Nucleotide sequence of the lantibiotic Pep5 biosynthetic gene cluster and functional analysis of PepP and PepC

Evidence for a role of PepC in thioether formation

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The biosynthesis of Pep5, a lanthionine-containing antimicrobial peptide, is directed by the 20-kbp plasmid pED503. We identified a 7.9-kbp DNA-fragment within this plasmid which covers the information for Pep5 synthesis in the homologous host *Staphylococcus epidermidis* 5 which has been cured of pED503. This fragment contained, in addition to the previously described structural gene *pepA* and the immunity gene *pepI* [Reis, M., Eschbach-Bludau, M., Iglesias-Wind, M. I., Kupke, T. & Sahl, H.-G. (1994) *Appl. Env. Microbiol. 60*, 2876−2883], a gene *pepT* coding for a translocator of the ABC transporter family, a gene *pepP* coding for a serine protease and two genes *pepB* and *pepC* coding for putative modification enzymes; the gene arrangement is *pepTIAPBC*. We analyzed the biosynthetic genes with respect to their function in Pep5 biosynthesis. Deletion of PepT reduced Pep5 production to about 10%, indicating that it can be partially replaced by other host-encoded translocators. Inactivation of PepP by site-directed mutagenesis of the active-site His residue resulted in production of incorrectly processed Pep5 fragments with strongly reduced antimicrobial activity. Deletion of *pepB* and *pepC* leads to accumulation of Pep5 prepeptide in the cells without excretion of processed peptide. A *pepC*-deletion clone did not excrete correctly matured Pep5 but it did produce fragments from which serine and threonine were absent. Only one of these fragments contained a single lanthionine residue out of three expected while the remaining, unmodified cysteine residues could be detected by reaction with Ellman's reagent. These results demonstrate that PepC is a thioether-forming protein and strongly suggest that PepB is responsible for dehydration of serine and threonine.

Keywords: lantibiotics; Pep5 biosynthetic gene cluster; PepC, thioether-forming enzyme; PepP, serine protease.

Pep5 is a tricyclic peptide produced by *Staphylococcus epidermidis* 5 (Sahl and Brandis, 1981) which belongs to the family of lantibiotics, a designation introduced to characterize lanthionine containing peptides with antimicrobial activity (Schnell et al., 1988). In contrast to conventional peptide antibiotics, which are synthesized by multienzyme complexes, lantibiotics derive from gene-encoded precursor peptides. These precursors consist of a leader sequence and a propeptide part which is posttranslationally modified to give the mature lantibiotic. It was proposed that in a first modification step the serine and threonine residues of the propeptide part are dehydrated to didehydroalanine (Dha) and didehydrobutyrine (Dhb) (Schnell et al., 1988). Such dehydrated prepeptides have been isolated from *S. epidermidis* 5 (Weil et al., 1990). In a second step thiol groups of cysteine

residues react with the double bonds of some of the Dha and Dhb residues to form lanthionine or methyllanthionine, respectively. Some lantibiotics contain further modified residues such as D-Ala (Skaugen et al., 1994) or the C-terminal S-aminovinyl-cysteine (Kupke et al., 1994). For nisin it has been demonstrated that, after modification of the propeptide part, the prelantibiotic is exported from the cell, followed by removal of the leader sequence (Van der Meer et al., 1993).

Obviously, such a maturation pathway requires a dedicated biosynthetic machinery composed of several proteins. The genes for these proteins have been found to be arranged in gene clusters as described for nisin (Engelke et al., 1992, 1994; Kuipers et al., 1993; Van der Meer et al., 1993), subtilin (Klein et al., 1992, 1993; Klein and Entian, 1994; Chung et al., 1992; Chung and Hansen, 1992) and epidermin (Schnell et al., 1992). Typically, these clusters include genes for specialized peptidases belonging to the subtilisin family of serine proteases for proteolytic processing, an export protein of the ABC transporter family, proteins involved in immunity, and two component regulatory proteins. In addition, in all of these clusters two genes designated *lanB* and *lanC* were found, the corresponding proteins of which do not have any similarity to known sequences. As dehydration of peptide hydroxyamino acids and thioether formation are hitherto undescribed reactions, novel catalysts were expected to be

found, thus making these LanB and LanC proteins likely candidates. However, no experimental evidence concerning the role of these two proteins has been reported so far.

