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Producer Immunity towards the Lantibiotic Pep5: Identification of the Immunity Gene *pepI* and Localization and Functional Analysis of Its Gene Product

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The lantibiotic Pep5 is produced by *Staphylococcus epidermidis* 5. Pep5 production and producer immunity are associated with the 20-kb plasmid pED503. A 1.3-kb *Kpn*I fragment of pED503, containing the Pep5 structural gene *pepA*, was subcloned into the *Escherichia coli-Staphylococcus* shuttle vector pCU1, and the recombinant plasmid pMR2 was transferred to the Pep5- and immunity-negative mutant *S. epidermidis* 5 Pep5$^-$ (devoid of pED503). This clone did not produce active Pep5 but showed the same degree of insensitivity towards Pep5 as did the wild-type strain. Sequencing of the 1.3-kb *Kpn*I-fragment and analysis of mutants demonstrated the involvement of two genes in Pep5 immunity, the structural gene *pepA* itself and *pepI*, a short open reading frame upstream of *pepA*. To identify the 69-amino-acid *pepI* gene product, we constructed an *E. coli* maltose-binding protein–PepI fusion clone. The immunity peptide PepI was detected in the soluble and membrane fractions of the wild-type strain and the immune mutants (harboring the plasmids pMR2 and pMR11) by immunoblotting with anti-maltose-binding protein–PepI antiserum. Strains harboring either *pepI* without *pepA* or *pepI* with incomplete *pepA* were not immune and did not produce PepI. Washing the membrane with salts and EDTA reduced the amount of PepI in this fraction, and treatment with Triton X-100 almost completely removed the peptide. Furthermore, PepI was hydrolyzed by proteases added to osmotically stabilized protoplasts. This suggests that PepI is loosely attached to the outside of the cytoplasmic membrane. Proline uptake and efflux experiments with immune and nonimmune strains also indicated that PepI may act at the membrane site.

Lantibiotics are lanthionine-containing, antimicrobial peptides exclusively produced by gram-positive bacteria (19). Pep5 (22), produced by *Staphylococcus epidermidis* 5, belongs to the subgroup of type A lantibiotics, further comprising nisin (14), subtilin (13), epidermin (1), and gallidermin (21). These amphiphilic cationic peptides share the same mode of action, characterized by the formation of voltage-dependent, short-lived pores which cause an efflux of ions and small molecules (27). In addition, for Pep5 and nisin, induction of autolysis by activation of cell wall-hydrolyzing enzymes has been demonstrated (3, 4). Biosynthesis of lantibiotics involves several posttranslational modifications of ribosomally synthesized precursor peptides (16–18, 30, 36, 46). The structural gene of Pep5, *pepA*, was shown to be plasmid encoded (20). The 60-amino-acid Pep5 prepeptide consists of a 26-residue leader peptide followed by a 34-residue propeptide region. In this propeptide part, serine and threonine residues are dehydrated to didehydroalanine and didehydrobutyrine, respectively; cysteine thiol groups are then added to the double bond of the didehydroamino acids to yield the characteristic lanthionine and methyllanthionine residues. Finally, the N-terminal leader peptide is cleaved off (57). Biosynthesis via posttranslational modifications of prepeptides clearly distinguishes lantibiotics from both peptide antibiotics synthesized by multienzyme complexes (e.g., gramicidin or valinomycin) and nonpeptide antibiotics, which are typically products of secondary metabolism (25). Lantibiotics do, however, have much in common with bacteriocins, characterized as proteins or complexes of proteins not active against the producer bacterium (26). The best-known bacteriocins are the colicins, proteins of molecular masses of 25 to 70 kDa which act on closely related bacterial strains via specific receptor proteins located in the outer membrane of gram-negative bacteria. Because of the absence of an outer membrane in gram-positive bacteria, receptor-mediated action is an exception (54). Consequently, the numerous bacteriocins of gram-positive bacteria which have been described have a broad action spectrum that is restricted to other gram-positive bacteria (51) and can under certain environmental conditions be extended to gram-negative bacteria (50). Bacteriocins of gram-positive bacteria are a heterogeneous group of antibacterial substances including autolytic enzymes, phospholipases, and, interestingly, a large number of small peptides in the same range of molecular masses as lantibiotics (2,000 to 8,000 Da) (35, 51–53). A common characteristic of bacteriocins is the existence of specific-immunity peptides or proteins, which protect the producer strains from the lethal action of their own products. Channel-forming colicins (e.g., colicins A, E1, and B) produced by *Escherichia coli* are known to be specifically antagonized by stoichiometric complex formation with immunity proteins residing in the cytoplasmic membrane (11, 47, 48). The Pep5 producer strain *S. epidermidis* 5 shows a significant degree of immunity, the genetic information for which appeared to be located on plasmid pED503 (9), which also harbors the structural gene *pepA* (20). The Pep5 immunity gene, *pepI*, could also be

