *Pediococcus* due to plasmid instability.

As pediocin PA-1 production and secretion has only been studied in *E. coli* and since this species is insensitive to pediocin, the immunity gene could not be identified. Most bacteriocins from lactic acid bacteria studied so far have their immunity determinants closely linked to the bacteriocin structural genes (Klaenhammer, 1993), making *pedB* and *pedC* the most likely candidates in the *ped* operon to specify immunity. However, immunity functions have also been reported to be
encoded by the chromosome in *P. acidilactici* PAC1.0 (Gonzalez and Kunka, 1987).

Pediocin PA-1 is translated as a 62-amino-acid precursor (Marugg *et al.*, 1992). This precursor is cleaved in vivo behind two glycine residues of the leader peptide, resulting in the formation of the mature pediocin PA-1 molecule of 44 amino acids (Henderson *et al.*, 1992). The processing site is conserved in several bacteriocins (Klaenhammer, 1993; Kok *et al.*, 1993), but the identity of the specific leader peptidase is still unknown.

Here we report an extensive analysis of the pediocin PA-1 operon in both *Pediococcus* and *E.coli* and identify both the pediocin immunity gene and the leader peptidase.

**RESULTS**

**Two transcripts are produced from the ped operon.**

Total RNA samples of *P. acidilactici* PAC1.0, as well as of *E. coli* harboring pSRQ220, which contains the entire ped operon, were hybridized to 5′-end labelled single-stranded antisense oligonucleotides specific for different regions of the ped operon (Marugg *et al.*, 1992) (Fig. 1). Two transcripts were detected in RNA preparations from both bacterial species with a probe complementary to pedA (data not shown). The most abundant transcript had a size of approximately 1.2 kb, while the size of a minor transcript was approximately 3.5 kb. With a pedC-specific probe both the 1.2-kb and the 3.5-kb transcript were detected in RNA preparations of *E. coli* (pSRQ220), while pedD-specific probes only detected the 3.5-kb transcript (data not shown). With single stranded probes complementary to the regions upstream or downstream of the ped operon no transcripts were detected (data not shown). The sizes and positions of the two transcripts are shown in Fig. 1.

**Analysis of the ped operon in Pediococcus.**

When pMC220, containing the entire ped operon (Fig. 1), was introduced in the non-producing, pediocin-sensitive *P. pentosaceus* strain PPE1.2, transformants produced pediocin PA-1 to which they were insensitive (results not shown), indicating that the insert encodes all information required for pediocin production, secretion, and immunity.

When *P. pentosaceus* PPE1.2 carried pMC5207 or pMC5205 in which pedA or pedD were disrupted, respectively, no pediocin activity was detectable in a halo assay, indicating that, as was observed in *E. coli*, pedA and pedD are essential for pediocin activity. To determine the role of pedC in pediocin production, we removed it from the ped operon (pMC118) or introduced a frameshift mutation (pMC119). *P. pentosaceus* PPE1.2 containing either pMC118 or pMC119 was not able to produce active pediocin in a plate assay, demonstrating that pedC is required for extracellular pediocin PA-1 activity (see Fig. 1).

When PPE1.2 was transformed with pMC5206, carrying ped with a mutated pedB gene, transformants were obtained, but they grew very poorly. After two transfers into fresh medium the cells started to grow normally, with normal pediocin production. When PPE1.2(pMC5206) was tested in agar spot tests for sensitivity to the bacteriocin, its immunity towards pediocin PA-1 was affected but not completely abolished: PPE1.2(pMC5206) was more sensitive to the bacteriocin than PPE1.2(pMC220), but was still insensitive to the low (< 6AU) concentrations of pediocin to which PPE1.2 is sensitive. To obtain a decisive answer on its role in immunity, pedB was cloned under control of the strong lactococcal promoter P32 (pMC113dc; Fig. 1). PPE1.2(pMC113dc) withstood high (>160 AU) concentrations of pediocin. The level of insensitivity was even higher than that of *P. acidilactici* PAC1.0. On the basis of these results we conclude that pedB encodes the immunity protein against pediocin PA-1.

