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Cloning, Expression, and Nucleotide Sequence of Genes Involved in Production of Pediocin PA-1, a Bacteriocin from *Pediococcus acidilactici* PAC1.0

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The production of pediocin PA-1, a small heat-stable bacteriocin, is associated with the presence of the 9.4-kbp plasmid pSRQ11 in *Pediococcus acidilactici* PAC1.0. It was shown by subcloning of pSRQ11 in *Escherichia coli* cloning vectors that pediocin PA-1 is produced and, most probably, secreted by *E. coli* cells. Deletion analysis showed that a 5.6-kbp *SalI-EcoRI* fragment derived from pSRQ11 is required for pediocin PA-1 production. Nucleotide sequence analysis of this 5.6-kbp fragment indicated the presence of four clustered open reading frames (*pedA*, *pedB*, *pedC*, and *pedD*). The *pedA* gene encodes a 62-amino-acid precursor of pediocin PA-1, as the predicted amino acid residues 19 to 62 correspond entirely to the amino acid sequence of the purified pediocin PA-1. Introduction of a mutation in *pedA* resulted in a complete loss of pediocin production. The *pedB* and *pedC* genes, encoding proteins of 112 and 174 amino acid residues, respectively, are located directly downstream of the pediocin structural gene. Functions could not be assigned to their gene products; mutation analysis showed that the PedB protein is not involved in pediocin PA-1 production. The mutation analysis further revealed that the fourth gene, *pedD*, specifying a relatively large protein of 724 amino acids, is required for pediocin PA-1 production in *E. coli*. The predicted PedD protein shows strong similarities to several ATP-dependent transport proteins, including the *E. coli* hemolysin secretion protein HlyB and the ComA protein, which is required for competence induction for genetic transformation in *Streptococcus pneumoniae*. These similarities suggest strongly that the PedD product is involved in the translocation of pediocin PA-1.

The pediococci are a diverse group of gram-positive homofermentative lactic acid bacteria often found as saprophytes on vegetable material (18, 35). Commercially, pediococci are used in the fermentation of vegetables (39) and meats (45). Some strains of *Pediococcus pentosaceus*, *P. cerevisiae*, and *P. acidilactici* contain resident plasmids, although the roles of most of these remain unknown (16, 19, 41). The association of raffinose fermentation and plasmid DNA has been reported (17), as has been the ability of *P. acidilactici* to ferment sucrose (18). Moreover, there have been several reports which associate the production of bacteriocins by pediococci with plasmid DNA (9, 18, 19, 26, 42). It was shown by Gonzalez and Kunka (18) that bacteriocin production was encoded by a 9.4-kb plasmid, designated pSRQ11, in *P. acidilactici* PAC1.0. Further work of Pucci et al. (40) demonstrated that the bacteriocin of *P. acidilactici* PAC1.0 was active against a wide spectrum of gram-positive lactic acid bacteria and also against *Listeria monocytogenes*. This antilisterial activity was observed in broth and on agar plates, as well as in some dairy products. Inhibition of *L. monocytogenes* by this bacteriocin, called pediocin PA-1, has also been noted in fermented semidry sausage (1) and fresh meat (37).

Despite the many investigations on the detection and characterization of bacteriocins produced by pediococci and other lactic acid bacteria, information on their genetics is still limited. The cloning of the genetic determinants of several bacteriocins from lactic acid bacteria has been described only recently. The structural gene for the precursor of nisin, a lantibiotic produced by a number of *Lactococcus lactis* subsp. *lactis* strains, has been cloned and sequenced (4, 12, 29). The genetic determinants encoding another bacteriocin also were cloned from the same subspecies (20). Recently, van Belkum et al. cloned and sequenced the genes involved in bacteriocin production and immunity of three different bacteriocins, designated lactococcins, from *Lactococcus lactis* subsp. *cremoris* 9B4 (48–50). In addition, the gene for a fourth lactococcin, from *Lactococcus lactis* subsp. *cremoris* LMG2130, was cloned and sequenced by Holo et al. (25). The sequence of the structural gene appeared to be identical to the sequence of one of the structural lactococcin genes from *Lactococcus lactis* subsp. *cremoris* 9B4 (25, 49). Finally, the structural genes for helveticin J from *Lactobacillus helveticus* 481, lactacin F from *Lactobacillus acidophilus* 11088, and leucocin A-UAL 187 from *Leuconostoc gelidum* UAL 187 have been cloned and sequenced (21, 28, 36).