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Characteristics	Reference
Strains		
S. epidermidis 5	Wild-type strain; contains 5 plasmids (pED501 to pED505); pED503 (20 kb) confers Imm^+ phenotype	9
S. epidermidis 5 $Pep5^-$	Cured variant of *S. epidermidis* 5, lacking pED503; Pep^- Imm^- phenotype	9
E. coli 71-18	F^- *lacI*^{q} (*lacZ*) M15 *proAB* (*lac-proAB*)*thi supE*	44
Plasmids		
pCU1	Amp^r Cm^r; 4.95-kb shuttle vector, contains pC194 and pUC19 sequences and multiple cloning site from pUC19	2
pCU1 derivatives		
pMR2	Amp^r Cm^r Pep^- Imm^+; contains 1.3-kb *Kpn*I fragment of pED503 in pCU1	39
pMR7	Amp^r Cm^r Pep^- Imm^-; contains 540-bp PCR-generated *pepA* subcloned into *Sma*I-linearized pCU1	39
pMR9	Amp^r Cm^r Pep^- Imm^-; contains 438-bp PCR-generated *pepI* subcloned into *Sma*I-linearized pCU1	39
pMR11	Amp^r Cm^r Pep^- Imm^+; contains 1.094-kb *Hae*III-*Hin*dIII fragment of pMR2 subcloned into *Sma*I-*Hin*dIII-cut pCU1	39
pMR13	Amp^r Cm^r Pep^- Imm^-; contains 660-bp *Alu*I fragment of pMR11 subcloned into *Sma*I-linearized pCU1	39

localized on this plasmid (39). For nisin, the most prominent lantibiotic, producer cell immunity and its genetic cotransfer with lantibiotic production have also been demonstrated (10). Recently, a gene encoding a 245-amino-acid protein to which an immunity function was ascribed has been detected in the nisin operon (28). Here we report the identification of *pepI* and its 69-amino-acid gene product, which provides immunity to the lethal, channel-forming action of the lantibiotic Pep5. Furthermore, the results indicate that both *pepI* and *pepA* are required for the expression of the immunity phenotype.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. The Pep5-producing wild-type strain *S. epidermidis* 5 and the nonproducing, Pep5-sensitive mutant *S. epidermidis* 5 $Pep5^-$, lacking plasmid pED503, were subcultured weekly on blood agar. *E. coli* 71-18 was used as the host for recombinant DNA. The 4.95-kb *E. coli-Staphylococcus* shuttle vector pCU1 was constructed from pC194 (33) and pUC19 (2, 56) and kindly provided by R. Rosenstein and F. Götz, Tübingen, Germany.

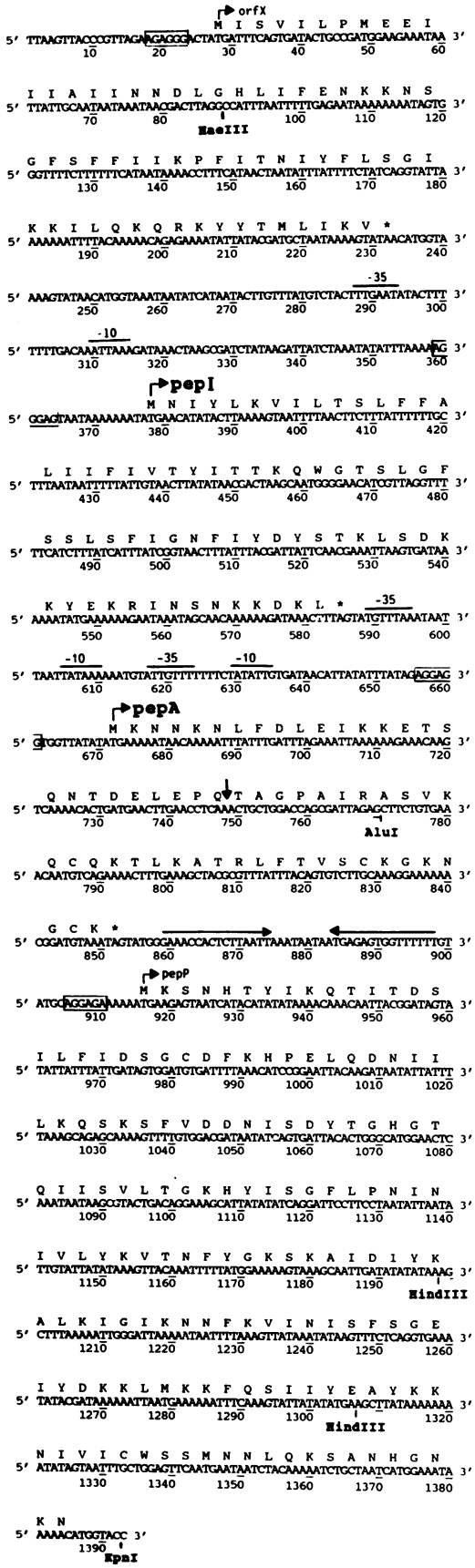
DNA cloning and sequencing. Plasmid DNA from *S. epidermidis* 5 and *S. epidermidis* 5 $Pep5^-$ cells was prepared according to the method in reference 5 but with an additional phenol-chloroform extraction and an RNase digestion. DNA from *E. coli* 71-18 derivatives was isolated by the method of Holmes and Quigley (15) or by buoyant density gradient centrifugation in cesium chloride-ethidium bromide (44). DNA cloning techniques and transformation of competent cells of *E. coli* 71-18 were performed as described previously (44). Transformation of *S. epidermidis* 5 $Pep5^-$ protoplasts was performed by the method of Götz and Schumacher (12). Nucleotide sequences were determined by sequencing both strands of double-stranded plasmid DNA by using the dideoxy chain termination method (45) and the T7 sequencing kit (Pharmacia, Uppsala, Sweden) with ^{35}S labeling. M13/pUC sequencing and reverse sequencing primers, as well as synthetic oligodeoxyribonucleotides, were used for sequencing and PCR. Synthesis was performed with 391 PCR Mate (Applied Biosystems, Weiterstadt, Germany). Sequence data were analyzed with the GENMON program, version 4.1 (GBF, Braunschweig, Germany).