**Overexpression of ped or pedD leads to increased bacteriocin production in Pediococcus.**

The entire ped operon was placed under control of promoter P32 by replacing the ped promoter region and pedA translation signals by those of P32/ORF32 (Chikindas *et al.*, 1995). The resulting plasmid pMC117 (Fig. 1) was introduced into *P. acidilactici* PAC1.0, its plasmid-free derivative *P. acidilactici* PAC1.14, and *P. pentosaceus* PPE1.2, and the strains were tested for pediocin production (Table 1). Cells containing pMC117 produced 2- to 4-fold more pediocin than PAC1.0(pMG36E) in which pediocin is produced from the endogenous plasmid pSRQ11. In pMC120 pedD is under control of promoter P32 (Fig. 1). The extracellular pediocin activity produced by PAC1.0(pMC120) was 2-fold higher than that by PAC1.0 (Table 1).
Figure 1: Genetic analysis of the pediocin PA-1 operon in _Pediococcus_. The position and orientation of the genes encompassing the ped operon is indicated by open arrows. Only relevant restriction enzyme sites are indicated. The construction of the indicated plasmids is detailed in the text. Plasmids between brackets: pBR322 derivatives that do not replicate in _Pediococcus_. All other plasmids replicate and were tested in _Pediococcus_. Thin lines: DNA fragments contained in the various plasmids used. The asterisks indicate the positions of point mutations. Halo: ability of _P. pentosaceus_ PPE1.2 carrying the indicated plasmids to produce active, secreted pediocin PA-1 in an overlay of _P. pentosaceus_ PPE1.2; +: halo is present, -: no halo. Insensitivity to pediocin PA-1 of _Pediococcus_ PPE1.2 containing the recombinant constructs is indicated by: (+), not sensitive; (-), sensitive; ND, not determined; P: promoter; T: terminator; P32: strong lactococcal promoter (Van der Vossen et al., 1987); T7: phage T7 RNA polymerase promoter (Studier and Moffatt, 1986); thick wavy arrow: size and location of major RNA transcript detected in the Northern hybridization analysis; thin wavy arrow: size and location of minor RNA transcript.
Table 1. Pediocin PA-1 production by *Pediococcus*.

<table>
<thead>
<tr>
<th>strain</th>
<th>pediocin PA-1 activity AU/l</th>
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<tbody>
<tr>
<td><em>P. acidilactici</em> PAC1.0 (pMG36E)</td>
<td>$6.40 \times 10^6$</td>
</tr>
<tr>
<td><em>P. acidilactici</em> PAC1.0 (pMC117)</td>
<td>$1.28 \times 10^7$</td>
</tr>
<tr>
<td><em>P. acidilactici</em> PAC1.0 (pMC120)</td>
<td>$1.28 \times 10^7$</td>
</tr>
<tr>
<td><em>P. pentosaceus</em> PPE1.2 (pMC117)</td>
<td>$2.56 \times 10^7$</td>
</tr>
<tr>
<td><em>P. acidilactici</em> PAC1.14 (pMC117)</td>
<td>$1.28 \times 10^7$</td>
</tr>
</tbody>
</table>

* Arbitrary units (AU) of activity were determined by spot testing supernatants of overnight cultures grown in MRS at $30^\circ C$ without aeration, on lawns of the indicator *Pediococcus pentosaceus* PPE1.2.

Figure 2: Bacteriocin activity specified by *ped* operon mutants. Supernatants (1 µl) and cell-free extracts (1 µl of 35 µg protein/ml) were subjected to tricine-SDS-PAA (16%) gel electrophoresis. Pediocin activity was visualized by the overlay technique using *Pediococcus pentosaceus* PPE1.2 as the indicator strain. Lanes: 1, supernatant of *P. acidilactici* PAC1.0; 2, 3, and 4, lysates of *E.coli* JM101 containing pSRQ220, pUR5206, and pUR5205, respectively; 5, 6, and 7, supernatants of *P. pentosaceus* PPE1.2 carrying pMC220, pMC5206, and pMC5205, respectively; 8 and 9, lysates of *E. coli* BL21/DE3(pMC122) without and with induction of the T7 promoter, respectively; 10, molecular size standard (Rainbow protein molecular weight marker; Amersham International, Amersham, England) of which the sizes (in kilodaltons) are shown on the right.
Expression of pedA alone is sufficient for pediocin activity in E. coli.