Here we report the cloning and nucleic acid sequencing of the DNA region of *P. acidilactici* plasmid pSRQ11, which is involved in the production of pediocin PA-1. We present evidence that the pediocin is produced and secreted in *Escherichia coli*. Furthermore, we show that pediocin PA-1 production depends on the presence of a protein which

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Remarks[a]	Reference
Strain		
P. acidilactici		
PAC1.0	Contains 9.4-kb PA-1 pediocin plasmid, pSRQ11	18
PAC1.14	PAC1.0 derivative cured of pSRQ11	18
P. pentosaceus		
FBB63C	Sensitive indicator strain for PA-1 pediocin	18
E. coli		
V850	Hypersensitivity to macrolide antibiotics	33
SK1592	Tetracycline sensitive	47
Plasmid		
pBR322	Ap^r Tc^r	3
pACYC184	Cm^r Tc^r	5
pVA891	Em^r	33
pSRQ11	9.4-kb PA-1 pediocin plasmid	18

[a] Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; r, resistance; Tc, tetracycline.

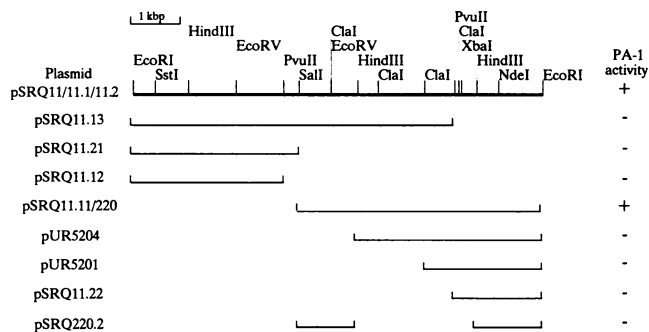

FIG. 1. Schematic overview of the subclones derived from pediocin PA-1-encoding plasmid pSRQ11 (18). The restriction enzyme map of pSRQ11 is shown. The inserts of the various subclones are represented by the horizontal lines. Pediocin PA-1 activities of *E. coli* cells containing these subclones are indicated.

shows very strong similarities to members of a family of ATP-binding membrane proteins, including several bacterial ATP-dependent transport proteins.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmid vectors used in this study are listed in Table 1. *Pediococcus* spp. were routinely maintained on MRS agar (Difco Laboratories, Detroit, Mich.). *E. coli* strains were routinely carried on Lennox L agar (GIBCO/Bethesda Research Laboratories, Gaithersburg, Md.). *E. coli* strains were also grown on modified MRS agar (no citrate or acetate) or in M9 medium (34) supplemented with 1% yeast extract (Oxoid, Ltd., Basingstoke, Hampshire, U.K.) and 1% Hy Case (Sheffield Products, Norwich, N.Y.) for pediocin assays. Selective antibiotic concentrations were as follows: ampicillin, 25 μg/ml; tetracycline, 10 μg/ml; erythromycin, 50 μg/ml; chloramphenicol, 25 μg/ml. All antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

Pediocin assays. Production of pediocin was assayed as described previously (18). One arbitrary unit of pediocin was defined as 5 μl of the highest dilution of culture supernatant yielding a definite zone of growth inhibition on the indicator lawn. The titer was expressed as the reciprocal of the highest dilution showing inhibition. Strains were patched on MRS agar or modified MRS agar for *E. coli* and incubated at 35°C for 18 h. The plates then were overlaid with soft agar (0.8%) seeded with indicator cells. Isolates which produced a clear, defined zone of inhibition were considered pediocin producers.

DNA manipulations. Covalently closed circular plasmid DNA was isolated from *E. coli* by the method of Clewell and Helinski (8). *E. coli* strains were screened for plasmid content as described previously (34). *Pediococcus* plasmid DNA was obtained by a scaled-up modification of the LeBlanc and Lee procedure (32) as described by Gonzalez and Kunka (16). Restriction endonuclease digestions were performed in low-, medium-, or high-salt buffers, as recommended by Maniatis et al. (34), or using buffer systems provided by the manufacturer. Restriction enzymes were obtained from Bethesda Research Laboratories, Biolabs, or Amersham International. DNA ligation reactions were carried out with T4 DNA ligase (Bethesda Research Laboratories) at 15°C for 18 h, conditions recommended by the manufacturer. All other molecular and microbiological techniques (e.g., electroelution, transformations, and agarose gel electrophoresis) were the procedures published by Maniatis et al. (34).