PCR and primers. PCRs were carried out as described elsewhere (43) with the modifications given in reference 39. *pepA* and *pepI* were amplified with *Taq* polymerase, pMR2 as the template DNA, and the following primers: *pepA* 5′ primer, 5′d(AACGACTAAGCAATGGGG)3′; *pepA* 3′ primer, 5′d(AAATCACATCCACTATCAATAA)3′; *pepI* 5′ primer, 5′d(ATACGATGCTAATAAAAGTATAAC)3′; and *pepI* 3′ primer, 5′d(ACCACCTCCTCTATAAATATAAT)3′. For construction of the *malE-pepI* fusions, *pepI* was amplified with Vent-DNA-Polymerase, 0.1 μg of pMR2 DNA as the template, and the following primers: 5′d(ATGAACATATACTT AAAAGTAATTTTAAC)3′, containing the ATG start codon of *pepI*, and 5′d(CATACTAAAGTTTATCTTTTTTGTTGC) 3′, with CTA representing the TAG stop codon of *pepI*.

Construction and purification of the MBP-PepI fusion protein. Construction and purification of the maltose-binding protein (MBP)–PepI fusion protein were performed according to the methods in reference 40, as described in detail for the MBP-EpiD fusion protein in reference 29.

Production of polyclonal antiserum. New Zealand White rabbits were injected subcutaneously with a total amount of 1 mg of purified MBP-PepI fusion protein dissolved in 1.5 ml of phosphate-buffered saline and emulsified with incomplete Freund's adjuvant (1:1) for the primary immunization. After 6 weeks, intramuscular booster injections (500 μg of MBP-PepI fusion protein in 0.15 M NaCl) were given at 1-week intervals. Anti-MBP-PepI antiserum was collected after the third intramuscular booster injection.

Preparation of staphylococcal cell fractions. The wild-type strain *S. epidermidis* 5 and mutants were grown in 2 liters of half-concentrated tryptose soy broth (Oxoid, Wesel, Germany) to an A_{600} of 1.0 to 1.5. Phenylmethylsulfonyl fluoride (2 mM) was added to the cultures 30 min before the cells were harvested by centrifugation (5 min at 1,000 × *g*). Cells were washed once with 50 mM sodium phosphate buffer–5 mM dithioerythritol (pH 7.0) and resuspended in a small volume of the same buffer with 2 mM phenylmethylsulfonyl fluoride. Then glass beads were added (1:1 by vol), and the cells were broken in a Braun MSK cell homogenizer (20 times for 30 s each at 0°C). Unbroken cells and glass beads were removed by centrifugation (5 min, 1,000 × *g*), and the pellet was washed twice in a small volume of 50 mM sodium phosphate buffer (pH 7.0)–50 μM phenylmethylsulfonyl fluoride and again sedimented by centrifugation (5 min, 4,300 × *g*). For separation of cell membranes, the supernatant was subjected to a



further centrifugation step (48,000 × *g*, 30 min). The resulting pellet was resuspended in 2.4 ml of 50 mM sodium phosphate buffer (pH 7.0) and stored together with the supernatant at −20°C. Both fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Cell membranes were washed five times in 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM dithioerythritol, twice in 5 mM $MgSO_4$, four times in 10 mM EDTA, twice in 0.01% Triton X-100, and twice in 0.1% Triton X-100, each dissolved in 50 mM sodium phosphate buffer (pH 7.0). The respective pellets were resuspended in sodium phosphate buffer, and 25-μl aliquots were subjected to SDS-PAGE and immunoblotting with anti-MBP-PepI antiserum. For localization of PepI, the wild-type strain *S. epidermidis* 5 was grown in tryptose soy broth to an A_{600} of 1.5. The culture was then divided, and one-half was supplemented with either 20% (wt/vol) sucrose or 30% (wt/vol) raffinose to prevent lysis of protoplasts and liberation of cytoplasmic proteins. Both parts received achromopeptidase (Wako Chemicals), lysostaphin (Sigma), and lysozyme (Sigma) in final concentrations of 100 μg ml^{-1}; none of these cell wall hydrolases alone is able to completely lyse cell walls of *S. epidermidis*. After 30 min of incubation at 35°C, proteinase K and chymotrypsin (10 μg ml^{-1} each, final concentration) were added to cleave proteins outside the cytoplasmic membrane in the protoplast suspension and all accessible protein of lysed cells, respectively. After 30 min of proteolysis, 5-ml samples of each culture were taken and centrifuged for 5 min at 1,000 × *g*. Trichloroacetic acid (final concentration, 20% [wt/vol]) was then added to the supernatants of samples and controls, and the precipitate was removed by centrifugation and dissolved in SDS-PAGE sample buffer. Insoluble debris was removed by centrifugation, and appropriate aliquots of the supernatants were subjected to SDS-PAGE and Western blot (immunoblot) analysis for the presence of PepI.