Lantibiotic gene clusters may be located on the chromosome as in the case of subtilin (Banerjee and Hansen, 1988), on plasmids as in the case of epidermin (Schnell et al., 1988), or on a transposon as shown for nisin (Dodd et al., 1990). The structural gene for Pep5 biosynthesis, *pepA*, is located on the 20-kbp plasmid pED503 in the producer strain *S. epidermidis* 5 (Kaletta et al., 1989). Upstream of *pepA*, the immunity gene *pepI* was found which confers protection against Pep5 to the producer strain, while downstream an open reading frame starts which codes for the N-terminal fragment of a putative protease (Reis et al., 1994). A 6.8-kbp fragment of pED503 that comprises *pepI*, *pepA* and a further 5.3-kbp of downstream sequence is sufficient for Pep5 biosynthesis in a variant of *S. epidermidis* 5 that has been cured of pED503, although the production rate of this clone is lower than that of the wild-type strain (Bierbaum et al., 1994). Here we report the nucleotide sequence of the 6.8-kbp fragment and of an additional 1.072-kbp upstream of *pepI*. We identified and functionally analyzed the processing protease PepP and the transporter PepT. In addition, we found the genes for the putative modification enzymes PepB and PepC and present, for the first time, experimental evidence for their role in lanthionine biosynthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains listed in Table 1 were kept as glycerol stock cultures at $-70\,°C$ or subcultured weekly on blood agar. Both *Escherichia coli* strains were used as hosts for recombinant DNA, *E. coli* BMH 71-18 *mut* S and the vector pALTER were used for site-directed mutagenesis. The plasmids pBR322, pEMBL18[+]/pEMBL19[+] and pUC19 were used for transformation of *E. coli*; pCU1 is an *E. coli*/*Staphylococcus* shuttle vector, which was kindly provided by R. Rosenstein and F. Götz (Tübingen; Augustin et al., 1992).

DNA cloning, sequencing and amplification by the polymerase chain reaction (PCR). Plasmid DNA of *S. epidermidis* 5 was purified as described previously (Reis et al., 1994). Plasmid DNA of *E. coli* strains was isolated by the methods of Holmes and Quigley (1981) or Birnboim and Doly (1979); DNA for sequencing was purified on Qiagen 20 columns (Diagen, Hilden, FRG). Restriction enzymes and T4 DNA ligase were supplied by Boehringer (Mannheim). *E. coli* strains and *S. epidermidis* 5 Pep5^{-} were transformed by electroporation (Augustin and Götz, 1990).

For determination of the sequence downstream of *pepP*, the 6.8-kbp insert of pGB7 was digested with the restriction enzymes *Hind*III and *EcoRI*. The fragments obtained were cloned in pUC19 or pEMBL19[+] and designated pCM4/1 – 4/5 (Table 1). In order to sequence the DNA upstream of *pepI*, deletion clones of pCM1 (designated pJS9 to pJS12, Table 1) were constructed using the double-stranded nested deletion kit from Pharmacia. The instructions of the manufacturer were modified as follows: the restriction enzymes *EcoRI* and *Aat*II were used to linearize the plasmid; deletion reactions were performed without NaCl at $30\,°C$; samples were taken at 1-min intervals; after the S1 nuclease digest, Klenow polymerase was used to obtain a sufficient number of blunt ends; ligation was performed in the presence of 0.25% Nonidet P40. The clones were hybridized with a PCR fragment covering *pepA* which was labelled with [32P]dATP using the random primed DNA labelling kit from Boehringer (Mannheim). Southern hybridization was performed according to established protocols (Sambrook et al., 1989). Clones lacking progressively about 300 bp were selected.