DNA sequencing. The DNA sequence of the 5.6-kbp *Sal*I-*Eco*RI DNA fragment, as present on plasmid pSRQ220, was established by the Sanger dideoxy chain termination procedure (44) with the modifications described by Biggin et al. (2), using α-^{35}S-dATP (2,000 Ci/mmol) and Klenow enzyme (Amersham), dideoxynucleoside triphosphates (Pharmacia-P-L Biochemicals), and deoxynucleoside triphosphates (Boehringer). The sequencing reaction products were separated on a denaturing polyacrylamide gel with a buffer gradient as described by Biggin et al. (2). Purified, double-stranded plasmid DNA of pSRQ220 served as the template in the sequence reaction, following the procedure described by Hattori and Sakaki (22). Site-specific 18-mer primers, based on newly sequenced DNA, were synthesized on a DNA synthesizer (Applied Biosystems 380A).

Computer analysis. DNA sequence analysis, sequence alignments, and sequence data base searching were conducted with programs contained within the Sequence Analysis Software Package (version 6.1) licensed from the Genetics Computer Group (University of Wisconsin, Madison) (11). The predicted amino acid sequences encoded by the different open reading frames were compared by the algorithm of Pearson and Lipman (38) (FASTA) with proteins in the SWISS-PROT Protein Sequence Database (release 18) and the EMBL Nucleotide Sequence Database (release 27).

Nucleotide sequence accession number. The DNA sequence was submitted to GenBank (Los Alamos, N.M.) and was given the accession number M83924.

RESULTS

Production of pediocin PA-1 in *E. coli*. Pediocin PA-1 production was previously shown to be associated with the

FIG. 2. Nucleotide sequence and predicted amino acid sequences of the 5,995-bp *Sal*I-*Eco*RI fragment of the *P. acidilactici* pediocin PA-1-encoding plasmid pSRQ11. The predicted amino acid sequences of the open reading frames (*pedA*, *pedB*, *pedC*, and *pedD*) are shown below the nucleotide sequence. The putative −35 and −10 promoter regions as well as the putative ribosome binding sites (RBS) are underlined. The vertical arrow in the amino acid sequence of PedA indicates the mature processing site. Restriction sites mentioned in the text are shown.

presence of a 9.4-kbp plasmid, designated pSRQ11 (18). Plasmid pSRQ11 was digested with a number of restriction endonucleases to generate the restriction site map shown in Fig. 1.

Plasmid pSRQ11 was linearized at the unique *Eco*RI site and ligated to *Eco*RI-digested pVA891 (33), which contains an erythromycin resistance marker expressed in both *E. coli* and lactococci. Recombinant plasmids were obtained with pSRQ11 inserted in both orientations and were designated pSRQ11.1 and pSRQ11.2 (Fig. 1). These *E. coli* strains were assayed for pediocin PA-1 activity as described previously (18). The strains were grown on modified MRS medium and overlaid with the indicator strain *P. pentosaceus* FBB63. *E. coli* strains containing pSRQ11.1 and pSRQ11.2 both produced zones of inhibition in the indicator lawn, while the control *E. coli* V850 strains showed no zone of inhibition. When the *E. coli* V850 strains carrying the recombinant clones were grown overnight in M9 medium supplemented with 1% yeast extract and 1% Hy Case, the filter-sterilized culture supernatant yielded approximately 400 arbitrary units of pediocin PA-1 per ml. This observation indicated that pediocin PA-1 was produced and, most likely, was secreted into the medium.

Deletion derivative analysis of pSRQ11 subclones. To localize the region encoding the pediocin gene(s), *Sal*I and *Pvu*II deletion derivatives of pSRQ11.1 and pSRQ11.2 were obtained. An overview of these (and other) deletions is given in Fig. 1. The *Sal*I deletion derivative of pSRQ11.1 (pSRQ11.11) retained activity, while neither of the *Pvu*II deletion derivatives (pSRQ11.12 and pSRQ11.13) displayed zones of inhibition against the indicator strain. Neither the *Pvu*II nor the *Sal*I deletion derivative of pSRQ11.2 (pSRQ11.21 and pSRQ11.22) expressed pediocin. These data indicated that all information for bacteriocin production was located on the approximately 5.6-kbp large *Eco*RI-*Sal*I fragment of pSRQ11 (Fig. 1).

