Heterologous Production of Antimicrobial Peptides in Propionibacterium freudenreichii

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Heterologous bacteriocin production in Propionibacterium freudenreichii is described. We developed an efficient system for DNA shuttling between Escherichia coli and P. freudenreichii using vector pAMT1. It is based on the P. freudenreichii rolling-circle replicating plasmid pME108 and carries the cmr(A)/cmx(A) chloramphenicol resistance marker. Introduction of the propionicin T1 structural gene (pctA) into pAMT1 under the control of the constitutive promoter (Pc) yielded bacteriocin in amounts equal to those of the wild-type producer Propionibacterium thoenii 419. The P. freudenreichii clone showed propionicin T1 activity in coculture, killing 90% of sensitive bacteria within 48 h. The pamA gene from P. thoenii 419 encoding the protease-activated antimicrobial peptide (PAMP) was cloned and expressed in P. freudenreichii, resulting in secretion of the pro-PAMP protein. Like in the wild type, PAMP activation was dependent on externally added protease. Secretion of the antimicrobial peptide was obtained from a clone in which the pamA signal peptide and PAMP were fused in frame. The promoter region of pamA was identified by fusion of putative promoter fragments to the coding sequence of the pctA gene. The Pc and P_pamA promoters directed constitutive gene expression, and activity of both promoters was enhanced by elements upstream of the promoter core region.

Propionic acid bacteria (PAB) are economically important bacteria used in the production of Swiss-type cheese. The influence of PAB in the cheese-making process has been extensively studied by microbiological and biochemical methods (10, 23). During the last few years, much of the scientific focus has been directed towards studies of the antimicrobial potential of PAB. The dairy PAB species have achieved a "generally recognized as safe" status, which makes their antimicrobial substances attractive as food preservatives (1, 2). For instance, propionic acid is commonly used as a mold inhibitor (2). PAB also have a potential use as protective cultures for inhibition of pathogens and food spoilage organisms (30, 31). The antimicrobial capacity of PAB is only partly due to the production of organic acids, and it has become evident that PAB also produce other biologically active substances such as bacteriocins (4, 7, 9, 13, 26). Recently, Faye et al. (9) characterized the propionicin T1 bacteriocin from Propionibacterium thoenii. The propionicin T1 gene locus is organized in an operon structure with a putative ABC transporter (orf2) immediately downstream of the bacteriocin structural gene pctA. Propionicin T1 is an unmodified peptide that contains a signal sequence probably recognized by the general secretory (sec) pathway. The bacteriocin is inhibitory to all dairy PAB species except Propionibacterium freudenreichii. Faye et al. (7) purified a bacteriocin-like peptide, protease-activated antimicrobial peptide (PAMP), from protease-treated culture supernatants of Propionibacterium jensenii LMGT 3032. Biochemical and genetic analysis revealed that the PAMP-encoding gene, pamA, encodes a 225-amino-acid preproprotein with a 27-residue leader peptide. Mature PAMP is comprised of the 64 C-terminal residues of the secreted 198-residue proprotein. P. jensenii LMGT 3032 constitutively produces pro-PAMP during growth in sodium lactate broth. Besides pamA, no genes connected to pro-PAMP production have been identified. The function of the PAMP prodomain remains elusive, but an involvement in protection of the producer has been suggested (7).

Genetic investigations of dairy PAB have been limited, and characterization of gene function has previously only been possible through reverse genetics or expression in heterologous hosts such as Escherichia coli (11, 21, 24, 28). About 30 PAB genes have been characterized with an assigned function (33). However, the recent publication of the genome sequence of Propionibacterium acnes (5), a nondairy pathogenic species, provides an important source of information for the genetic study of dairy propionibacteria. Furthermore, the current improvements in tools for genetic manipulation of PAB will develop functional genetic characterization of dairy propioni-bacteria (16, 18, 19). Nevertheless, PAB transformation has proved to be difficult, especially with DNA prepared from E. coli (16, 18). This trait represents a major obstacle, since most cloning requires E. coli as an intermediate host. Compared to other bacterial transformation systems, the number of Propionibacterium shuttle vectors (including expression vectors) is limited, all of which originate from two theta-type replication plasmids (16, 18, 26). In this work, we have developed a new E. coli-Propionibacterium shuttle vector based on rolling-circle replication in propionibacteria and designed an efficient
method for transformation of \textit{P. freudenreichii} with plasmids constructed via \textit{E. coli}. This protocol was used to study heterologous expression of the propionicin T1 and pro-PAMP-encoding genes in \textit{P. freudenreichii}.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains, vectors, and media}.

