

Cloning and Expression of the Pediocin Operon in *Streptococcus thermophilus* and Other Lactic Fermentation Bacteria

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Abstract. Production of pediocin in *Pediococcus acidilactici* is associated with pMBR1.0, which encodes prepediocin, a pediocin immunity protein, and two proteins involved in secretion and precursor processing. These four genes are organized as an operon under control of a single promoter. We have constructed shuttle vectors that contain all four structural genes, the chromosomal promoter ST_{P2201} from *Streptococcus thermophilus*, and *repA* from the 2-kbp *S. thermophilus* plasmid pER8. The recombinant plasmid, pPC318, expressed and secreted active pediocin in *Escherichia coli*. *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, and *Enterococcus faecalis* were electrotransformed with pPC418, a modified vector fitted with an erythromycin resistance tracking gene. Pediocin was produced and secreted in each of the lactic acid bacteria, and production was stable for up to ten passages. The expression of pediocin in dairy fermentation microbes has important implications for bacteriocins as food preservatives in dairy products.

Streptococcus thermophilus is a significant industrial microbe, since it functions as a biocatalyst in the production of yogurt as well as Italian- and Swiss-style cheeses. During the fermentative process, the accumulated biomass of this innocuous microbe remains in the product and is consumed. Since ingestion of this organism is harmless, *S. thermophilus* has been classified as a "food-grade" microorganism. Thus, *S. thermophilus* is an attractive host for the expression of heterologous genes, e.g., bacteriocins that could protect dairy foods from contamination by undesirable microorganisms.

Pediocin is a bacteriocin produced by *Pediococcus acidilactici*, which has attracted interest as a potential biopreservative in food systems. Pediocin is initially synthesized as a precursor, and the genes for prepediocin and associated products are organized within an operon structure [11]. Within the operon, the genes *pedA*, *pedB*, *pedC*, and *pedD* encode prepediocin, an immunity protein, a membrane-bound protein required for secretion,

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and a protein involved in the processing of the active peptide, respectively [26].

Pediocin exhibits a broad spectrum of bactericidal activity against Gram-positive bacteria responsible for food spoilage and pathogenesis [8, 15–17], including *Listeria monocytogenes*, the causative agent of listeriosis that may be associated with a variety of dairy foods [10]. Thus, the availability of lactic acid bacteria (LAB) engineered to produce pediocin as components of starter culture mixes and to provide a bioprotective barrier against listeriosis in dairy products would be particularly advantageous from a microbiological, as well as a marketing standpoint. The validity of this position was recently underlined in a report by Buyong et al. [3], who described the control of *L. monocytogenes* in Cheddar cheese by lactococci engineered to produce pediocin during the cheese-making process.

In this paper, we report the cloning of the pediocin operon from *Pediococcus acidilactici* into the *Escherichia coli* shuttle vector pPC418 and the expression of this vector in dairy fermentation (*S. thermophilus*, *Lactococcus lactis* subsp. *lactis*) and other types of LAB (*Enterococcus faecalis*).

Materials and Methods

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* DH5 α was obtained from Life Technologies, Inc. (Gaithersburg, MD). *Lactococcus lactis* subsp. *lactis* ML3 and SLA1.1, *Streptococcus thermophilus* ST128, *Enterococcus faecalis* DL3, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and other strains of *S. thermophilus* were from an in-house laboratory culture collection. The pediocin producing *Pediococcus acidilactici* F and *E. coli* JM109 harboring pMBR1.0 were supplied by B. Ray (Univ. of Wyoming, Laramie, WY). *P. acidilactici* Ev4c and *P. pentosaceus* Ev5c were gifts from E. Vedamuthu (Quest International, Rochester, MN). The vector pUC18 was purchased from Life Technologies, Inc.

Escherichia coli strains were grown routinely in Luria-Bertani (LB) broth (Life Technologies, Inc.) while *Lactococcus* sp. and *Streptococcus* sp. were cultured in tryptone–yeast extract–lactose (TYL) medium [22]. All strains of *Pediococcus* sp. were grown in MRS medium (Difco Laboratories, Detroit, MI). *E. coli* transformants were selected for by growth in LB media containing carbenicillin (200 μ g/ml) or erythromycin (Em, 300 μ g/ml) when appropriate. Lactic acid bacteria (LAB) were cultured in TYL broth containing Em (15 μ g/ml) when appropriate.