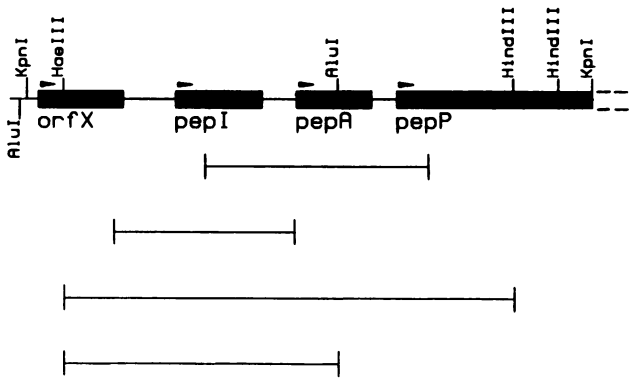
SDS-PAGE and immunoblotting. SDS-PAGE was performed in 15% polyacrylamide gels according to the method described by Laemmli (31). Conditions for Western blotting were as described in reference 57, except that 1% (vol/vol) skim milk was used for blocking; blots were incubated in diluted anti-MBP-PepI antiserum (1:500 in phosphate-buffered saline) and then treated with anti-rabbit immunoglobulin G-biotin conjugate (1:5,000; 1 h) and with streptavidin-biotinylated horseradish peroxidase complex (1:3,000; 20 min). PepI was visualized by enhanced chemiluminescence with Hyperfilm-ECL.

Uptake and efflux of radioactively labeled amino acids. Experiments measuring uptake and efflux of radioactively labeled amino acids were performed as described by Ruhr and Sahl (41).

Pep5 sensitivity (immunity) test. Pep5 sensitivity tests and detection of Pep5 production were carried out as described in reference 39. In addition, MICs of Pep5 for *S. epidermidis* 5 Pep5$^-$ and *S. simulans* 22 were determined in routine broth dilution tests using Mueller-Hinton broth in microtiter plates.

Nucleotide sequence accession number. The nucleotide sequences presented here have been deposited at GenBank under accession number L23967.

FIG. 1. Nucleotide and deduced amino acid sequences of the 1.3-kb *Kpn*I fragment of pED503. Putative ribosome binding sites are boxed. The processing site of pre-Pep5 and the putative rho-independent terminator downstream of *pepA* are indicated by arrows. Cut sites of restriction enzymes used and putative −35 and −10 sequences upstream of *pepI* and *pepA* are indicated.



Plasmid	Production of Pep5	Production of pre-Pep5	Production of PepI	Immunity Phenotype
pMR2	−	+	+	+
pMR7	−	+	−	−
pMR9	−	−	−	−
pMR11	−	+	+	+
pMR13	−	−	−	−

FIG. 2. Construction and analysis of *S. epidermidis* 5 Pep5$^-$ variants, with regard to pre-Pep5 and PepI production and immunity phenotype. Mutants were generated by PCR (pMR7 and pMR9) or by restriction digestion of the 1.3-kb *Kpn*I fragment (pMR11 and pMR13) (see Table 1). Cut sites of restriction enzymes used are indicated, and the resulting fragments subcloned are shown by solid bars. Pep5, pre-Pep5, and PepI production and immunity of the mutants are shown on the right. The immunity phenotype was tested as described in Materials and Methods.

RESULTS

Localization and sequencing of the Pep5 immunity-coding DNA region. The structural gene *pepA* was localized on a 2.1-kb *Bgl*II fragment of the 20-kb plasmid pED503 by hybridization (20). A further restriction digestion of this fragment yielded a 1.3-kb *Kpn*I fragment, still harboring *pepA*, which was subsequently subcloned into the *E. coli-Staphylococcus* shuttle vector pCU1. The recombinant plasmid, designated pMR2, was then transferred into the nonproducing and Pep5-sensitive strain *S. epidermidis* 5 Pep5$^-$ (devoid of plasmid pED503) by protoplast transformation. The resulting clones did not produce active Pep5 but showed the same degree of insensitivity towards Pep5 as did the wild-type strain. In quantitative sensitivity tests with purified Pep5, both strains were sensitive only at concentrations higher than 160 μg of Pep5 ml^{-1}, in contrast to the plasmid-cured strain *S. epidermidis* 5 Pep5$^-$, which was already inhibited at 0.605 μg of Pep5 ml^{-1}. Thus, the immunity phenotype appeared to be completely restored in the transformants harboring plasmid pMR2.