The bacterial strains and vectors are shown in Table 1. \textit{E. coli} was cultivated at 37°C in LB medium supplemented with 100 µg/ml of ampicillin or 50 µg/ml kanamycin where appropriate. Propionibacteria were grown anaerobically at 30°C in sodium lactate broth (SLB) (4). Lactobacilli were propagated anaerobically in MRS medium (Oxoid, Basing-stoke, Hampshire, United Kingdom) at 30°C. Determination of the MIC of chloramphenicol was performed for propionibacteria on solidified LB medium containing 1.6% agar with E-test strips (AB Biodisk, Sweden).

\textbf{General methods}.

General molecular biological techniques used in this study were performed as described previously by Sambrook et al. (29), unless otherwise stated. Transformation of \textit{E. coli} was performed according to a method described previously by Inoue et al. (15). Plasmid DNA for cloning was purified with QiAprep spin columns, while plasmid DNA for transformation of \textit{P. freudenreichii} was prepared by use of Midi Prep columns (QIAGEN, Hilden, Germany). Restriction enzymes and DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.) or Fermentas (Vilnius, Lithuania). DNA amplified by PCR for cloning was done in 100-µl reaction mixtures using 2.5 units of Taq polymerase (Invitrogen, Paisley, United Kingdom) and 100 pmol of each primer. The PCR conditions included a polymerase activation/tempplate denatura-tation step at 94°C (3 min) followed by 35 cycles of denaturing at 94°C (15 s), annealing at 57 to 60°C (30 s), and polymerization at 68°C. Taq polymerase (QIAGEN) was used to add single-nucleotide 3'-A overhangs to PCR products. DNA fragments from PCR amplification or restriction digests were analyzed by agarose gel electrophoresis and purified on QiAquick purification columns (QIAGEN). DNA sequencing was performed with the BigDye V.3.1 Terminator cycle sequencing ready reaction kit and an Applied Biosystems (Foster City, Calif.) model 3100 genetic analyzer. All products were used according to the manufacturers' instructions.

\textbf{DNA transformation of propionibacteria}.

Electrocompetent \textit{P. freudenreichii} isolates were prepared from cells precultivated overnight in SLB. This preculture was diluted 1:50 in SLB and further incubated for 18 h (A$_{620}$~0.7), placed on ice for 30 min, and then harvested by centrifugation at 5,000 × g for 4 min. The cells were washed twice in 1 volume of ice-cold distilled water and once in 1 volume of 10% glycerol. Finally, the cells were suspended in 70-µl aliquots and stored at −80°C. Electroporation was performed with a Gene Pulser apparatus (Bio-Rad, Hercules, Calif.) using 35 µl of the cell suspension mixed with DNA in a cooled 1-mm electroporation cuvette. An electric pulse was delivered at 200-fs resistance and 25-µF capacitance at 20 kV/cm. Immediately after the pulse, 930 µl of SLB medium was added to the cell suspension. The cells were further incubated at 30°C for 3 h before appropriate volumes were plated onto SLB agar supplemented with 3.4 µg/ml and 10 µg/ml chloramphenicol for \textit{P. freudenreichii} IFO12426 and \textit{P. freudenreichii} ATCC6207, respectively. The plates were incubated at 30°C under anaerobic conditions, and transformants could be detected after 5 to 10 days.

\textbf{DNA preparation from Propionibacterium freudenreichii cells}.