In plasmid pMC122 pedA is under inducible control of the strong T7 promoter (Studier and Moffat, 1986) (Fig. 1). Although no pediocin activity could be detected in the supernatant of IPTG-induced E.coli(pMC122) (see below), activity was observed in lysates of these cells (Fig. 2, lane 9). Pediocin was not present in lysates of non-induced E.coli(pMC122) (Fig. 2, lane 8). The electrophoretic mobility of pediocin produced by E. coli(pMC122) was lower than that of mature pediocin PA-1 present in E.coli(pSRQ220) lysates, and that in culture supernatants of P. acidilactici PAC1.0 and P. pentosaceus PPE1.2(pMC220) (Fig. 2, compare lane 9 with lanes 2,1 and 5, respectively), suggesting that it is the non-processed form of the bacteriocin. In extracts of E.coli(pUR5205) or Pediococcus (pMC5205), both carrying an intact pedA but mutated pedD gene, neither pediocin nor its precursor were observed (Fig. 2, lanes 4 and 7), presumably because the ped-specific promoter driving pedA expression on these plasmids is too weak.

PedD is the processing enzyme for pediocin PA-1.

As β-galactosidase was not detectable in the supernatant of E.coli BL21/DE3(pSRQ220) (data not shown), extracellular pediocin activity in E. coli is not caused by lysis, but a consequence of the action of PedC and/or PedD. Moreover, PedC and/or PedD are essential for pediocin maturation. To examine which of the two proteins was responsible for processing, their genes were introduced separately in E.coli BL21/DE3(pMC122) producing pediocin. Figure 3I, stab A shows that, as expected, strain BL21/DE3(pSRQ220), containing the complete ped operon, secretes mature pediocin PA-1. BL21/DE3(pMC122) and BL21/DE3(pMC122, pMC113) do not secrete active pediocin (Fig. 3I, stab B and C, respectively). However, pediocin activity is present in the cell lysates of these strains (Fig. 3II, lane b and c). Also, BL21/DE3(pMC122, pUKpedD) does not secrete pediocin activity (Fig. 3II, stab D). The cell lysate of this strain contains, apart from pediocin, also an activity at the position of mature pediocin (Fig. 3II, lane d). These results convincingly show that PedD is involved in the processing of pediocin to its active form.

Coomassie staining of a tricine-SDS-PAA gel containing lysates from E.coli BL21/DE3(pMC122) and BL21/DE3(pMC122, pUKpedD) revealed that only a small amount of prepediocin is converted into the active form (Fig. 3III, compare lanes 1 and 2); whereas no band of stained protein is present at the position of mature pediocin (m), the band of prepediocin (p) is clearly visible.

PedD shows homology with other bacteriocin ABC-transporters. When the amino acid sequences (excluding the ATP-binding boxes) of a subgroup of these transporters, namely those that are involved in the secretion of bacteriocins containing the double glycine leader motif, were aligned, most of the homology was found in the N-terminal first 190 amino acids (Fig. 4). DNA fragments encoding the N-terminal 172 or 192 amino acids were amplified by PCR and cloned behind promoter P32 (pD172 and pD192; Fig. 1) When introduced in the prepediocin producing E.coli BL21/DE3 (pMC122) the N-terminal fragments of PedD specified by pD172 and pD192 were capable of processing prepediocin (Fig. 3III, lanes e and f, respectively).

DISCUSSION

Two overlapping transcripts are initiated from a DNA region upstream of the ped operon previously postulated to carry the promoter. Their 3'-ends would agree with two possible terminator structures identified previously (Marugg et al., 1992). Deletion of the downstream region of ped did not affect pediocin production in E.coli (pMC220dr in Fig. 1). Surprisingly, deletion of the 724-bp SalI-EcoRV DNA fragment located 352 bp upstream of pedA (pMC220dl) resulted in complete loss of pediocin production in P.pentosaceus (data not shown), suggesting that the upstream region carries some as yet unidentified control element(s). Such elements are often present upstream of promoters in several other bacterial genera (Klier et al. 1992). As ped production was also abolished in E.coli(pMC220dl), it is conceivable that one of the ped gene products regulates expression of the operon in both microorganisms by interacting with this upstream element.

As P.pentosaceus PPE1.2 carrying pedB was insensitive to pediocin PA-1, we conclude that pedB encodes the immunity protein against the bacteriocin. The observation that PPE1.2(pMC113dc) has an even higher level of insensitivity than PAC1.0 is caused
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Figure 3: Analysis of *pedC* and *pedD* involvement in pediocin transport and processing.