Double-stranded plasmid DNA was sequenced by using the dideoxynucleotide chain-termination method (Sanger et al., 1977), the T7 sequencing kit (Pharmacia) and [35S]dATP[αS] (Amersham). Oligonucleotides used for sequencing and amplification were synthesized with a 391 PCR Mate (Applied Biosystems). Sequence data were analyzed with ClustalW (kindly provided by D. Higgins; Higgins et al., 1992; Thompson et al., 1994), readseq (kindly donated by D. Gilbert), the GENIUS HUSAR program suite at the DKFZ (Heidelberg) and the Genetic Data Environment program suite (kindly provided by S. Smith).

RNA isolation and Northern hybridization. RNA was prepared by the minilysate method of Holmes and Quigley (1981) as modified by Oskouian and Stewart (1990) for isolation of RNA from staphylococci. *S. epidermidis* 5 cells were grown in tryptic soya broth medium (Oxoid, Basingstoke, England: 17 g/l Bacto tryptone, 3 g/l Bacto soytone, 2.5 g/l Bacto dextrose, 5 g/l NaCl, 2.5 g/l Na₂HPO₄) containing 0.7% (mass/vol.) glycine to an absorbance of 1.0 at 578 nm, pelleted, suspended in 50% sucrose, 20% Triton, 0.5 M EDTA, 1 M Tris, pH 6.8 and incubated with lysostaphin (0.71 mg/ml) for 40 s at $100\,°C$. After addition of 20 U RNasin (Promega Corporation, Heidelberg), the cold sample was centrifuged and the RNA was precipitated from the supernatant with ethanol/3 M sodium acetate pH 4.8. After digestion with DNase I (Boehringer, Mannheim) according to Ambulos et al. (1987) and denaturation with glyoxal/dimethylsulfoxide (McMaster and Carmichael, 1977), total RNA was separated on a 1.75% agarose gel (Seaplaque, FMC Corporation, Rockland, USA) using 10 mM sodium phosphate, pH 7. Vacuum blotting was performed with a Vacu Gene XL (Pharmacia) within 90 min and a suction of 50 cm H₂O. Complete RNA transfer was controlled by staining with ethidium bromide. After a short incubation in 1 mM EDTA, 10 mM Tris pH 8 at $96\,°C$, the filter was dried for 2 h at $80\,°C$.

As hybridization probes [32P]dATP randomly primed PCR fragments of *pepI* and *pepA* were used. These were synthesized according to Saiki et al. (1988) using pMR4 as a template; the primers were: pepI-5′ [5′(ATGAACATATACTTAAAAG-TAAT)3′], pepI-3′ [5′(TAAAGTTTATCTTTTTTGTTGC)3′]; pepA-5′ [5′(TATTTATAGAGGAGGTGGTTA)3′], pepA-3′ [5′(GAGTGGTTTCCCATACTATT)3′]. Hybridization was performed using the formamide protocol and Zeta-probe membranes (BioRad).

For transcription start site analysis, primer extension studies were performed using the following oligonucleotides: *pepA*-5′ with the sequence 5′-GGTTCAAGTTCATCAGTG-3′ and *pepI*-5′ with the sequence 5′-CCATTGCTTAGTCGTTA-3′; both were labelled with the 5′-labelling kit from Boehringer (Mannheim; Maxam and Gilbert, 1980). The primer extension was performed with reverse transcriptase (Boehringer, Mannheim) as described by Poolman et al. (1990). The half-life of the *pepI* and *pepA* mRNA was measured by the rifampicin (0.1 mg/ml) procedure of Ambulos et al. (1987).