Double-stranded DNA sequencing of the 1.3-kb *Kpn*I fragment yielded the entire nucleotide sequence shown in Fig. 1. In addition to the structural gene *pepA*, three other open reading frames (ORFs) within this sequence were identified and were designated ORFX, *pepI*, and *pepP*. ORFX and *pepI* are short ORFs located upstream of *pepA*, sharing the same transcription direction and coding for putative 68- and 69-amino-acid peptides, respectively. All ORFs are preceded by putative ribosome binding sites located an appropriate distance from the methionine start codons. The downstream ORF *pepP* is incomplete, as it stops at the cloning restriction site. Its ATG start codon is separated only by 62 bp from the TAG (amber) stop codon of *pepA*. Within this short noncoding region an inverted repeat structure with the typical characteristics of a rho-independent terminator was identified. This stem-loop structure has a free energy of approximately −13.6 kcal (ca. −56.9 kJ), as calculated according to the method of Zuker and Stiegler (58). The close location of this ORF to the 3′ end of the structural gene *pepA* and lack of apparent promoter sequences upstream support the idea that it is probably transcribed under the control of the *pepA* promoter. Since preliminary sequence alignments of the first 157 amino acids of this ORF indicated homology to serine proteases, it was named *pepP*. *pepP* is currently being sequenced and studied with regard to its function in Pep5 biosynthesis. No homologies to known peptides or proteins could be found for the putative gene product of ORFX, and any possible function in Pep5 biosynthesis remains to be elucidated. Possible −35 and −10 sequences for *pepA* and *pepI* were identified upstream of the respective structural genes (Fig. 1).

Genes involved in Pep5 immunity. To identify the immunity gene, *pepI* and *pepA* were amplified by PCR. The respective PCR products were subcloned into the *Sma*I-linearized shuttle vector pCU1, and the resulting recombinant plasmids pMR9 (harboring the *pepI* PCR product) and pMR7 (harboring the *pepA* PCR product) were then transferred into the cured variant of *S. epidermidis* 5. Neither plasmid pMR7 nor plasmid pMR9 was able to confer the immunity phenotype to the Pep5-sensitive strain *S. epidermidis* 5 Pep5$^-$. The tested clones showed the same degree of sensitivity towards Pep5 as the plasmid-cured strain (MIC of 0.605 μg of Pep5 ml^{-1}). However, *S. epidermidis* 5 Pep5$^-$(pMR7) produced the inactive Pep5 prepeptide (42). This clearly demonstrates the transcription and expression of the *pepA* PCR product in this mutant strain. Pre-Pep5 could also be isolated from *S. epidermidis* 5 Pep5$^-$(pMR2) (see Fig. 2). We then successively shortened the 1.3-kb insert of pMR2. The construction and analysis of the respective clones are shown in Fig. 2. A 1.09-kb *Hae*III-*Hin*dIII restriction fragment harboring only intact *pepI* and *pepA* and the further shortened *pepP* was subcloned into *Sma*I-*Hin*dIII-cut pCU1. The resulting recombinant plasmid pMR11 was transferred to the plasmid-cured *S. epidermidis* 5 Pep5$^-$ strain. This mutant was immune, showing the same degree of insensitivity towards Pep5 as did the wild-type strain. To inactivate the structural gene *pepA*, we digested the insert of pMR11 with *Alu*I. The resulting blunt-end 0.66-kb *Alu*I fragment, covering only intact *pepI*, was again subcloned into *Sma*I-linearized pCU1. The respective recombinant plasmid pMR13 was transferred to the plasmid-cured variant of *S. epidermidis* 5, but transformants were sensitive to Pep5 to the same extent as the plasmid-cured strain. This is in agreement with the results obtained with pMR9, supporting the idea that both genes, *pepI* and *pepA*, have to be complete to restore the immunity phenotype.

Characterization and detection of the immunity peptide PepI. As deduced from the DNA sequence (Fig. 1), *pepI* encodes a 69-amino-acid peptide with a calculated mass of

8,065 Da. In the N-terminal part of the peptide two Lys residues at positions 6 and 27 flank a 20-amino-acid region of apolar residues, which may indicate that the peptide is membrane associated. The C-terminal region is very hydrophilic with a net positive charge. Both terminal regions of the peptide are predicted to adopt an α-helical conformation. In order to obtain more information about the cellular localization of the immunity peptide, we searched for the respective gene products in cell extracts of *S. epidermidis* 5 and mutant strains. Synthetic peptides comprising residues 53 to 67 and 47 to 69, coupled to bovine serum albumin, failed to raise antibodies in rabbits. Therefore, we constructed an MBP-PepI fusion protein, which was expressed in *E. coli* TB1 and purified by amylose affinity chromatography. The purified protein with a molecular size of 51 kDa was used for immunization of New Zealand White rabbits. Cytoplasmic and membrane fractions of *S. epidermidis* 5, *S. epidermidis* 5 Pep5$^-$(pMR2), *S. epidermidis* 5 Pep5$^-$(pMR7), *S. epidermidis* 5 Pep5$^-$(pMR9), *S. epidermidis* 5 Pep5$^-$(pMR11), and *S. epidermidis* 5 Pep5$^-$(pMR13) were subjected to SDS-PAGE and subsequent immunoblotting with the anti-MBP-PepI antiserum. Specific positive blot signals in the 8-kDa range were obtained with soluble and membrane fractions of the wild-type strain and of clones bearing pMR2 and pMR11 (Fig. 3A and B). Soluble as well as membrane fractions seemed to contain equal amounts of the immunity peptide. No specific antibody reaction occurred with the clone bearing pMR7 (Fig. 3A and B). With the clone bearing pMR13 we observed faint bands of incorrect size in both fractions. These could be due to unspecific antibody reactions which regularly occur with polyclonal rabbit sera. However, as such cross-reactivities were not observed with the clone bearing pMR7 (Fig. 3B, lanes 3 and 4), we cannot rule out the possibility that the faint bands represent a small amount of truncated peptide in the cytoplasmic fraction and incorrectly processed peptide of higher molecular mass in the membrane fraction, respectively. This point needs further studies, as it could provide information on the molecular basis for expression of the immunity phenotype.