I: Halo formation on the indicator strain *P. pentosaceus* PPE1.2 by *E. coli* containing the plasmids pSRQ220 (A), pMC122 (B), pMC122 + pMC113 (C), and pMC122 + pUKpedD (D), respectively.

II: Tricine SDS-PAA-gel (16%) overlaid with the same indicator strain. Lanes a through d contain lysates of the corresponding *E. coli* clones in part I of the figure; lanes e and f contain lysates of *E. coli* containing the plasmids pMC122 + pD172 and pMC122 + pD192, respectively; lane m: Molecular weight marker, the sizes of which are indicated in the right margin.

III: Coomassie stained tricine-SDS-PAA gel (16%). Lanes 1, 2 and 3 contain lysates of *E. coli* carrying the plasmids pMC122, pMC122 + pUKpedD, and pT712, respectively. Lane m: Molecular weight marker, the sizes of which are indicated in the right margin. p: position of prepediocin; m: position of mature pediocin.
by the fact that the copy number of pMC113dc is higher compared to pSRQ11 (unpublished results) and that pedB expression on pMC113dc is higher due to promoter P32. P. pentosaceus PPE1.2(pMC5206) with a mutated pedB is still insensitive to low pediocin concentrations. As has been observed previously for lactococcin (Van Belkum et al., 1992) pediocin production can lead to pediocin tolerance of the producer cells. This insensitivity is different from the immunity specified by pedB. This explains why, although pSRQ11 was eliminated from P. acidilactici PAC1.0, the resulting strain PAC1.14 was still insensitive to pediocin (Gonzalez and Kunka, 1987). Apparently, PAC1.0 had acquired tolerance, possibly by a spontaneous chromosomal mutation (e.g. leading to loss of the receptor for pediocin).

The present study shows that PedC is essential for pediocin PA-1 secretion, both in Pediococcus and in E. coli. This is in contrast to the results obtained by Bukhtiyarova et al. (1994) who concluded that in E. coli only papA and papD are required for extracellular pediocin activity. However, these authors have not examined whether the extracellular activity resulted from lysis of PedD carrying cells. Although PedC and HlyD differ by approximately 300 amino acids the two proteins are structurally similar, in the sense that both molecules contain one N-terminal transmembrane helix (Schülein et al., 1992, E. Emond, pers. comm.). Analogous to the presumed function of HlyD, which is thought to span the two membranes of E. coli to form a "channel" for hemolysin A secretion (Schülein et al., 1992), we speculate that PedC is involved in channel formation. It is of interest to note that the product of a partial open reading frame preceding the lactacin F structural gene (Fremaux et al., 1993), and LcnD, which is essential for the production of lactococcins (Stoddard et al., 1992), are also structurally related to PedC, suggesting that a common mechanism exists for secretion of these small bacteriocin molecules.

The last gene of the ped operon, pedD, is only poorly transcribed. Pediocin activity could be enhanced by placing ped behind a strong promoter, or by
overexpressing pedD alone, indicating that production of PedD is a limiting factor. As PedD is involved in bacteriocin secretion, we presume that the increase in PedD production leads to a more efficient maturation and/or translocation of pediocin.

Here we define a subgroup of ABC-transporters that contain a conserved N-terminal domain (Fig. 4). All of these transporters are assumed to be involved in the secretion of certain bacteriocins, namely those containing the double glycine leader motif. These include class II bacteriocins of Gram positive and Gram negative bacteria, as well as some of the lantibiotics (Havarstein et al., 1994). The N-terminal domain of PedD is located in the cytoplasm (E. Emond, pers. comm.). In this study we convincingly demonstrate that this intracellular domain is responsible for processing of prepediocin. This process involves cleavage of the leader sequence behind the glycine doublet at the processing site (Henderson et al., 1992). By analogy, we presume that the other examples of this subgroup of bacteriocin ABC-transporters are also involved in proteolytic maturation of their respective bacteriocins. In the case of pediocin PA-1 studied here, this proteolytic cleavage leads to a huge increase in activity, as a very small amount of mature pediocin produces a halo similar in size to that caused by a clearly visible amount of prepediocin (Fig. 3III). The fact that the N-terminal domain of PedD is still capable of processing prepediocin and that under these circumstances the mature pediocin is found intracellularly demonstrates that processing can be uncoupled from pediocin secretion. However, a considerable quantity of unprocessed prepediocin remained in the cell under the conditions employed. Therefore, it would seem that processing is more efficient when it proceeds concomitantly with export.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and media.**