Site-directed mutagenesis. Site-directed mutagenesis of PepP was performed with a commercial phagemid system as previously described for Pep5 mutant peptides (Bierbaum et al., 1994). The following mutagenic oligonucleotide was used to exchange the active-site His for Pro (the mismatch is underlined): 5′(GATTACACTGGGCCTGGAACTCAAATAATA)3′.

Purification of Pep5 peptides and Western blot analysis of pre-Pep5. For purification of Pep5 peptides 2 l tryptic soya broth was directly inoculated with the respective *S. epidermidis* clone and incubated to an absorbance of 3 – 4 at 600 nm. Cells were removed by centrifugation and the supernatant directly used for purification which included adsorption chromatography on XAD2 adsorbent (Serva), cation-exchange chromatography and HPLC reverse-phase chromatography as described pre-

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Characteristics	References
S. epidermidis 5	wild-type strain; contains five plasmids (pED503−505); pED503 (20 kbp) confers Pep$^+$Imm$^{\mathsf{r}}$ phenotype	Sahl and Brandis, 1981
S. epidermidis 5 Pep5$^-$	cured variant of *S. epidermidis* 5, lacking pED503; Pep$^-$Imm$^{\mathsf{r}}$ phenotype	Ersfeld-Dreßen et al., 1984
S. carnosus TM300	cloning host and indicator strain	Schleifer and Fischer, 1982
S. simulans 22	indicator strain	Sahl and Brandis, 1981
Micrococcus luteus ATCC 4698	indicator strain	Sahl and Brandis, 1981
E. coli 71-18	F$^-$lacL^q(lacZ) M15 proAB(lac-proAB) thi supE	Sambrook et al., 1989
E. coli BMH 71-18 *mut* S	variant strain of *E. coli* 71-18, defective in DNA mismatch repair	Zell and Fritz, 1987
E. coli JM 83	thi ara (lac-proAB) strA (lacZ) M15	Vieira and Messing, 1982
E. coli JM 109	supE$^+$ (lac-proAB) hsdR17 dam[F′traD36 pro AB$^+$ lacI^{q}I(lacZ) M15]	Yanisch-Perron et al., 1985
pBR322	Amp^R Tet^R 4.36-kbp low-copy vector	Bolivar et al., 1977
pEMBL18+/19+	Amp^{R}lacZ, 3.96-kbp vector	Dente et al., 1983
pUC19	Amp^{R}lacZ, 2.68-kbp high-copy vector	Vieira and Messing, 1982
pCU1	Amp^{R}Cm^R, 4.95-kbp shuttle vector	Augustin et al., 1992
pALTER	Amp^{R}Tet^R, 5.68-kbp phagemid	Dotto et al., 1981
pMR4	2.0-kbp *Bgl*II fragment of pED503 cloned into pCU1	this study
pMR7	540-bp PCR fragment cloned into pCU1	Reis and Sahl, 1991
pSZ1	8.3-kbp *Bam*HI fragment of pED503 cloned into pEMBL18+	Bierbaum et al., 1994
pGB7	6.8-kbp *Sph*I−*Bam*HI fragment of pSZ1 in pCU1	Bierbaum et al., 1994
pGB9	4.8-kbp *Kpn*I−*Bam*HI fragment of pSZI subcloned into pCU1	Bierbaum et al., 1994
pGB10R	6.2-kbp *Kpn*I−*Bam*HI fragment of pED503 in pCU1	Bierbaum et al., 1994
pGRO1	pGB10R with the mutation H53P in PepP	this study
pCM1	5.0-kbp *Hind*III fragment of pED503 in pBR322	this study
pCM4/1	2.0-kbp *Hind*III fragment of pGB7 subcloned into pUC19	this study
pCM4/2	2.2-kbp *Eco*RI fragment of pGB7 subcloned into pUC19	this study
pCM4/3	2.0-kbp *Hind*III fragment of pGB9 subcloned into pUC19	this study
pCM4/4	1.6-kbp *Eco*RI fragment of pGB7 subcloned into pUC19	this study
pCM4/5	0.9-kbp *Hind*III fragments of pGB7 subcloned into pEMBL19+	this study
pJS9−pJS12	deletion clones of pCM1	this study
pCM7	3.8-kbp *kpn*I−*Spe*I fragment derived from pGB9 and 1.39-kbp *Bgl*II−*Kpn*I fragment derived from pMR4 subcloned into pCU1	this study