Molecular cloning techniques. Restriction enzymes, T4 ligase, and DNA-modifying enzymes were purchased from either New England Biolabs (Beverly, MA) or Life Technologies, Inc. Transformation of competent *E. coli* DH5 α cells was by the heat-shock protocol recommended by the supplier (Life Technologies, Inc.). LAB cultures were electrotransformed as previously described [23]. Plasmid mini prep isolation from *E. coli* was by the alkaline lysis method as described by Sambrook et al. [18]. Large-scale plasmid isolations from *E. coli* were performed with the Qiagen midi-prep system (Valencia, CA). Plasmids from LAB were isolated by the mini-prep method of O’Sullivan and Klaenhammer [14], and large-scale plasmid isolations were performed according to Somkuti and Steinberg [22], with the CsCl ultracentrifugation protocol of Stougaard and Molin [25]. DNA was analyzed by agarose gel electrophoresis (AGE) in a TBE buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA, pH 8). DNA was eluted from agarose with GeneElute agarose spin columns (Supelco, Bellefonte, PA).

Pediocin expression by *E. coli*. Expression of pediocin in *E. coli* was monitored by an assay procedure described by Miller et al. [12], with modifications. *E. coli* transformants carrying the pediocin plasmid were grown overnight at 37°C, with shaking, for 16 h in LB broth containing antibiotic. The 16-h-old cultures were serially diluted in LB broth lacking antibiotic and plated in soft LB agar (0.8%, 7 ml per dish) to yield ca. 100–200 colony forming units (cfu). Plates were incubated overnight at 37°C. The plates were then overlaid with 7 ml of MRS soft agar (0.8% concentration) inoculated with 50 μ l of an overnight culture of the pediocin-sensitive *P. pentosaceus* assay organism grown at 30°C. Solidified agar plates were incubated for 16 h at 30°C and examined for zones of inhibition around individual colonies.

Alternatively, 15 μ l of transformed *E. coli* cultures grown overnight at 37°C were spotted onto LB agar plates lacking antibiotic, allowed to air dry, and then overlaid with 7 ml of molten MRS soft agar (0.8%) inoculated with 50 μ l of a 16-h culture of *P. pentosaceus* grown at 30°C. Plates were held overnight at 30°C and examined for zones of inhibition.

Pediocin expression by LAB. Colonies of *S. thermophilus*, *E. faecalis*, and *L. lactis* subsp. *lactis* electrotransformed with appropriate plasmid DNA were toothpicked from TYL-Em medium into TYL-Em broth in microtiter plates, followed by overnight growth at 32°C. Samples (5 μ l) were then transferred into TYL broth without Em in microtiter plates

and incubated at 32°C overnight. Bacterial suspensions (100 μ l) were loaded into precast wells in Petri plates with MRS-agar (1.3%) inoculated with *P. pentosaceus* at a final concentration of 0.5%. Plates were equilibrated at 4°C for 16 h, incubated overnight at 30°C, and then examined for zones of inhibition.

The amount of pediocin produced by LAB was measured by the method of Henderson et al. [6], with 2-mm-deep MRS agar (1.3%) plates inoculated with *P. pentosaceus*. The pediocin content of cell-free supernatants of 16-h growth media was estimated by serial twofold dilutions with sterile dH₂O followed by spotting 5- μ l samples on indicator lawns. One arbitrary activity unit (AU) was defined as the highest dilution showing inhibition of the indicator organism. Total antimicrobial activity of supernatants was calculated as the reciprocal of the highest dilution and expressed as AU.ml⁻¹.

Segregational stability of pediocin⁺ phenotype (PED⁺). PED⁺ LAB transformants were grown in TYL-Em broth, followed by 2 \times daily transfer in TYL without Em, with incubation at 32°C (lactococci) or 34°C (*S. thermophilus*, *E. faecalis*). After each cycle, samples were taken and serially diluted with 0.5% peptone broth. Plating was in both TYL-Em and TYL agar, and colony counts were established after 16 h at 32°C or 34°C.

Results

Construction of plasmids. The schematic diagram of the construction of the *E. coli*–*St. thermophilus* shuttle vectors pPC318 and pPC418 is shown in Fig. 1. The vectors pPC318 and pPC418 were constructed in a two-step and three-step process, respectively. The first step resulted in pUC19St and pPC218, while the product of the second step was pPC318. Finally, pPC418 was assembled from pPC318 and pMEU5a in the third step.