The results obtained with clone pMR9 were identical to those obtained with clone pMR13 (not shown). The membrane fraction of *S. epidermidis* 5 was then washed with sodium phosphate buffer and subsequently with 5 mM $MgSO_4$, 10 mM EDTA, and 0.01 and 0.1% Triton X-100 and analyzed for the presence of PepI by Western blotting (Fig. 3C). A considerable amount of the immunity peptide was removed from the membranes already after EDTA washing. Only a small portion of PepI (approximately 10%) remained membrane attached, and it could be almost totally removed by Triton X-100 treatment. These results point to a weak membrane association rather than insertion of the immunity peptide. Moreover, the immunity peptide could not be released by mild lysostaphin treatment of whole cells, indicating that the peptide may be attached to the membrane.

To localize PepI more precisely, we evaluated if the peptide is sensitive to proteases after removal of the cell wall. To this end, we prepared intact protoplasts in the presence of sucrose and of raffinose and compared these with nonstabilized protoplasts which should release cytoplasmic protein upon lysis. Figure 3D demonstrates that PepI was equally destroyed by proteolysis regardless of whether sucrose was present. Results with raffinose were identical (not shown). This experiment suggests that in protoplasts the immunity peptide is accessible to external proteases and thus may be located on the outer surface of the cytoplasmic membrane.

Functional analysis of PepI. To confirm our hypothesis that PepI—in analogy to channel-forming colicin immunity proteins—may operate at the site of the cytoplasmic membrane, we tested the influence of PepI on membrane depolarization by Pep5. Therefore, we compared the influence of Pep5 on active transport of the sensitive plasmid-cured variant *S. epidermidis* 5 Pep5$^-$ with that of the immune clone *S. epidermidis* 5 Pep5$^-$(pMR2) (Fig. 4). Cells of *S. epidermidis* 5 Pep5$^-$ pretreated with Pep5 were unable to carry out active transport of radioactively labeled L-proline, in contrast to the untreated control. The addition of Pep5 to control cells, which had accumulated the amino acid, led to a rapid efflux. On the contrary, no significant inhibition of accumulation of