viously (Sahl and Brandis, 1981; Sahl et al., 1985). Amino acids of peptide hydrolysates were analyzed after derivatization with *o*-phthaldialdehyde (Sahl et al., 1985).

The purified peptides were characterized by electro-spray mass spectrometry and by Edman degradation as previously described (Weil et al., 1990). For determination of free thiol groups, peptides were treated with Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid). The reagent was dissolved in ethanol (1 mg/ml) and mixed with an equal volume of buffer (0.4 M ammonium acetate pH 8.0) shortly before use. Aqueous solutions of the peptides were supplemented with an equal volume of the reagent mixture and incubated for 30 min at room temperature before mass determination. For reduction of disulfide bonds prior to Ellman derivatization, peptides were treated with 2 mM dithioerythritol for 30 min followed by addition of a threefold excess of Ellman's reagent. Reduction of disulfide bonds before HPLC was achieved by treating the peptides with 0.2 mM dithioerythritol in 0.2 M ethylmorpholine pH 8.

For semiquantitative comparison of Pep5 production by different clones a rapid one-step procedure for Pep5 enrichment and subsequent SDS/PAGE has been described (Bierbaum et al., 1994); Western blots of prepeptides were performed according to Reis et al. (1994).

Nucleotide sequence accession number. The nucleotide sequences presented here have been deposited at the EMBL data bank under accession number Z49865.

RESULTS

The Pep5 biosynthetic gene cluster. Previous work has shown that Pep5 production in *S. epidermidis* 5 depends on the presence of plasmid pED503 (Ersfeld-Dreßen et al., 1984) and that, in a pED503-cured variant, production can be restored with a 6.8-kbp fragment of this plasmid (Bierbaum et al., 1994). We further subcloned this fragment using the restriction sites indicated (Fig. 1) and obtained its entire sequence (Fig. 2). Downstream of the immunity gene *pepI* and the Pep5 structural gene *pepA* (Reis et al., 1994) we identified three further open reading frames which, on the basis of sequence similarity to proteins in other lantibiotic gene clusters, were designated *pepP*, *pepB* and *pepC*. Moreover, we detected the start of a fourth open reading frame on the opposite strand upstream of *pepI*, of which the full sequence could not be determined due to the length of the fragment. This gene was completed by sequencing deletion clones pJS9 to pJS12 derived from pCM1 (Fig. 1), and the corresponding protein was found to have sequence similarity to lantibiotic translocators of the ABC transporter family. Thus, the Pep5 biosynthetic gene cluster covers approximately 7.9 kbp and comprises *pepTIAPBC* (Fig. 1, 2).