This 5.6-kbp *Eco*RI-*Sal*I fragment then was cloned into *E. coli* plasmid pBR322 (3), which was digested with *Eco*RI and *Sal*I. The resulting chimeric plasmid, designated pSRQ220 (Fig. 1), was used to transform *E. coli* cells. *E. coli* cells

containing pSRQ220 were assayed and found to express pediocin, as expected. Three additional deletion derivatives of pSRQ220, i.e., a plasmid derivative lacking a 2.7-kbp *Hin*dIII fragment (pSRQ220.2), a plasmid derivative lacking a 1.3-kbp *Hin*dIII-*Sal*I fragment (pUR5204), and a plasmid derivative lacking a 2.9-kbp *Sal*I-*Cla*I fragment (pUR5201), were assayed, and all were found to be negative for pediocin production.

Since the *Xba*I restriction site is unique on both pSRQ11 and pSRQ220 and is positioned within the putative pediocin-encoding region, it was chosen as a site to insert a foreign DNA fragment and interrupt transcription of a gene(s) involved in pediocin production. Plasmid pACYC184 (5), approximately 4 kbp in size and also containing a single *Xba*I site, was cloned into the *Xba*I site on pSRQ220. The strain containing the resulting recombinant plasmid, pSRQ221, was assayed for pediocin production and proved negative. When the pACYC184 insert was removed by *Xba*I digestion, followed by religation, activity was once again restored. Together these results showed that the region needed for pediocin PA-1 production stretches out beyond the *Hin*dIII site (left side) and the *Xba*I site (right side) both present within the 5.6-kbp *Sal*I-*Eco*RI fragment (Fig. 1).

Nucleotide sequence analysis of pSRQ220. The DNA sequence of the 5.6-kbp *Sal*I-*Eco*RI DNA fragment, as present on plasmid pSRQ220, was established. The complete sequence of 5,595 bp is shown in Fig. 2.

The DNA sequence, when translated in all possible reading frames, revealed at least four open reading frames, each preceded by a putative ribosome binding site (Fig. 2). The first open reading frame (*pedA*) encodes a protein which consists of 62 amino acid residues and is followed by a TAG stop codon (Fig. 2). The second open reading frame (*pedB*), positioned just downstream of *pedA*, codes for a protein which consists of 112 amino acid residues and is also followed by a TAG stop codon (Fig. 2). A third open reading frame (*pedC*), located directly behind the *pedB* reading frame, starts with the less frequently used TTG initiation codon and encodes a protein of 174 amino acid residues with a TAG stop codon (Fig. 2). Further downstream, the fourth open reading frame (*pedD*) predicts a protein consisting of 724 amino acid residues with a TAG stop codon (Fig. 2). A putative promoter was found directly upstream of the *pedA* gene. The sequences of its −35 and −10 regions (TTGACA and TAGAAT, respectively) as well as their spacing (18 nucleotides) correspond closely to the sequences and spacing found in other (gram-positive) constitutive promoters (51). No putative promoter sequences were found in the regions directly upstream of the *pedB*, *pedC*, and *pedD* genes.

Amino acid residues 19 to 62 of the predicted PedA protein correspond entirely to the amino acid sequence of a protein designated pediocin PA-1, which was isolated previously from *P. acidilactici* PAC1.0. When separated on a polyacrylamide gel, it inhibited *P. pentosaceus* FBB-63 effectively in an overlay experiment (23). This indicates that the *pedA* gene encodes a precursor of pediocin PA-1 containing an 18-amino-acid N-terminal peptide which is cleaved off during the process of synthesis or secretion.

Both the *Pvu*II deletion derivative pSRQ11.13 and the *Hin*dIII deletion derivative pSRQ220.2 (Fig. 1), as well as the *Xba*I insertion derivative pSRQ221 (Fig. 3), resulted in a loss of pediocin production. As these deletions disturb either the *pedD* gene or the *pedB*, *pedC*, and *pedD* genes together, but not the pediocin-encoding gene (*pedA*), it was concluded that the presence of *pedB*, *pedC*, or *pedD* (or combinations of these three genes) is required for pediocin production.