Plasmid mini-preparations from \textit{P. freudenreichii} were performed using cells from a 5-ml overnight culture. The cells were washed in 1 volume of STE buffer (100 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl at pH 8.0) before they were suspended in 0.25 ml GTE buffer (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl at pH 8.0) containing 100 µg/ml RNase (Sigma, St. Louis, Mo.) and 10 mg/ml lysozyme (Sigma). The cell suspension was incubated at 37°C for 15 min prior to the addition of 0.25 ml of alkaline lysis solution P2 (1% sodium dodecyl sulfate, 0.2 N NaOH) and further incubated at room temperature for 5 min. Next, 0.35 ml of neutralization buffer P3 (3 M potassium acetate, 2 M acetic acid, pH 5.4)
was added before the cell debris was removed by centrifugation (13,000 rpm, 10 min). The resulting supernatant was applied onto a QIAprep spin column (QIAGEN). Subsequent steps in the procedure were performed according to the plasmid Mini Prep protocol of QIAGEN. Large-scale plasmid preparations from 200-ml PAB cultures were performed with the Nucleobond AX 500 kit (Macherey-Nagel) including an additional lysis step by incubating the cells with 10 mg/ml lysozyme and 30 U/ml mutanolysin at 37°C for 30 min. Isolation of total DNA from *P. freudenreichii* was done from 5-ml overnight cultures (A600 ~0.5) using Advamix beads according to manufacturer’s recommendations (Advanced Genetic Technologies Corp., Gaithersburg, Md.).

**Construction of Propionibacterium-E. coli shuttle vector pAMT1.** A 1.5-kb PCR fragment containing the cmx(A) and cmx(A) genes was amplified from the *Corynebacterium striatum* pTP10 plasmid (32) using primer pair cmx1-cmx2 (Table 2). This fragment was cloned into Sim-digested pUC18 DNA. The resulting plasmid was cut at the Sall site and ligated with XhoI-digested *(P. freudenreichii)* resulting plasmid was cut at the SalI site and ligated with XhoI-digested pLME108, resulting in the pAMT1 vector (Table 1 and Fig. 1).

**TABLE 2. List of primers used in this study**

| Primer      | Sequence (5’–3’) | Relevant characteristics
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>PAMP1</td>
<td>GG CGC TGG CAG ATG GTA GGA</td>
<td>pAM1 sec, Rev</td>
</tr>
<tr>
<td>PAMP3</td>
<td>CAT CGG GCC TGG CCC TCC</td>
<td>pAM1 promoter, Rev</td>
</tr>
<tr>
<td>PAMP5</td>
<td>GA GGA GGC CCA GCC CCG ATG AAG AAG ACC CTC CTA CGA AGT</td>
<td>pctA-Ppamp fusion, Fwd</td>
</tr>
<tr>
<td>PAMP2</td>
<td>TCC TAC CAT CTC CCA CGG CC AAG GCC AGG GCG CCC CTC CAA AAG</td>
<td>sec-PAMP fusion, Fwd</td>
</tr>
<tr>
<td>PAMP6</td>
<td>GAA GTC TGT CAG TAC TGG GGT TCC CTT TTC</td>
<td>pamA, Rev</td>
</tr>
<tr>
<td>PAMP7</td>
<td>CCG CAC GAT ATG GTT TGG GGT GTG AGA ATT CCA GTC ACG ACT AC</td>
<td>pam-Af1 promoter fusion, Fwd</td>
</tr>
<tr>
<td>PCTA1</td>
<td>CCG CAC GAT ATG GTT TGG GGT GTG AGA ATT CCA GTC ACG ACT AC</td>
<td>pam-Af1 promoter fusion, Fwd</td>
</tr>
<tr>
<td>PAMP4</td>
<td>GATC TCA TGG GGT TTC TCC GCT GAC</td>
<td>pctA-Ppamp promoter fusion, Fwd</td>
</tr>
<tr>
<td>P4A</td>
<td>ACC CGG ACA CAT ATC GTC CG</td>
<td>Pp promoter, Rev</td>
</tr>
<tr>
<td>P4B</td>
<td>CGA AGG CCT CCG AAA ATG C</td>
<td>Pp promoter, Fwd</td>
</tr>
<tr>
<td>P4C</td>
<td>TCG AGT TGC AGG CGG AGG</td>
<td>Pp promoter, Fwd</td>
</tr>
<tr>
<td>419PC</td>
<td>GTC TCA TGG GGT TTC TCC CTT TTT</td>
<td>pctA, Rev</td>
</tr>
<tr>
<td>419PG</td>
<td>ACC TCC CAC CAA GAT CCA ACC</td>
<td>Ppct promoter, Fwd</td>
</tr>
<tr>
<td>419PS</td>
<td>ACC CAC TGA TGG CCA AGT G</td>
<td>Ppct promoter, Fwd</td>
</tr>
<tr>
<td>cmx1</td>
<td>GAT GGC TCA TCA ATT GGC CTC</td>
<td>Complete cmx(A)</td>
</tr>
<tr>
<td>cmx2</td>
<td>CTC CAC CGC AGC ACA TGT CG</td>
<td>Complete cmx(A)</td>
</tr>
</tbody>
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* Rev, reverse primer; Fwd, forward primer.