One ligation product of the initial step was pUC19St (4.44 kb) constructed from pUC19 and a 1.76-kb *Bam*HI fragment of p5aGFP2201a. This *Bam*HI fragment contained the Gram-positive *repA* from pER8, a 2.1-kbp cryptic plasmid of *S. thermophilus* ST108 [22], and the chromosomal promoter ST_{P2201} from *S. thermophilus* ST128 [21]. Both elements are necessary for optimum expression of heterologous genes in LAB hosts [20].

The other ligation product of the first stage was pPC218 (6.16 kb), constructed from *Pst*II/*Eco*RI fragments of pUC18 and pMBR1.0. The latter is an *E. coli*–*P. acidilactici* shuttle vector that contains the structural genes for pediocin production, as well as erythromycin (Em) and chloramphenicol (Cm) resistance marker genes [2]. The 3.48-kb *Eco*RI–*Pst*I fragment from pMBR1.0 contained all four genes of the pediocin operon but not its promoter element. Thus, pPC218 carried all four structural genes for pediocin expression aligned in such manner that allowed the strategic upstream positioning of an LAB promoter (ST_{P2201}) to control transcription of the pediocin operon in dairy fermentation bacteria.

Once pPC218 and pUC19St were available, it was then possible to assemble a shuttle vector capable of replicating and expressing pediocin in both LAB and *E.*