Transcriptional aspects. Closer inspection of the DNA sequence suggested the presence of two inverted repeats with features of rho-independent transcriptional terminators. The first
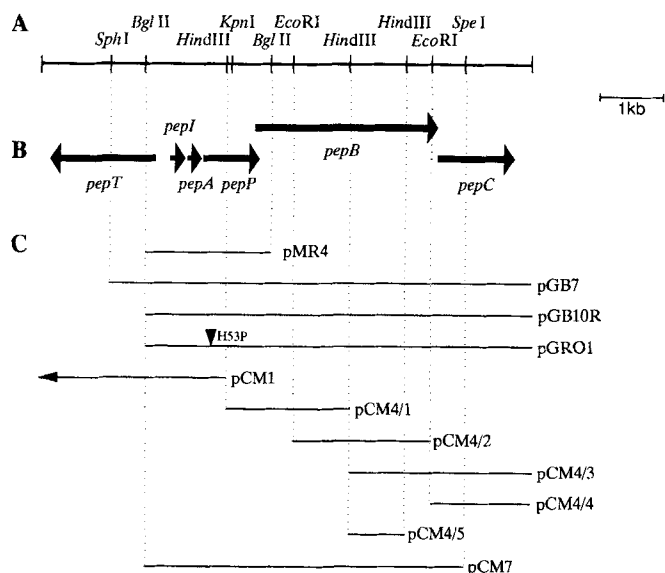
Fig. 1. Organization of the Pep5 biosynthetic gene cluster. (A) Restriction map of the 7.9-kbp segment of the plasmid pED503 covering the DNA sequence information for Pep5 production. (B) Organization of the Pep5 biosynthetic genes. (C) Subcloned fragments of the gene cluster; dotted lines indicate the restriction sites used for cloning.

putative terminator with a calculated free energy of −56.9 kJ/mol is located in the short non-coding segment between *pepA* and *pepP* and may allow partial readthrough (Reis et al., 1994), while the second inverted repeat follows 110 bp downstream of *pepC* and is probably strong enough (−78.7 kJ/mol) for transcription termination (Fig. 2). Potential promoters with reasonable similarity to published consensus sequences were only detected in front of *pepI* and *pepA*. Northern blot analysis with probes for both genes did not detect any transcripts longer than expected for monocistronic mRNA (Fig. 3). The *pepI* transcript was approximately 300 bases long, had a half-life of 1.5 min (Fig. 3) and started 42 bases in front of the respective ATG codon (Fig. 2). The *pepA* mRNA was of similar size (Fig. 3), but more stable (half-life of 7−10 min) and started 52 bases in front of the *pepA* start codon. On the basis of these start sites, promoter sequences are proposed as indicated (Fig. 2). The −35 region of the potential *pepI* promoter (TTGACA) is identical to the *E. coli* consensus sequence and the −10 region for the *pepA* promoter (TATAAA) has reasonable similarity to lactococcal −10 sequences (e.g. Chassy and Murphy, 1993).

The protease PepP is necessary for correct processing of Pep5. Adjacent to *pepA*, separated by 61 base pairs, follows *pepP* coding for a 285-amino-acid gene product with a calculated mass of 32.4 kDa (Fig. 2). PepP shows sequence similarities to subtilisin-like serine proteases, especially the conserved regions containing the amino acids of the active-site Asp20, His53, Ser228 and Asn145 of the oxyanion hole (Fig. 4). PepP is an overall hydrophilic protein without any clusters of hydrophobic amino acids. A prepro-sequence which is common with most other serine proteases (Siezen et al., 1991) is also missing, suggesting that it is not exported but localized in the cytoplasm.

In order to determine whether PepP is active and necessary for Pep5 biosynthesis, the protease was inactivated by site-directed mutagenesis exchanging the active-site His53 for Pro. The resulting clone *S. epidermidis* 5 Pep5⁻ pGRO1 accumulated unprocessed prepeptide inside the cells but did not excrete any prepeptide into the culture medium (Fig. 5B, lanes 4, 5, 6; com-