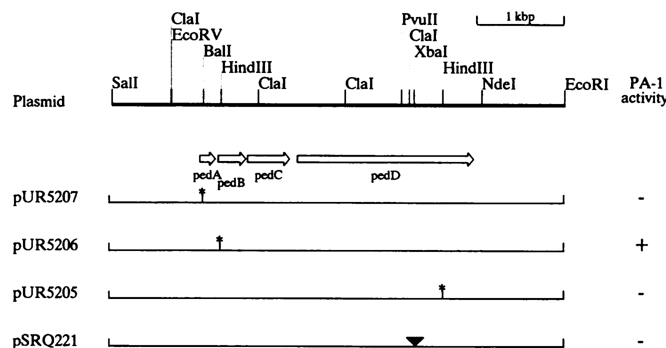

FIG. 3. Schematic overview of mutation derivatives of plasmid pSRQ220. The restriction enzyme map of the 5.6-kbp *Sal*I-*Eco*RI fragment of pSRQ220 as well as the locations of the *pedA*, *pedB*, *pedC*, and *pedD* open reading frames are shown. The horizontal lines represent the inserts of the various plasmids. Asterisks (*) indicate the mutations in the open reading frames (see text for details). The arrowhead represents the pACYC184 insertion of plasmid pSRQ221. Pediocin PA-1 activities of *E. coli* cells containing these plasmids are indicated.

Site-specific mutagenesis of genes involved in pediocin production. The specific role in pediocin production of each of the open reading frames was determined by introduction of frameshift mutations in the various genes.

Plasmid pSRQ220 contains two sites for the restriction enzyme *Bal*I. One is situated in the pBR322 part of the plasmid, whereas the other is positioned within the *pedA* gene, which encodes pediocin (Fig. 3). A frameshift mutation in *pedA* was introduced by insertion of a double-stranded oligonucleotide linker fragment with the sequence 5′-TGCATGGATCCTGATC-3′ into the latter *Bal*I site. Plasmid pSRQ220 was therefore partially digested with *Bal*I, generating linear blunt-ended DNA molecules, and was allowed to ligate with the linker fragment. Insertion of the linker fragment disrupted the *Bal*I site but introduced a new and unique *Bam*HI site into the plasmid that was used for identification of the desired mutant. After transformation of *E. coli* with the ligation mixture, plasmid DNA was isolated from the transformants and screened for the presence of a *Bam*HI site, concomitant with the loss of a *Bal*I site. In this way, plasmid pUR5207, which carried the desired linker insertion within *pedA*, was identified. Introduction of the mutation was confirmed by determination of the nucleotide sequence around the restriction site of the mutant. *E. coli* cells containing pUR5207 were assayed for pediocin production and found to have lost this property. This result is in good agreement with the deletion data obtained previously, and it again suggests that the presence of *pedA* is essential for pediocin production.

Restriction enzyme *Hin*dIII has only two restriction sites in pSRQ220, one of which is positioned in *pedB* and the other of which is positioned in *pedD* (Fig. 3). These sites were therefore well suited for introduction of mutations in these genes. The two *Hin*dIII sites were filled in separately, using Klenow enzyme, as described in Materials and Methods. The accuracy of the process was checked by the inability to cleave the plasmid DNA derived from each clone at the original *Hin*dIII restriction site and by determination of the nucleotide sequence around the *Hin*dIII restriction site (data not shown). In this way, plasmid pUR5206, which

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PedD   1 MWTQKWHKYYTAQVDENDCGLAALNMILKYYGSDYMLAHLRQLAKTTADG 50
ComA   1 MKFGKRH..YRPQVDQMDCGVASLAMVFGYYGSYYFLAHLRELAKTTMDG 48

PedD  51 TTVLGLVKAAKHLNLNAEAVRADMDALTASQLPLPVIVHVFKKNKLPHYY 100
ComA  49 TTALGLVKVAEEIGFETRAIKADMTLFDLPDLTFPFVAHVLKEGKLLHYY 98