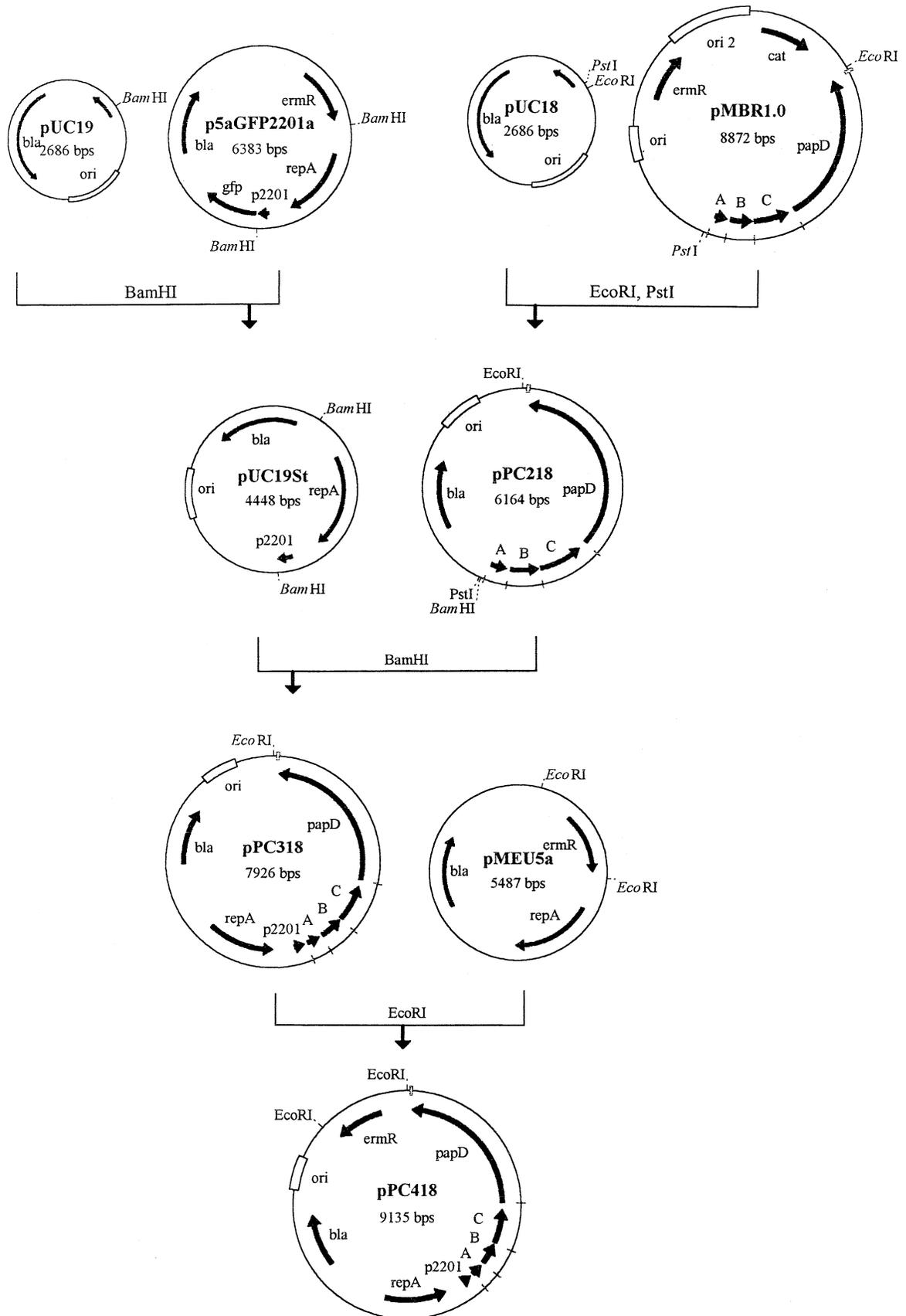


Fig. 1. Construction of vectors pPC318 and pPC418 harboring the pediocin operon (only the pertinent restriction enzyme sites are indicated).

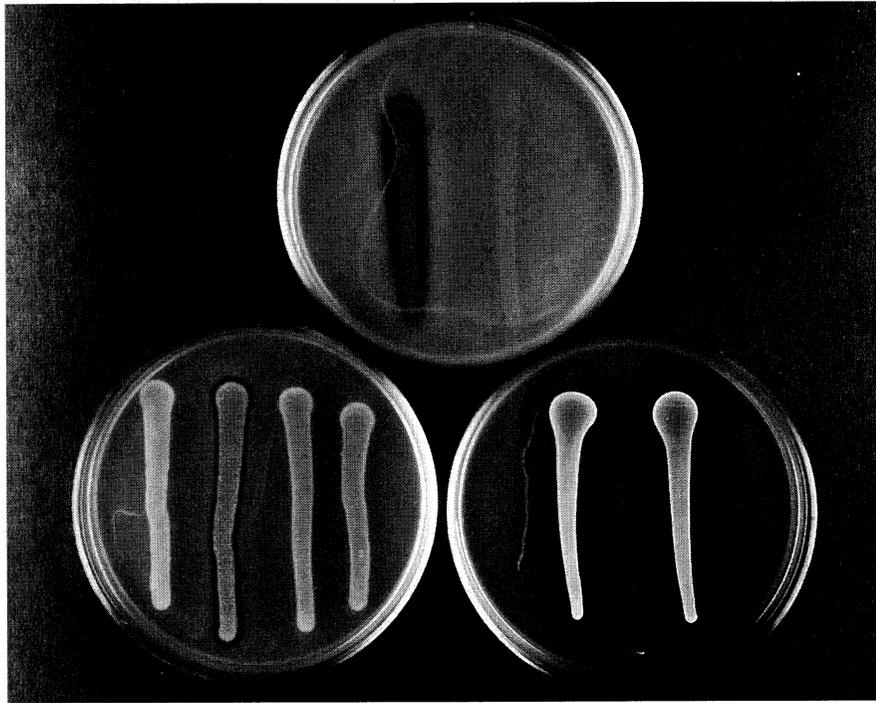


Fig. 2. Expression of pediocin in *E. coli* and pediococci. Bacterial strains harboring various plasmids are as follows (left to right): top plate, *E. coli* JM109/pMBR1.0 and JM109; bottom left plate, DH5 α /pUC19St, DH5 α /pPC318, DH5 α /pPC218, and DH5 α ; bottom right plate, *P. acidilactici* F and *P. pentosaceus* Ev4c.

coli. In the second step, the recombinant pPC318 was assembled by insertion of the 1.76-kb *Bam*HI fragment from pUC19St into the *Bam*HI site immediately upstream of the pediocin operon. This *Bam*HI fragment contained the *repA* and ST_{P2201} genes from *S. thermophilus*. The new 7.93-kb plasmid, pPC318, included all the structural genes for pediocin production, a functional promoter, and a gene (*repA*) necessary for plasmid replication in *S. thermophilus*.