A fragment containing the propionicin T1-encoding gene (pctA) and 75 bp of the putative promoter (PpctE) and the pctA gene was amplified from *P. thoenii* 419 using primers 419P5 and 419PC and cloned into pAMT1, resulting in the pTD110 plasmid. The putative promoter region of the *pctA* gene was analyzed for its ability to direct expression of the *pctA* gene. The *PpctE* fragment, which covers 150 bp upstream from the *pctA* initiation codon, was amplified using primers PAMP8.

A fragment containing the *pctA* 5’-untranslated leader peptide. These two fragments were mixed and served as a template in the second round of PCR with primers P4B and PAMP6, which generated a new prebacteriocin gene where the *pctA* sec leader is fused to the N terminus of the mature PAMP peptide. Thus, in the pTD114 plasmid, the *PpctE* promoter directly expresses of *pctA* with an in-frame deletion of the prodomain-encoding part of the gene.

**Construction of propionicin-T1 and PAMP expression plasmids.** A number of propionicin T1 and PAMP expression plasmids were devised and introduced into *P. freudenreichii* IFO12426. First, the desired promoter and bacteriocin gene fragments were generated by PCR, cloned into the pCR2.1 Topo AT vector (Invitrogen), and subsequently cloned as XbaI-SpeI or XbaI-BamHI fragments in the E. coli-PAB shuttle vector pAMT1 (Table 1 and Fig. 2).

A fragment containing the propionicin T1-encoding gene (pctA) and 75 bp of the putative promoter (PpctE) was amplified from *P. thoenii* 419 using primers 419PG and 419PC and cloned into pAMT1, resulting in the pTD101 plasmid. Next, a fragment encompassing 400 bp of the putative propionicin promoter (PpctE) and the pctA gene was amplified from *P. thoenii* 419 with primers 419PS and 419PC and used to construct the pTD102 plasmid. The PAMP structural gene (pamA) with its putative short *pamA* promoter, Rev and Fwd, was amplified from *P. jensenii* and *P. thoenii* upstream from the pamA gene. The *PpampS* fragment, which covers 150 bp upstream from pamA initiation codon, was amplified using primers PAMP8 and PAMP6 and cloned into pAMT1, resulting in the pTD112 plasmid with primers PAMP1 and P4B. Next, the PAMP-encoding part of *pamA* was amplified with primers PAMP2 and PAMP6, which produces a 20-bp add-on complementary to the pamA leader peptide. These two fragments were mixed and served as a template in the second round of PCR with primers P4B and PAMP6, which generated a new prebacteriocin gene where the *pamA* sec leader is fused to the N terminus of the mature PAMP peptide. Thus, in the pTD114 plasmid, the *PpctA* promoter directs expression of *pctA* with an in-frame deletion of the prodomain-encoding part of the gene.
and PAMP3. The elongated P_{pamp} (P\_pamp\_el) fragment, which encompasses 480 bp upstream from the pamA initiation codon, was amplified using primers PAMP3 in combination with PAMP4. Subsequently, these fragments were fused to the pctlA gene amplified with primers PAMP5 and 419PC. The resulting promoter-gene fusions were used to construct plasmids pTD110 and pTD116, respectively.

All constructs were electroporated into P. freudenreichii IFO12426 where correct transformants were confirmed by restriction fragment analysis and DNA sequencing and subsequently screened for bacteriocin production.

**Propionicin T1 and PAMP bioassays.** P. freudenreichii IFO12426 carrying different bacteriocin expression constructs was grown on SLB plates without antibiotic for 120 or 240 h. A lawn of 5 ml SLB soft agar containing 500 μl of an overnight culture of the indicator organism was then poured over the plates for propionicin T1 expression, the standard indicator was P. acidipropionici ATCC 4965 as the amount of bacteriocin that produced 50% growth inhibition of the indicator organism was measured spectrophotometrially (A_{600}) using a microtiter plate reader (Multiscan Ascent; Labsystems, Finland). One bacteriocin unit (BU) was defined as the amount of bacteriocin that produced 50% growth inhibition of the indicator bacterium compared to a culture without added bacteriocin.