*Pediococcus acidilactic* PAC1.0, *P.acidilactic* PAC1.14 and *P.pentosaceus* PPE1.2 (Marugg et al., 1992) were grown in MRS broth (Difco Laboratories, Detroit, Mich.) at 37°C without aeration, or on MRS agar plates. *E. coli* 294 (Backman et al., 1976), *E. coli* JM101 (Yanisch-Perron et al., 1985) and *E. coli* BL21/DE3 (Studier and Moffat, 1986) were grown either in TY broth (Rottlander and Trautner, 1970) or on TY agar. For growth (30°C, without aeration) of *L. lactis* IL1403 (Chopin et al., 1984), M17 broth (Terzaghi and Sandine, 1975) or M17 agar were used. MRS broth and MRS agar were used for determination of pediocin production. Selective antibiotic concentrations were as follows: 250 µg/ml of ampicillin, 100 µg/ml of erythromycin and 20 µg/ml of kanamycin for *E. coli*, and 5 µg/ml of 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 in Maniatis et al. (1982). A modification of this procedure (Leenhouts et al., 1989) was used for isolation of plasmid DNA from *Pediococcus* and *L. lactis*. All enzymes were purchased from Boehringer GmbH (Mannheim, Germany) and were used as recommended by the supplier. All manipulations with DNA were carried out essentially as described by Maniatis et al. (1982). Electroporation was used to transform *E. coli* (Dower et al., 1988) and *L. lactis* (Holo and Nes, 1989). Electrottransformation of *Pediococcus* was conducted by the method of Holo and Nes (1989) with the following modifications. An overnight culture of *Pediococcus* in MRS broth with 0.5 M sucrose was diluted in fresh MRS broth containing 0.5 M sucrose and 3.5% glycine, and grown to an optical density of 0.2 at 600 nm. Cells were harvested and washed as described and resuspended in electroporation buffer (0.5 M sucrose, 1 mM MgCl₂, 1 mM KPi pH 7.0). After electroporation using a BioRad gene pulser (Bio-Rad Laboratories, Richmond, Calif.), the cells were placed for 2 hours at 37°C in MRS broth containing 20 mM MgCl₂ and 2 mM CaCl₂ and subsequently plated on selective MRS agar.

**Plasmid constructions.**

The construction of plasmids pMC115 and pMC117 (Fig. 1) has been described elsewhere (Chikindas et al., 1995).

The 5.6-kbp *EcoRI-SalI* fragments from plasmid pSRQ220 and those of the pSRQ220-derivatives pUR5205, pUR5206, and pUR5207 (Marugg et al., 1992), were cloned in the *EcoRI-SalI* sites of pIL253 (Simon and Chopin, 1988), which resulted in pMC220, pMC5205, pMC5206, and pMC5207, respectively (Fig. 1).

To clone pedB under control of the strong lactococcal promoter P32 in the expression vector pMG36E (Van de Guchte et al., 1989), the 2060-bp *NheI-XbaI* DNA
fragment of pSRQ220 was cloned into the XhoI site of pUC19 (Yanisch-Perron et al., 1985). The SalI-SalI DNA fragment of the resulting plasmid pMC112 was cloned into the SalI and SalI sites of pMG36E. From the resulting plasmid pMC113 (Fig. 1) the two Clal DNA fragments of 1076 bp and 790 bp were deleted. The resulting plasmid pMC113dc (Fig. 1) only carries pedB under control of promoter P32. All cloning steps involving pMG36E were carried out in L. lactis IL1403.

To obtain pMC118 (Fig. 1), lacking pedC, plasmid pMC117b (Fig. 1; a pMC117 derivative with a mutated BamHI site in the multiple cloning site) was cleaved with AhaI and XhoI, treated with Klenow enzyme, followed by self ligation. To obtain a frame shift in pedC, pMC117 was partially digested with ClaI and the Clal site in pedC was filled in with Klenow enzyme giving pMC119 (Fig. 1). pMC120, carrying only pedD, was constructed by digesting pMC117b with XhoI and Smal, flushing the cohesive ends with Klenow enzyme, followed by self-ligation (Fig. 1). The pedA gene was placed under control of the T7 promoter by cloning the 274-bp SalI-XhoI DNA fragment from pMC115 into the SalI and XbaI sites of pT712 (Tabor and Richardson, 1985) using E. coli BL21/DE3 as a host. This resulted in plasmid pMC122 (Fig. 1).