In the final step, pPC418 was constructed from pPC318 and pMEU5a. The 1.21-kb *Eco*RI fragment from pMEU5a [20], which contains the erythromycin resistance gene (*erm*) of pPV141 (2.3 kb) from *Staphylococcus chromogenes* [24], was inserted into the *Eco*RI site downstream of the pediocin operon in pPC318 with the direction of transcription of *erm* in alignment with genes of the pediocin operon. The presence of *erm* in the new recombinant plasmid made the selection of LAB electrotransformants containing the pediocin operon more convenient in TYL-Em agar.

Pediocin expression in *E. coli*. Once pPC318 was available, this plasmid and other vectors used in its construction were transformed into *E. coli* DH5 α in order to assay for the production and secretion of pediocin, with *P. pentosaceus* as the pediocin-sensitive target organism. Figure 2 shows pediocin production by several *E. coli* DH5 α transformants harboring various plasmids. Pediocin production was detectable only in DH5 α transformants with either a plasmid carrying the pediocin

operon with its native promoter (pMBR1.0) or a plasmid in which the pediocin operon was fitted with the ST_{P2201} chromosomal promoter of LAB origin (pPC318). Figure 2 also shows that pediocin was produced by the control *P. acidilactici* strains F and Ev4c, each carrying a plasmid that incorporated genes for pediocin production as well as immunity.

Pediocin expression in LAB. Em^R clones of LAB were screened after electrotransformation with pPC418. By random selection, 48 transformants of each *S. thermophilus* ST128 and *E. faecalis* DL3 were transferred into TYL-Em broth in 96-well microtiter plates and incubated at 34°C. After overnight growth they were transferred into antibiotic-free TYL broth for 16 h and tested for pediocin production. Of the initially Em^R transformants, 20 clones of ST128 and 32 clones of DL3 produced pediocin as shown by the agar-well assay (Fig. 3).

Since pediocin was produced by *S. thermophilus* ST128 transformed with pPC418, we also tested pediocin operon expression by two different strains of the mesophilic dairy bacterium *L. lactis* subsp. *lactis*. Following the electrotransformation of strains ML3 and SLA1.1 with pPC418, Em^R clones were picked from TYL-Em agar after 48–72 h of incubation at 30°C and transferred into TYL-Em broth. After 16–24 h, transfers were made into TYL broth without antibiotic, with overnight incubation at 30°C. Of the 24 Em^R clones of each strain tested for pediocin production against *P. pentosaceus*, all of the ML3 clones but only six of the SLA1.1 clones produced

pediocin at a high enough level to result in clear zones of inhibition in MRS agar-well test plates.

The amount of pediocin produced by LAB transformed with pPC418 was strain dependent and ranged from 3,000 to 12,000 AU.ml⁻¹, as measured by the spot test against *P. pentosaceus* as the test organism.

Segregational stability of the Ped⁺ phenotype in streptococci and lactococci. To investigate the segregational stability of the Ped⁺ phenotype in LAB transformed with pPC418, broth cultures of streptococci (eight of each ST128 and EF-DL3) and lactococci (eight of ML-3 and six of SLA1.1) were transferred daily in TYL-Em medium. After each transfer cycle, subculturing was done in antibiotic-free medium, followed by incubation for 24 h at 34°C (*S. thermophilus*, *E. faecalis*) or 30°C (*L. lactis* subsp. *lactis*), and checking cell-free supernatants for pediocin activity in MRS-agar plates inoculated with *P. pentosaceus*. The capacity to produce pediocin at a high enough level to result in clear zones of inhibition by the agar-well assay persisted in *S. thermophilus* ST128 after five or more daily transfers, depending on the particular clone. Similar results were obtained with transformants of *E. faecalis* DL-3 and *L. lactis* subsp. *lactis* ML3. On the other hand, after two daily transfers in TYL-Em broth, all six Ped⁺ *Lactococcus* SLA1.1 clones lost the capacity to produce pediocin, while retaining the Em^R phenotype.