pared to the wild-type strain, lane 2). Also, Pep5 could not be found in the supernatant (Fig. 5A, lane 5), yet some antibacterial activity was detectable after partial enrichment. Using the Pep5 purification protocol (Sahl et al., 1985), a number of peaks were found to elute from the HPLC C18 column at a lower concentration of acetonitrile as compared to Pep5. These peaks contained mixtures of peptides with molecular masses lower than 3488 Da which were identified as fragments of Pep5 by N-terminal sequencing and mass spectroscopy. The major components were Pep5 17−34 (with a mass of 1899.24 Da and a N-terminal sequence of Leu-Lys-Ala), Pep5 6−34 (3106.15 Da, Ile-Arg-Ala) and Pep5 15−34 (2110.5 Da, Lys-block). Moreover, *S. epidermidis* 5 Pep5⁻ pGB10R (*pepIAPBC*) produces, in addition to correctly dehydrated Pep5, significant amounts of a Pep5 peptide with a surplus water molecule that corresponds to the presence of a Ser residue in amino acid hydrolysates (Bierbaum et al., 1994); corresponding peptide fragments with an additional mass of 18 Da were also detected (Pep5 17−34, 1917.5 Da; Pep5 6−34, 3124.5 Da; Pep5 15−34, 2128.5 Da). Thus, in the absence of functional PepP, the Pep5 prepeptide is processed at different sites by other host proteases present, indicating that PepP is essential for correct processing of Pep5. Additionally, only in the PepP-deficient clone can a peptide be detected by immunoblotting with anti-leader peptide antiserum which has approximately half the mass of the prepeptide (Fig. 5, lane 4). Apparently, this peptide represents the incorrectly processed leader peptide which is not subjected to rapid degradation when additional amino acids are attached to its C-terminus due to processing at an incorrect site in the propeptide part of pre-Pep5. Activity determinations of the Pep5 fragments against *Staphylococcus simulans* 22 gave minimal inhibitory concentrations of 0.5−3.0 mg peptide/ml as compared to 1.2 ng/ml for native Pep5, i.e. native Pep5 is six orders of magnitude more active than the fragments; this corresponds to the almost complete loss of production of antibacterial activity by the PepP-deficient clone as compared to the wild-type strain.

The modification enzymes PepB and PepC. Downstream of *pepP*, with an overlap of 14 bp and shifted by +1 base pair, a large open reading frame starts which codes for a protein of 967 amino acids and of a calculated molecular mass of 116 kDa (Fig. 2). This putative protein PepB has sequence similarity to NisB (Engelke et al., 1992), EpiB (Schnell et al., 1991) and SpaB (Gutowski-Eckel et al., 1994). Although the overall amount of identical amino acids between these LanB proteins is only in the range of 20%, several segments are highly conserved (Fig. 6). Such segments are between residues 80 and 110 (RS/TTPF/YGXXS) and the C-terminal YXXEXXRYGG motif around position 850 with two further conserved Glu residues in its close vicinity; however, any predictions as to the functional importance of these segments seem premature. Hydrophobicity plots indicate that PepB in accordance with other LanB proteins is rather hydrophilic and that it has only one major hydrophobic stretch in the C-terminal part.

Downstream of *pepB* and with a −1 base pair shift follows *pepC* coding for a putative protein of 398 amino acids with a calculated molecular mass of 42.2 kDa (Fig. 2). PepC has significant sequence similarity to the other LanC proteins described so far with identity values slightly higher than calculated for PepB. Amino acid sequence alignments reveal several segments with significant similarity. Most of these segments contain amino acids with inert side chains and a number of regularly positioned Gly residues which could be of structural importance. However, towards the C-terminus some conserved sequence clusters contain residues which may be functionally relevant such as the NXGXAHGXXG around position 230, the