PedD 101 VVYQVTENDLIIGDPDPTVKTTKISKSQFAKEWTQIAIIIAPTVKYKPIK 150
ComA  99 VVTGQDKDSIHIADPDPGVKLTKLPRERFEEEWTGVTLFMAPSPDYKPHK 148

PedD 151 ESRHTLIDLVPLLIKQKRLIGLIITAAAITTLISIAGAYFFQLIIDTYLP 200
ComA 149 EQKNGLLSFIPILVKQRGLIANIVLATLLVTVINIVGSYYLQSIIDTYVP 198

PedD 201 HLMTNRLSLVAIGLIVAYAFQAIINYIQSFFTIVLGQRLMIDIVLKYVHH 250
ComA 199 DQMRSTLGIISIGLVIVYILQQILSYAQEYLLLVLGQPLSIDVILSYIKH 248

PedD 251 LFDLPMNFFTTRHVGEMTSRFSDASKIIDALGSTTLTLFLDMWILLAVGL 300
ComA 249 VFHLPMSFFATRRTGEIVSRFTDANSIIDALASTILSIFLDVSTVVIISL 298

PedD 301 FLAYQNINLFLCSLVVVPIYISIVWLFKKTFNRLNQDTMESNAVLNSAII 350
ComA 299 VLFSQNTNLFFMTLLALPIYTVIIFAFMKPFEKMNRDTMEANAVLSSSII 348

PedD 351 ESLSGIETIKSLTGEATTKKKIDTLFSDLLHKNLAYQKADQGQQAIKAAT 400
ComA 349 EDINGIETIKSLTSESQRYQKIDKEFVDYLKKSFTYSRAESQQKALKKVA 398

PedD 401 KLILTIVILWWGTFFVMRHQLSLGQLLTYNALLAYFLTPLENIINLQPKL 450
ComA 399 HLLLNVGILWMGAVLVMDGKMSLGQLITYNTLLVYFTNPLENIINLQTKL 448

PedD 451 QAARVANNRLNEVYLVESEFSKSREITALEQLNGDIEVNHVSFNYGYCSN 500
ComA 449 QTAQVANNRLNEVYLVASEFEEKKTVEDLSLMKGDMTFKQVHYKYGYGRD 498

                       * *** ********* ***
PedD 501 ILEDVSLTIPHHQKITIVGMSGSGKTTLAKLLVGFFEPQEQHGEIQINHH 550
ComA 499 VLSDINLTVPQGSKVAFVGISGSGKTTLAKMMVNFYDPSQ..GEISLGGV 546
                                  NB

PedD 551 NISDISRTILRQYINYVPQEPFIFSGSVLENLLLGSRPGVTQQMIDQACS 600
ComA 547 NLNQIDKKALRQYINYLPQQPYVFNGTILENLLLGAKEGTTQEDILRAVE 596

                                  ********** ******    ****
PedD 601 FAEIKTDIENLPQGYHTRLSESGFNLSGGQKQRLSIARALLSPAQCFIFD 650
ComA 597 LAEIREDIERMPLNYQTELTSDGAGISGGQRQRIALARALLTDAPVLILD 646
                                              TP
         * ** **
PedD 651 ESTSNLDTITEHKIVSKLLFMKDKTIIFVAHRLNIASQTDKVVVLDHGKI 700
ComA 647 EATSSLDILTEKRIVDNLIAL.DKTLIFIAHRLTIAERTEKVVVLDQGKI 695

PedD 701 VEQGSHRQLLNYNGYYARLIHNQE 724
ComA 696 VEEGKHADLLAQGGFYAHLVNS   717
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FIG. 4. Alignment of *P. acidilactici* PedD and *S. pneumoniae* ComA (24) deduced protein sequences. The sequences were aligned by using the program GAP (11). Broken lines indicate identical residues. Closely related residues are indicated by colons, while less related residues are indicated by dots. Potential membrane-spanning segments (27, 31) are over- and underlined. The short segment marked with NB indicates the nucleotide binding site (13, 24). TP indicates a region that is conserved in most members of the ATP-dependent transport family (24). Conserved residues are marked with an asterisk (*).

carried a mutation at the *Hin*dIII site in *pedB*, and plasmid pUR5205, which carried a mutation at the *Hin*dIII site in *pedD*, were identified (Fig. 3). *E. coli* cells containing pUR5206 were assayed and found to produce pediocin, whereas *E. coli* cells containing pUR5205 were negative for pediocin production. From these data we conclude that the presence of the *pedA* gene, as well as the presence of an intact *pedD* gene, is needed for pediocin production.