These results indicated that damage to the structural integrity or loss of the pediocin operon, but not the erythromycin resistance gene, may have occurred in electrotransformed LAB strains. The anticipated *Hind*III fragments were obtained after two transfer cycles from plasmid DNA from transformed ST128, ML3, and EF-DL3 clones transformed with pPC418, while AGE analysis of the plasmid isolated from SLA1.1 showed only one recognition site for this enzyme (data not shown). This suggested that a critical portion of the pediocin operon in pPC418 was already compromised in the SLA1.1 host after two transfer cycles, while the capacity to produce pediocin persisted longer in the other transformed clones of LAB.

However, serial transfer in TYL-Em medium resulted in the gradual decline of pediocin productivity in nearly all electrotransformed LAB clones, leading to the loss of Ped⁺ phenotype after ten or more daily transfers. Analysis by AGE of restriction fragments smaller than expected plasmids from several Ped⁻.Em^R transformants indicated deletion events. These deletions occurred primarily within the pediocin operon (data not shown), which apparently impaired the functionality of the pediocin operon but not that of *erm* in pPC418. Yet, after storage

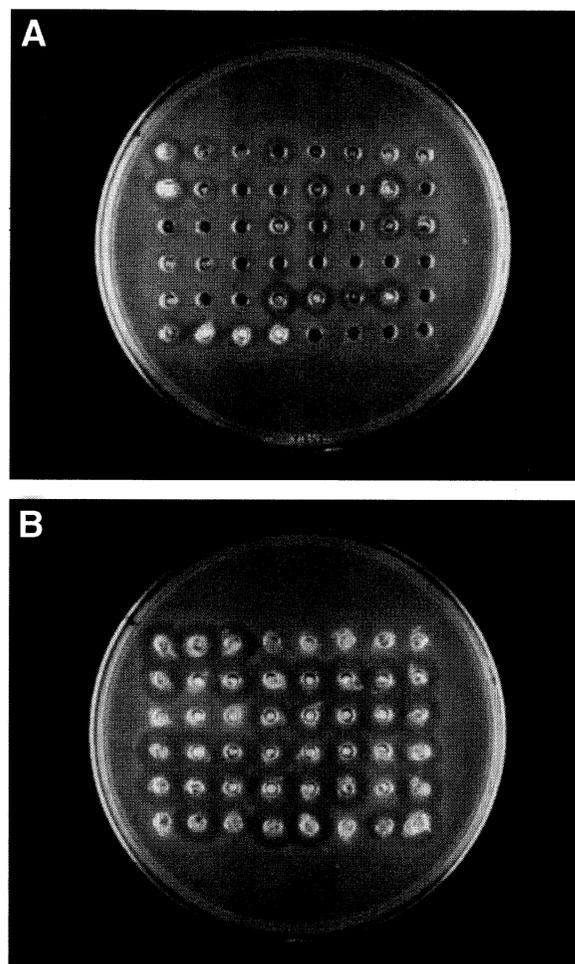


Fig. 3. Expression of pediocin in electrotransformed lactic acid bacteria. Top plate, *S. thermophilus* 128/pPC418; bottom plate, *E. faecalis* DL3/pPC418.

for 3 months or more at -70°C, Ped⁺.Em^R clones retained the Ped⁺ phenotype.

Pediocin sensitivity of other lactic acid bacteria. Susceptibility to pediocin was tested with 12 strains of *S. thermophilus* and 10 strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*, the companion of the former species in starter cultures used in yogurt production. Without exception, all 22 strains grew normally in agar plates when challenged with the culture filtrate of a Ped⁺ ST128 strain (ca. 6400 AU.ml⁻¹ against *P. pentosaceus*), indicating a natural resistance of these two LAB species to pediocin.

Discussion

We have constructed two shuttle vectors capable of harboring and expressing the pediocin operon in *E. coli* DH5α and LAB species *S. thermophilus*, *E. faecalis* and

Lactococcus lactis subsp. *lactis*. These vectors, pPC318 and pPC418, are equipped with *ori* and *repA* genes that allow for replication in *E. coli* and LAB backgrounds, respectively. Both plasmids harbor all four structural genes of the pediocin operon (*pedA*, *pedB*, *pedC*, *pedD*) that encode prepediocin, pediocin immunity, pediocin secretion, and pediocin translocation respectively [4, 26] but not the promoter element of *pedA* from *P. acidilactici*. Instead, in pPC318 and pPC418, the ST128 chromosomal promoter ST_{P2201} was cloned upstream to *pedA* to facilitate the transcription of the pediocin gene cluster in both *E. coli* and LABs. The successful application of ST_{P2201} once again demonstrated the versatility of this promoter, which had been used previously in lactic acid bacteria to promote expression of cholesterol oxidase from *Streptomyces* and the green fluorescent protein from jellyfish [20, 21].