**RESULTS AND DISCUSSION**

Development of vector and transformation procedure for efficient E. coli-Propionibacterium gene shuttling. In a previous work, we did an extensive search for plasmids in propionibacteria (25). Plasmid pLME108 (2,051 bp) was isolated from Propionibacterium freudenreichii subsp. shermanii, and its replicon was identified by comparative DNA analysis. It contained a putative replicase gene (rep) showing an identity of 42% to the rep gene of the Arcanobacterium pyogenes plasmid pAP1, which uses the rolling-circle mechanism for replication (3). The replicon of pLME108 was fused with the E. coli replicon from pUC18 and the cml(A)/cmx(A) chloramphenicol resistance marker genes (Table 1) from Corynebacterium striatum (32). The resulting construct (pAMT1) (Fig. 1) was successfully transformed into E. coli JM110 and P. freudenreichii subsp. freudenreichii ATCC 6207 using ampicillin and chloramphenicol selection, respectively. The MIC of chloramphenicol could be augmented by about 100-fold from 0.05 to 4 to 6 μg/ml considering the MIC of nontransformed recipient strains of Propionibacterium. Electroporation of P. freudenreichii ATCC 6207 with pAMT1 from E. coli only gave 10 to 20 transformants/μg DNA, while a high efficiency of 10^8 transformants/μg DNA was obtained when the vector was prepared from P. freudenreichii. The difference is probably due to the presence of restriction-modification systems in PAB (16, 18). The low number of transformants achieved with DNA prepared from E. coli represented a major obstacle for studying gene function in PAB. In a previous study, Kiatpapan et al. (18) described transformation of P. freudenreichii using the E. coli/PAB shuttle vector pPK705, which contains a hygromycin B selection marker. According to the authors, this vector could be propagated in E. coli and then transformed into a P. freudenreichii subsp. shermanii strain with an efficiency of 10^7 transformants/μg vector DNA. However, we experienced a high background of nontransformed colonies using hygromycin B selection, which hampered the use of the pPK705 vector. In contrast, the cml(A)/cmx(A) chloramphenicol resistance marker of pAMT1 provided efficient selection without any background. The data did, however, indicate that the efficiency of the restriction barrier to foreign plasmid DNA could be strain dependent. Based on these premises, we devised an optimized protocol for transformation of P. freudenreichii with vector DNA from E. coli. Electroporation of competent P. freudenreichii subsp. shermanii IFO12426 cells produced 10^6 and >10^7 transformants/μg DNA with vector prepared from E. coli and P. freudenreichii, respectively. With DNA from E. coli,
this is an improvement in transformation efficiency by 3 orders magnitude compared to the transformation of P. freudenreichii ATCC 6207. Accordingly, all constructions made in subsequent cloning experiments were based on pAMT1, and P. freudenreichii subsp. shermanii IFO12426 served as the recipient for the bacteriocin expression plasmids.

**Heterologous expression of propionicin T1 in P. freudenreichii.** As part of a continued effort to study and exploit the antimicrobial potential of PAB, we used P. freudenreichii as a host for heterologous expression of a P. theoenii bacteriocin, propionicin T1 (9). The propionicin T1-encoding gene pctA was cloned in pAMT1 with either 75 or 400 bp of the upstream promoter region, resulting in expression plasmids pTD101 and pTD102, respectively. As shown in Table 3, the resulting P. freudenreichii clones did not produce any detectable amounts of propionicin T1. This could indicate that expression from the native propionicin T1 promoter is dependent on regulatory factors that are not present in P. freudenreichii. We therefore investigated if expression from the constitutive P. freudenreichii P₄ promoter (20) improved bacteriocin production. The pctA gene was cloned behind either a short (P₄₅) or extended (P₄₈₅) version of the P₄ promoter. As shown in Table 3, the resulting plasmids were able to facilitate propionicin T1 production in P. freudenreichii. Thus, the pctA structural gene encodes the information required for production and secretion of propionicin T1 in P. freudenreichii. The propionicin T1 locus contains an ABC transporter (orf2) directly downstream of the pctA gene (9). The presence of a sec leader in prepropionicin T1 and the fact that transport in P. freudenreichii occurred independently of orf2 indicate that the orf2 ABC transporter does not function as part of the propionicin T1 secretion apparatus. On agar plates, P. freudenreichii harboring the pTD104 plasmid produced large inhibition zones, while zones of inhibition with pTD103 were minute (Fig. 3). The difference in bacteriocin production between these clones was quantified in SLB cultures, where P. freudenreichii IFO12426 transformed with pTD103 and pTD104 reached a maximum propionicin T1 activity of 80 and 320 BU/ml, respectively (Table 3). Thus, the P₄₅ promoter directed bacteriocin activity that was approximately five times higher than that of P₄₈₅. Since the P₄₈₅ fragment contains the predicted ribosome binding site and the −10 and −35 promoter elements, it appears that the P₄₅ fragment contains unidentified elements upstream of the core promoter that contribute to activity.