Plasmids pD172 and pD192 were obtained by cloning PCR products obtained with primers PedD1 and PedD2, or PedD1 and PedD3 behind P32 in pMG36CT, respectively (Fig. 1). PedD1, PedD2 and PedD3 are complementary to the region just upstream of the RBS of pedD, the region just upstream of the sequence encoding the first putative transmembrane sequence (TMS), and the region just downstream of the sequence encoding the first TMS, respectively. The sequences of the primers were as follows (the restriction endonuclease sites used for cloning are underlined): PedD1: 5'- GAGCTTACAGATCCTGGTTTTGCTTA- TG -3'; PedD2: 5'- AAGCTTACATTACCAATTTTTT- TTTG -3'; PedD3: 5'- AAGCTTACTGAAAGAAAT- ATGCACC -3'.

Plasmid pUKPedD, a pUK21 (Vieira and Messing, 1991) derivative containing pedD as an Asp718 fragment from pMC117, was a generous gift from E. Emond. Plasmid pMG36CT is a derivative of pMG36E, in which the erythromycin resistance marker has been replaced by a chloramphenicol resistance marker (lab collection).

Pediocin assay.

Screening of Pediococcus, and E. coli transformants for pediocin PA-1 production in media (in arbitrary units, AU) and on plates was performed as described by Henderson et al. (1992).

T7 RNA polymerase-directed expression of pediocin PA-1 in E. coli

pedA expression from pMC122 was obtained using the procedure of van Belkum et al. (1992). Briefly, cultures were induced with 0.4 mM IPTG at OD600 of approximately 0.4, and grown for 1.5 hrs. Then rifampicin (200 µg/ml, final concentration) was added and the cells were grown for another 1 to 1.5 hrs. Lysates were obtained using the ‘Shake it baby’ (Biospec Products, Bartlesville, OK), as described previously (Venema et al., 1994). To determine biological activity of pediocin in gels, the procedure of Bhunia et al. (1987) was employed with the following modifications. Tricine-SDS-PAGE (Schägger and Von Jagow, 1987) was used instead of SDS-PAGE. Fixation of the gel was done by shaking in 20% isopropanol, 10% acetic acid in water for 30 min at room temperature. Subsequently the gels were washed with demineralized water for 1 hr at room temperature (with shaking), overnight at 4 °C (without shaking), and 1 hr at 4 °C (without shaking) using three times 50 ml per gel.

Isolation of RNA.

RNA was isolated from E. coli, L. lactis, and Pediococcus by the method of van der Vossen et al. (1987) with the following modifications: after extracting the aqueous phase twice with phenol (saturated with 0.2 M sodium acetate, pH 5.0, 1% SDS, 10 mM EDTA), the RNA was precipitated with 2.5 volumes of cold (-20°C) ethanol. The RNA pellet was resuspended in 2 ml of sodium acetate (20 mM, pH 5.0) with 1 mM EDTA and 0.5% SDS) to which an equal volume of 5 M LiCl was added. This solution was incubated overnight at 0°C. The RNA was collected by centrifugation, washed twice with cold 70% ethanol, dried under vacuum and dissolved in 100 µl distilled water. Ethanol-precipitated aliquots were stored at -80°C

Northern blot analysis.

RNA (10 µg) was separated on denaturing formamide/formaldehyde agarose gels as described (Marugg et al., 1992). Transfer to Genescreen plus membrane (NEN/DuPont, Boston, MA) and hybridization to 5'-end labelled oligonucleotides using poly-nucleotide kinase (Boehringer) and [γ-32P]-dATP (Amersham) followed the procedures of the manufacturers. The probes
used are primer 76 (pedA-specific) and primer 30 (pedD-specific) (Marugg et al., 1992).

**NOTE ADDED IN PROOF**

Very recently, it was shown that a fragment containing the 150 N-terminal amino acids of LagD (the lactococcin G ABC-transporter) was able to cleave its cognate prebacteriocin in vitro (Havarstein et al. 1995. *Mol. Microbiol.* 16: 229-240).

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**REFERENCES**


Marugg, J.D., Gonzalez, C.F., Kunka, B.S., Ledeboer,