Electrotransformants of all four types of LAB used in this study produced active pediocin when the pediocin operon lacking its native promoter was fitted with the *S. thermophilus* promoter sequence ST_{P2201}. This was especially encouraging in light of a report by Venema et al. [26] proposing that pediocin expression required a region of DNA 352 nt upstream of *pedA*, suggesting the presence of an unidentified control element in this region. From our results, it appears that the presumed control element was not actually required for the production of active pediocin irrespective of *E. coli*, lactococcal, enterococcal, or streptococcal backgrounds.

The ability to express pediocin in mesophilic lactococci was anticipated in light of the similarities of pediocin to lactococcin A, a bacteriocin from *Lactococcus lactis* subsp. *lactis* bv. *diacetylatis* WM4 [7]. These investigators expressed a hybrid gene encoding the lactococcal promoter and leader regions from the *lcnA* gene and the mature portion of the pediocin gene. Presumably, the lactococcal secretory mechanism allowed for the expression of this hybrid in *Lactococcus lactis* subsp. *lactis* IL1403. In addition, the PedC and PedD proteins involved in pediocin secretion are homologous to the LcnC and LcnD proteins encoded in the lactococcin operon [7]. However, these investigators found that the genes for lactococcin A (*lcnA*) and immunity (*lcnB*) were the minimal requirements for bacteriocin production in lactococci.

Segregational studies indicated that pPC418 was relatively stable (up to 8–10 transfer cycles) in *S. thermophilus* ST128, *E. faecalis* DL3, and *L. lactis* subsp. *lactis* ML3, but not in *L. lactis* subsp. *lactis* SLA1.1. However, continued serial transfers resulted in the gradual loss of Ped⁺ phenotype in all host systems tested. The variable stability of pPC418 in different LAB hosts was not unexpected, since strain-dependent instability of the

cloning vector pMEU5a, the source of pPC418, was reported previously in transformants of both *E. coli* and *S. thermophilus* [19]. Precedent for similar instability was also observed by Kok et al. [9], who reported that the low-copy-number plasmid pWV01 and the high-copy-number plasmid pSH71 were both capable of replication in some strains of *E. coli*. Since erythromycin resistance and plasmids of reduced size persisted in Ped⁻ LAB transformants through several generations, it is most likely that the loss of Ped⁺ was due to deletion events involving one or more key components of the pediocin operon, which was also evidenced by altered plasmid restriction patterns.

The apparent reduced production of pediocin in *E. coli* harboring pPC318 in comparison with *P. acidilactici* was probably owing to variation in secretory systems and perhaps the pH environment of the media. Biswas et al. [1] reported that processing of prepediocin to active pediocin by *P. acidilactici* requires a final pH of 5.0 or less in the culture medium to ensure processing efficiency [1]. Presumably, the lactic acid production of the *P. acidilactici*, but not *E. coli*, would lower the environmental pH and thus enhance processing of prepediocin to mature pediocin. This environmental pH effect has important implications for the in situ production and activation of pediocin as a bioprotective agent in dairy products against susceptible foodborne pathogens.

The expression of pediocin by genetically engineered *S. thermophilus* and *Lactococcus lactis* subsp. *lactis* is significant since both of these organisms are constituents of normal starter cultures used in the production of fermented dairy products. Expression of the pediocin operon in dairy lactic bacteria is particularly important since the natural producer of pediocin, *P. acidilactici*, which is primarily associated with vegetable fermentations, grows poorly in dairy foods in which lactose is the predominant fermentable carbohydrate [13]. *Listeria monocytogenes* is a food-borne organism of major concern to the dairy industry since it can proliferate in various dairy products at low pH and temperature [5]. Pediocin has strong antimicrobial activity against this microbe, and the successful use of pediocin-producing *L. lactis* subsp. *lactis* to control the growth of *Listeria* in cheese was recently reported [3]. It is anticipated that future studies will concentrate on the construction of pediocin-producing LAB strains eligible for receiving GRAS (“generally recognized as safe”) classification, which will permit specific applications of these bioprotective cultures in foods.

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