**The naturally occurring pctA-A allele encodes an inactive propionicin T1 variant.** A recent survey on the prevalence of the pctA gene revealed that 13 of 24 P. jensenii strains contained this gene. However, only five strains produced antimicrobial activity corresponding to propionicin T1 (8). Intriguingly, six of the propionicin T1-negative P. jensenii strains contained a G→A transition mutation in the pctA gene, resulting in the amino acid substitution G₃₅D in the mature bacteriocin (8). In order to investigate the biological activity of this propionicin T1 variant, the mutated gene (pctA-A) was cloned under the control of the P₄₅ promoter in the pAMT1 vector. Transfer of the resulting plasmid, pTD105 (Fig. 2A), into P. freudenreichii IFO12426 was confirmed by restriction analysis and DNA sequencing. This clone showed no antimicrobial activity in agar overlay assays (Fig. 3B) or in liquid cultures (Table 3). Thus, the point mutation in the pctA-A allele results...
in drastic changes of the antimicrobial properties of the encoded peptide. In vitro mutagenesis studies of the bacteriocin pediocin Ac-H have demonstrated that most amino acid substitutions that change either structural or physicochemical properties of the peptide greatly influence its antimicrobial properties (27). Similarly, amino acid substitutions that reduce the net positive charge of sakacin P result in a less potent bacteriocin (17). The G23D substitution only slightly reduces the net positive charge of propionicin T1, but it is possible that introduction of the negatively charged aspartate residue causes a structural change that diminishes the antimicrobial activity of the peptide.

**Antagonistic activity of a propionicin T1-producing** *P. freudenreichii* **in cocultures.** Purified propionicin T1 has been demonstrated to kill sensitive bacteria (9). We investigated whether it was possible to achieve the same antagonistic effect in situ from a propionicin T1-producing *P. freudenreichii* towards sensitive bacteria. A vector stability experiment was conducted on the *P. freudenreichii* IFO12426(pTD104) clone where all tested colonies were chloramphenicol resistant and produced propionicin T1 (data not shown). Encouraged by the fact that the expression plasmid and bacteriocin production were stably maintained in the culture, we designed a cocultivation competition assay. SLB broth was inoculated with ~5 × 10^6 CFU/ml of a spontaneous erythromycin-resistant mutant strain of *P. acidipropionici* ATCC 4965 was inoculated with 5 × 10^6 CFU/ml of *P. freudenreichii* IFO12426 in SLB medium. At 10^9 CFU/ml of *P. acidipropionici* ATCC 4965*Ery* only; □, *P. acidipropionici* ATCC 4965*Ery* cocultured with *P. freudenreichii* IFO12426(pAMT1); △, *P. acidipropionici* ATCC 4965*Ery* cocultured with *P. freudenreichii* IFO12426(pTD104). Appropriate dilutions of the cultures were plated out on SLB plates containing 10 μg/ml of erythromycin and incubated for 5 days before cell numbers of *P. acidipropionici* ATCC 4965*Ery* were determined. The results represent the averages of three independent experiments, and standard deviations are indicated.

shown in Fig. 4, *P. freudenreichii* IFO12426(pAMT1) did not prevent growth of *P. acidipropionici*. In contrast, the *P. freudenreichii* IFO12426(pTD104) clone efficiently prevented growth of *P. acidipropionici*. This effect appeared to be immediate and resulted in a 90% reduction in *P. acidipropionici* cell counts after 48 h. The effect was sustained throughout the test period and led to a 5-log reduction in *P. acidipropionici* viable counts compared to those of the *P. acidipropionici*- *P. freudenreichii* IFO12426(pAMT1) control culture. The fact that the propionicin T1 expression plasmid was stably maintained without selection and rendered high levels of bacteriocin production demonstrates the potential of propionicin T1-producing *P. freudenreichii* for practical applications. Growth of nonstarter pigmented and psychrophilic PAB in Swiss-type cheeses may cause brown spots and “anomalous blowing,” resulting in devaluated products and economic losses (6, 22). The use of a propionicin T1-producing *P. freudenreichii* as a secondary starter would be a convenient method to prevent growth of nonstarter PAB without affecting the lactic acid bacterial culture and facilitate a more controlled ripening of the cheese.