DISCUSSION

As reported previously (18), *P. acidilactici* PAC1.0 produces a bacteriocin designated pediocin PA-1, which was shown to be encoded by the 9.4-kbp plasmid pSRQ11. Here we present data on cloning and sequencing of a DNA region of this plasmid involved in pediocin PA-1 activity. The results of the deletion analysis described above show that a substantial part of a 5.6-kbp *Sal*I-*Eco*RI fragment derived from pSRQ11 is required for production and excretion of the bacteriocin. The nucleotide sequence analysis of this 5.6-kbp fragment indicated the presence of four clustered genes (*pedA*, *pedB*, *pedC*, and *pedD*). Analysis of the nucleotide sequence showed the presence of a single putative promoter directly upstream of *pedA* (Fig. 2). Promoter sequences could not be detected upstream of three other genes, which indicates that the four genes may be organized in an operonlike structure. Preliminary transcription analysis data show that transcription is initiated at the *pedA* promoter, yielding a transcript sufficiently large to cover all four genes (data not shown).

The *pedA* gene appears to encode a 62-amino-acid precursor of pediocin PA-1, from which an 18-amino-acid N-terminal peptide is removed. This was concluded after comparison of the gene-derived sequence with the direct sequence data for pediocin PA-1, which was purified from *P. acidilactici* PAC1.0 previously (23). The mutation analysis confirmed these findings, as the introduction of a frameshift mutation in *pedA* resulted in a complete loss of pediocin production. Pediocin PA-1 appears to be a small heat-stable protein (4.6 kDa, 44 amino acids) which contains two disulfide bridges in the mature molecule. Amino acid composition analyses of purified pediocin PA-1 did not reveal the presence of unusual residues such as lanthionines found in the lantibiotic group of bacteriocins (e.g., nisin and subtilin) (23).

Directly downstream of the structural gene for pediocin is located a second gene (*pedB*) which encodes a protein of 112 amino acids. Mutation analysis showed that this *pedB* gene is not involved in pediocin production in *E. coli*. For many well-characterized bacteriocins it has been found that immunity genes are in close association with bacteriocin production genes (10, 49). This suggests that the PedB product is involved in bacteriocin immunity. In *P. acidilactici* PAC1.0, however, immunity determinants seem to be located chromosomally, as curing of the bacteriocin-encoding plasmid pSRQ11 resulted in a loss of pediocin production but did not affect the resistance of PAC1.0 cells towards pediocin PA-1 (18). Since the expression of pediocin genes was investigated only in *E. coli* cells, which are insensitive to pediocin PA-1, immunity functions could not be determined. Analysis of the mutant derivatives in sensitive host cells (e.g., *Pediococcus* spp.) will reveal the putative role of the *pedB* gene in pediocin immunity.

Further downstream of the pediocin structural gene is positioned the relatively large *pedD* gene specifying a polypeptide of 724 amino acids. Disruption of *pedD*, by deletions or by introduction of site-specific mutations which did not affect the pediocin structural gene, resulted in a complete loss of pediocin production. This clearly demonstrates that the PedD product is essential for pediocin production in *E. coli*. The predicted PedD protein consists of an N-terminal half which is largely hydrophobic in character, while the C-terminal half is mainly hydrophilic. When protein sequence data bases were searched for homology to the complete predicted PedD sequence, a high degree of amino acid sequence similarity was found between the PedD protein and a family of ATP-binding proteins, including several bacterial ATP-dependent transport proteins (24), and a group of eucaryotic proteins involved in multidrug resistance (6). One of the highest scores was found for the comparison

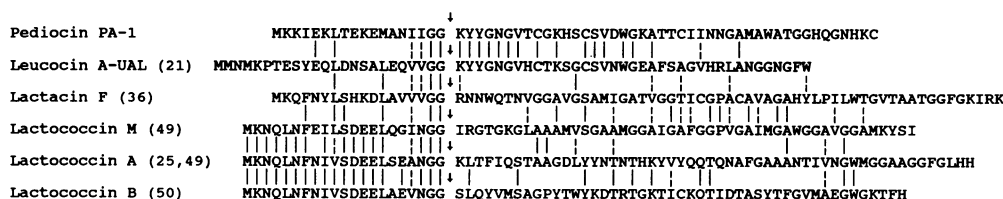

FIG. 5. Sequence comparison of several bacteriocin precursors. Identical residues are indicated by vertical lines, while similar residues are indicated by broken lines. The vertical arrows indicate the processing sites.

with HlyB, an *E. coli* membrane protein required for the export of hemolysin A (14).