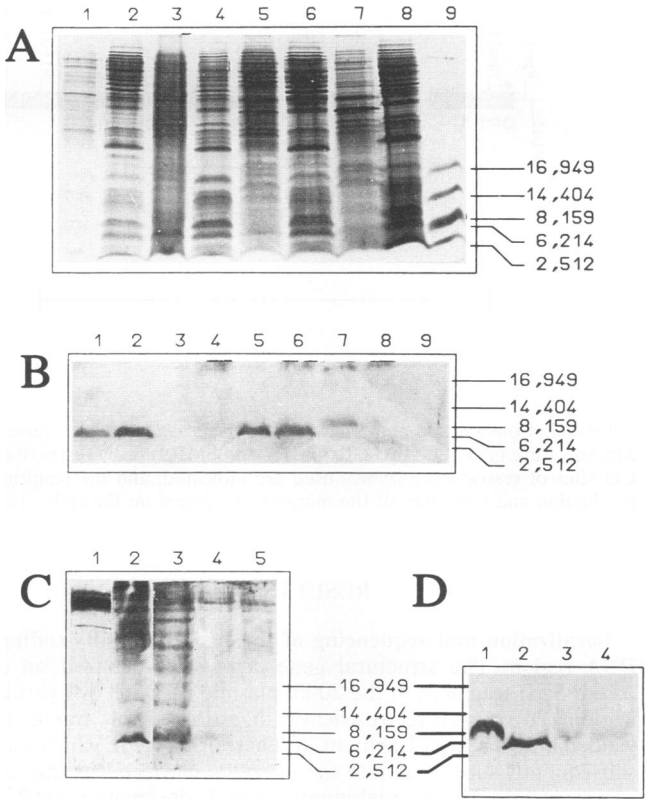


FIG. 3. Identification and localization of the immunity peptide PepI. (A) Coomassie-stained SDS-PAGE gel of membrane and soluble cytoplasmic fractions of *S. epidermidis* 5 (lanes 1 and 2), the clone bearing pMR7 (lanes 3 and 4), the clone bearing pMR11 (lanes 5 and 6), and the clone bearing pMR13 (lanes 7 and 8). The results obtained with clone pMR11 were identical to those obtained with clone pMR2. Standard peptides of the indicated molecular masses (in kilodaltons) are shown in lane 9. (B) Immunoblot analysis of panel A with anti-MBP-PepI antiserum. (C) Immunoblot analysis of washed membranes with anti-MBP-PepI antiserum. Lane 1, 0.005 μg of MBP-PepI; lanes 2 to 5, membranes of *S. epidermidis* 5 after washing with $MgSO_4$ (lane 2), EDTA (lane 3), 0.01% Triton X-100 (lane 4), and 0.1% Triton X-100 (lane 5). Membranes were resuspended in identical volumes of sodium-phosphate buffer, pH 7.0, after each washing step; 25-μl aliquots were applied in lanes 2 to 5. (D) Western blot analysis of PepI in protoplasts and lysed cells after incubation with proteinase K and chymotrypsin. Cells were enzymatically lysed as described in Materials and Methods, and protoplasts were obtained by addition of 20% sucrose. Lanes 1 and 2, trichloroacetic acid-precipitated protein after cell wall hydrolysis in the presence (lane 1) and in the absence (lane 2) of sucrose; lanes 3 and 4, trichloroacetic acid-precipitated protein after additional incubation with proteases for 30 min (protoplasts [lane 3] and lysed cells [lane 4]). The difference in migration of the immunity protein in lane 1 may be caused by overloading effects.

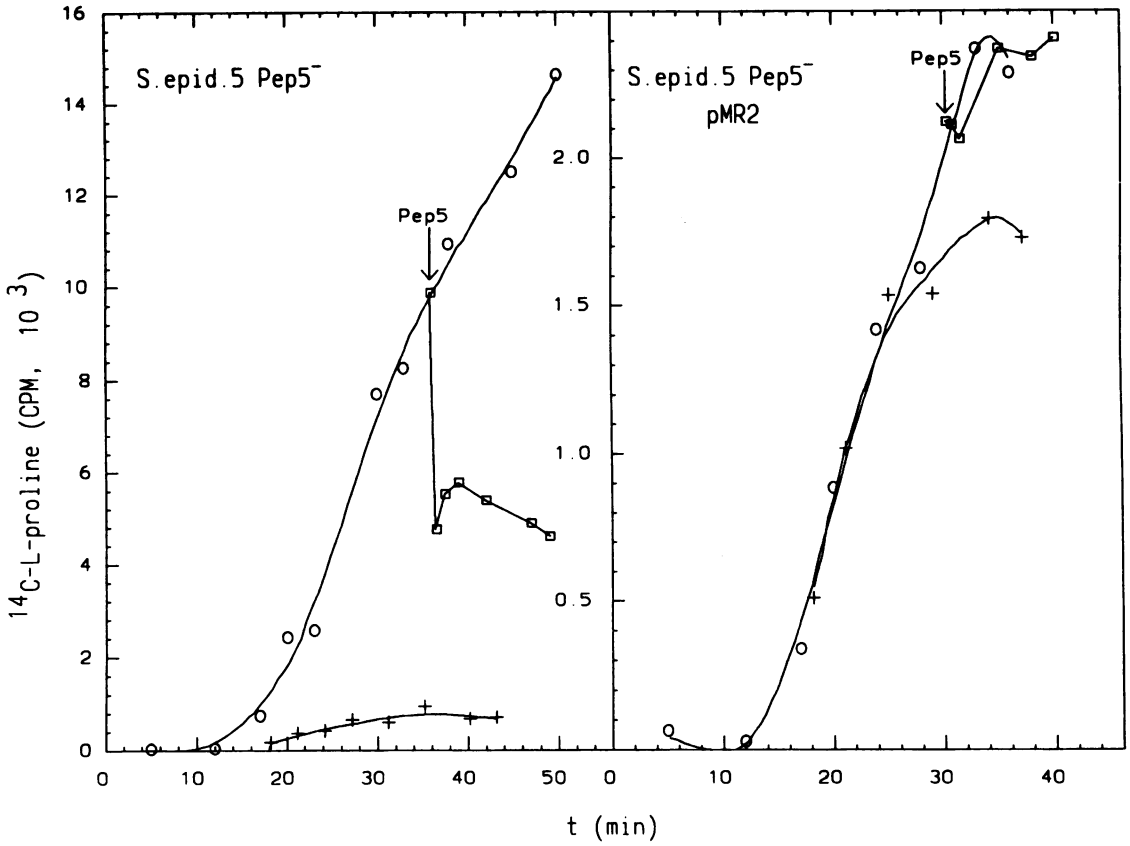


FIG. 4. Accumulation of L-[^{14}C]proline (○) by *S. epidermidis* 5 Pep5$^-$ (left) and the immune mutant strain (harboring pMR2) (right), efflux upon addition of Pep5 (□), and uptake of L-[^{14}C]proline by Pep5-pretreated cells (+). For experimental details, see reference 41.

L-[^{14}C]proline in the presence of Pep5 could be observed with cells of the immune mutant strain *S. epidermidis* 5 Pep5$^-$(pMR2). Also, no significant efflux of the accumulated amino acids was noticed after Pep5 addition to untreated control cells of the mutant strain. These results clearly demonstrate the antagonizing effect of the immunity peptide, which prevents the lethal, pore-forming action of Pep5. Because of these results in combination with those of the localization studies, we presume that the cytoplasmic membrane is the site where the immunity peptide interacts with Pep5; the molecular nature of this interaction and the question of whether PepA is directly taking part in immunity are currently being investigated.