**Heterologous expression of pro-PAMP and PAMP in *P. freudenreichii.** Faye et al. (7) reported that *P. jensenii* LMG 3032 secretes large amounts of the 20-kDa pro-PAMP protein. Processing of pro-PAMP by protease K produces the bacteriocin-like peptide PAMP. The production of pro-PAMP is prevalent among strains of *P. jensenii* and *P. thoenii* (8). It has been suggested that the secretion of an inactive probacteriocin, whose activation relies on proteases in the environment, might represent a novel strategy for production of antimicrobial peptides and producer self-protection (7). We investigated the PAMP system by cloning pamA under the control of the P_as or
P<sub>4</sub>F promoter fragment in plasmids pTD112 and pTD113, respectively. In P. freudenreichii IFO12426, both plasmids conferred protease-dependent inhibition of L. sakei NCDO 2714 (Fig. 2B and 3C). The same pattern of antimicrobial activity was observed in liquid culture, but the amounts produced were less than 10% of that produced by the wild-type producer P. jensenii LMG 3032. The pTD110 plasmid contains the pamA gene with a frameshift mutation that results in the C-terminal deletion of 54 amino acids corresponding to the PAMP-specific part of pro-PAMP. This clone did not produce any antimicrobial activity (Fig. 2B and Table 3). Next, we designed another deletion variant of the pamA gene that encodes the pamA leader peptide fused directly to the N terminus of mature PAMP. The resulting gene (pamA<sub>∆</sub>pro) expressed from the P<sub>4</sub>F promoter was cloned in pTD114. In contrast to the pTD112 and pTD113 clones, protease activation was not necessary. The pTD114 clone displayed reduced growth capacity in broth, and only low levels of bacteriocin activity were produced. Since P. freudenreichii IFO12426 is sensitive to PAMP, it is possible that the growth reduction was caused by suicide expression. In terms of specific activity (BU·ml<sup>-1</sup>·A<sub>620</sub><sup>-1</sup>), pro-PAMP expression by pTD113 was approximately 20 times higher than PAMP production by pTD114 (data not shown). These results indicate that the presence of the prodomain protected P. freudenreichii IFO12426 from the antimicrobial activity of PAMP.

Identification of the pamA promoter region. The promoter region of pamA was analyzed using the pctA gene as a reporter. To achieve this, different segments of the putative PAMP promoter region were fused to the pctA gene and ligated into the pAM1 vector (Fig. 2A). The fact that P<sub>4</sub>F promoter activity depended on elements only present in the extended version of the P<sub>4</sub>F promoter encouraged us to investigate if the putative pamA promoter inherited similar features. The short promoter fragment P<sub>pampE</sub><sup>initiation</sup> was designed to encompass the ribosome binding sites and −10 and −35 promoter sequences predicted previously by Faye et al. (7), while the P<sub>pampE</sub><sup>leader</sup> fragment includes 480 bp upstream of the pamA initiation codon. The P. freudenreichii strain carrying the extended promoter fragment P<sub>pampE</sub> produced the most bacteriocin. In liquid culture, P<sub>pampE</sub> directed bacteriocin production that was eight times higher than that of P<sub>pampS</sub> (Table 3). Hence, like P<sub>4</sub>F, the PAMP promoter appears to contain upstream sequence elements that contribute to increased transcriptional activity. The nature of these signals remains elusive, but a detailed investigation of such is beyond the scope of this study. Nevertheless, the identification of the PAMP promoter demonstrates the potential of the pctA gene as an in vivo reporter for quantitative assessment of promoter activity in P. freudenreichii. Hopefully, more detailed knowledge on transcriptional regulation and promoter structure in PAB will be available in the near future.

Concluding remarks. This work describes the first successful cloning and heterologous expression of bacteriocins in P. freudenreichii. The results demonstrate the utility of the described genetic manipulation system for the study of gene function in P. freudenreichii and a potential for generation of strains with improved genetic features.

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