Recently, the *comA* gene, required for competence induction for genetic transformation in *Streptococcus pneumoniae*, was sequenced (27). Homology analysis showed that the ComA protein is another member of this family of ATP-binding proteins (24, 27). Alignment of the predicted PedD protein with the ComA protein revealed an even higher degree of similarity than that found in the comparison of the PedD protein with HlyB: 69.6% of the amino acid sequence was conserved, including identities in 50.4% positions. On the basis of homology to the ComA and HlyB proteins, six potential membrane-spanning segments (31), clustered in the N-terminal half of the sequence, can be identified in the predicted PedD protein (Fig. 4). In the C-terminal part of the protein, an ATP-binding motif (GMSGSGKTT) at positions 519 to 527 and a region unique to proteins of the active transport group are highly conserved (Fig. 4) (13, 24). Comparable similarities to the HlyB protein were found for the SpaT (30) (also designated SpaB [7]) protein which is involved in the production of subtilin, a lantibiotic from *Bacillus subtilis* ATTC 6633.

The extensive similarities of the predicted PedD product to bacterial transport proteins suggest a comparable role for this protein in the synthesis and secretion of pediocin PA-1. This view is in good agreement with the experimental evidence presented here. By using several plasmid subclones, it was shown that the pediocin genes are expressed properly in *E. coli*. The expression of the bacteriocin genes as such is not very unusual since a number of cloned gram-positive genes have been shown to express well in *E. coli* (15, 43, 46). However, since these *E. coli* clones produce inhibitory zones on plate assays and that activity is also observed in the supernatant after centrifugation of the cells, it is concluded that pediocin probably also is secreted by the cells. Mutants which carry a (single) mutation in PedD no longer exhibit pediocin production. Considering the predicted role of the PedD protein in transport, we conclude that secretion of pediocin PA-1, rather than its synthesis, is disturbed in these mutants.

Homology analysis further revealed low sequence similarities between the predicted PedC protein and the SpaB (30) (=SpaD [7]) protein, which is also involved in subtilin biosynthesis (data not shown). This homology suggests that PedC is involved in pediocin biosynthesis too. The exact role of the *pedC* gene is being investigated.

Comparison of the predicted protein sequences of PedA and PedB with protein sequences of data bases did not show any significant similarities. However, recently, the characterization of several bacteriocins from other lactic acid bacteria, as well as that of their structural genes, has been reported (21, 25, 36, 49, 50). The different lactococcins from *Lactococcus lactis*, as well as lactacin F from *Lactobacillus acidophilus* and leucocin A-UAL 187 from *Leuconostoc gelidum*, are produced as precursor proteins with N-terminal extensions of 18 to 24 amino acids which are removed to yield the mature bacteriocin. Comparison of these different precursor sequences, including the one for pediocin PA-1, shows very few similarities in the mature parts of the proteins, with the exception of the extensive homology between the N-terminal halves of mature pediocin and mature leucocin (Fig. 5). However, significant similarities among all precursors are found in the leader peptide, particularly near the cleavage site. The precursor of pediocin PA-1 is cleaved behind two adjacent glycine residues of the leader peptide. This cleavage site appears to be conserved in bacteriocin precursors from lactic acid bacteria as similar cleavage sites are present in the precursors of lactacin F, leucocin A-UAL 187, and the three lactococcins (Fig. 5). These cleavage sites are different from typical signal peptidase cleavage sites ('−3, −1' rule) (52), suggesting that a separate processing enzyme(s) is needed for maturation of small heat-stable bacteriocins in lactic acid bacteria.

This study deals with production and excretion of pediocin PA-1 in *E. coli*. At present, we are investigating expression in several lactic acid bacteria, including *Pediococcus* spp. and *Lactococcus* spp. Since the latter species are resistant to pediocin PA-1, expression is particularly interesting with respect to the development of improved dairy starter cultures.

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