DISCUSSION

Bacteriocin-producing cells protect themselves from the hazardous action of their own products by a specific self-protection mechanism, generally designated immunity. Detailed models on the molecular mechanism of immunity, especially for the channel-forming colicins (32, 34) and colicins with nuclease activity (7), exist. The channel-forming activity of colicins A and E1 is blocked by specific integral membrane proteins which are supposed to directly interact with the channel-forming C-terminal domains of the respective colicins (11, 47, 48).

The Pep5 immunity gene, *pepI*, was localized immediately upstream of the structural gene *pepA* on the 20-kb plasmid pED503 of *S. epidermidis* 5 (39). The close localization of *pepI* to the structural gene reflects the direct relation between Pep5 production and immunity and with regard to the genetic organization points to a situation quite similar to that for the aforementioned colicins. In addition, there is obviously a regulatory mechanism preventing PepI synthesis when the structural gene *pepA* is absent; mutants harboring only intact *pepI* without *pepA* or with incomplete *pepA* were not able to express the immunity phenotype and did not produce the *pepI* gene product. Presently the molecular basis for this regulatory phenomenon is unknown. Clearly, *pepA* has its own functional promotor, as shown by the fact that clone *S. epidermidis* 5 Pep5$^-$(pMR7) produces pre-Pep5 in amounts similar to those produced by the wild-type strain (42). This makes it unlikely that *pepA* and the upstream-positioned *pepI* form a transcriptional unit. It seems possible, however, that pre-Pep5 regulates *pepI* transcription or that *pepI*-derived mRNA is unstable when *pepA* is incomplete or missing. In this context a report on the lantibiotic nisin in which the expression of the structural gene *nisA* and the level of nisin immunity were also correlated is interesting (38). In this case, a strongly reduced level, but not a complete absence, of nisin immunity was observed in a mutant strain in which *nisA* transcription did not take place. Such a graduated level of immunity expression could not be seen in the case of Pep5, as mutants either were able to express the Pep5 immunity phenotype to the same extent as the wild-type strain or were as sensitive as the pED503-cured nonproducing and nonimmune variant *S. epidermidis* 5 Pep5$^-$. Recently, the nisin immunity gene has been identified, cloned, and expressed in *E. coli*, in which it caused a significant

decrease in nisin sensitivity (28). *Lactococcus lactis* clones showed a low level of nisin immunity, while clones additionally expressing the structural gene *nisA* were fully protected. However, the putative *nisI* gene product is a 245-amino-acid protein with a lipoprotein signal sequence and no homology to PepI (28), indicating a different molecular mechanism of immunity. It appears that the Pep5 immunity peptide and the molecular mechanism of immunity are more closely related to the immunity phenomenon found for the nonlantibiotic peptide bacteriocins of lactic acid bacteria (for a review, see reference 23) than to that for nisin. Recently, the immunity protein of lactococcin A, produced by *L. lactis* subsp. *cremoris*, was isolated. This hydrophilic and cationic peptide with a molecular mass of approximately 11 kDa seems to be a peripheral membrane protein, possibly attached to an integral membrane protein on the inside of the cell (37). The existence of a 20-amino-acid hydrophobic region in the N-terminal part of PepI suggested that PepI could also be membrane associated. Similar to the lactococcin immunity peptide but in contrast to colicin immunity proteins (48), PepI should not be an integral membrane protein but rather should be weakly associated with the membrane. This is further indicated by the experiments showing the distribution of PepI in the soluble and membrane fractions and by the membrane washing experiments. For functional purposes it seems more likely that the interaction between Pep5 and the immunity protein takes place outside the cells. In fact, the level of PepI was significantly reduced by protease digestions performed with osmotically stabilized protoplasts, suggesting that PepI is attached to the outer surface of the cytoplasmic membrane. Once outside the cell, the immunity peptide can exert its antagonizing function by preventing membrane depolarization caused by the pore-forming action of Pep5. This effect was clearly demonstrated by the ^{14}C-labeled L-proline uptake and efflux experiments, performed with the Pep5-sensitive mutant *S. epidermidis* 5 Pep5$^-$ and the immune mutant, harboring pMR2.

An extracellular location of PepI raises the question of how the peptide is secreted. PepI has no typical signal peptide sequence which could direct the peptide to the outside, and therefore it should require a translocator similar to those found in other lantibiotic biosynthesis gene clusters (e.g., the gene products of *nisT* [8, 49] and *spaT* [6, 24]). These are supposed to export the modified but uncleaved prelantibiotics (55). Such a hypothetical transporter for PepI should be encoded on the chromosome of *S. epidermidis* 5, because the mutant strain containing pMR11 (i.e., the pED503 variant harboring only *pepI* and *pepA*) was fully immune to Pep5. Additional information about the location of PepI and the molecular mechanism of the immunity phenotype is expected from current molecular biological studies, e.g., site-directed mutagenesis within *pepI*, and from electron microscopy using immunogold labeling techniques.

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