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       TATCTCAATTAGTCATGTTTATTCATATAATTTTTGGAAATTGAATTACCCTCTCAATAATACTATGAAAATATAACAATAATTTATAATTAATTATCTAACTAACCTTTGTTTGCATAA  120
PepT                                                                            *  N  I  S  Q  N  S  V  F  T  N    571
       TATAAACAATACTAAAAATTAATTTATTACTACTCAAGGATATGGGCATTAAAAGGGTAATAGATTTTTATATTAAACTAGACGCCAAGAATTATCAGCTATTCGCTACGGTTAATATTA  240
PepT Y  K  N  H  N  K  I  L  Y  H  H  T  G  I  G  T  I  K  G  N  D  L  F  I  I  Q  D  A  T  R  L  L  R  Y  A  I  G  I  I  I    561
       GCATCAGGATAAAACTTTATTCAATAATTCACGTAAAACTTAAAAAAAAAGTGAAAGTGATAGATTTGATCGGCAACGAAGTAGGTTGTTATTCTATAGTCCTAAAAATTAATTTAAAGA  360
PepT T  T  R  N  Q  F  L  N  N  L  A  N  Q  I  K  K  E  S  E  S  D  L  S  A  T  A  E  D  L  L  L  I  D  P  N  K  I  L  N  R    521
       ACGTTATAATTAGGAGACAAAAACAGGGGGTCTTTCAAAATACGGCGAAAGAGGTTAAACTCTCAGCATAACTACTCCATTTAATGATTATTTTAGTACTCTTAAACGGTTTTATCTTAT  480
PepT A  I  N  I  R  Q  K  Q  G  G  S  L  K  I  G  S  E  G  I  Q  S  D  Y  Q  H  P  L  N  S  I  F  D  H  S  N  A  L  I  S  Y    481
       CATAAGATTAAGTAGTAGTCTATATAATAATCTATGAGGTATGTTTTACAATAGAGATTAACTGGGCAAGTAGTAATAATAATAAAACGTAATGCATAGGGTTAAATTATGCGGTAAAAAA  600
PepT Y  E  L  E  D  D  S  I  N  N  S  V  G  Y  L  I  N  D  R  I  S  G  N  M  M  I  N  N  Q  M  V  Y  G  L  K  I  R  W  K  K    441
       ATTTAGATATAGTATTTATCTTACAGGGGGTATTATTTAGGGAGGTGATAGTCGAAATATGTCAGATGATTAATTTAAATTCTATCATCTGAAAGGACTAGGACTTCCTGGTTGTTATCG  720
PepT L  D  I  D  Y  I  S  H  G  G  Y  Y  I  G  G  S  D  A  K  Y  L  R  S  I  L  N  L  I  T  S  K  G  S  G  S  P  G  V  I  A    401
       ACAAAAAAATAAATATCCTTGTCGCTTTCTTTGTAAAAGGTCATGTAAAAATCTTAGTATTCTTTTTATATGACTTAATTTTTACTCAAGAAATAAAACCCCATATAAAAGATTTAAGTG  840
PepT T  K  K  N  I  P  V  A  F  S  V  N  E  L  V  N  K  S  D  Y  S  F  Y  V  S  N  F  I  L  E  K  N  Q  P  I  N  E  L  N  V    361
       GAATAAAAGAAGATGCCCGAGTAAATTTTAAGGTATTTAAGCAAATGACCGAGGGAATCTTGAAAATATGAGCCAATAATTACAACTTTAAAGACTTTAACCACTTGACTCAACTTATTG  960
PepT K  N  E  E  V  P  E  N  L  I  G  Y  I  R  K  S  A  G  K  S  S  K  Y  E  T  I  L  T  S  I  E  S  I  P  S  S  L  Q  I  V    321
       TATTTTATAGTATCTTTGATTTCATGGTCTTCATTACCGCGGATGACGTTAAGATATAGGGGGTATACTATCATAATATGGTTAGTAATGATTATCCTCTTACGATCATTTTCCAACATT  1080
PepT Y  F  I  M  S  V  L  T  G  S  T  I  A  G  V  A  I  R  Y  G  G  Y  S  L  I  I  G  I  M  V  L  L  L  I  S  T  F  P  Q  L    281
       ATAACGATAATAGAATCGTACGAATTACAAATCAAATATTTATGACCTATCAAAAAGAAAACACAATAAATTGAGTTTGAGAAGTAATCATCTTTAAAATTGATTTGCTTATCGACTATA  1200
PepT I  A  I  I  K  A  H  K  I  N  L  K  Y  I  S  S  L  K  E  K  T  N  N  L  E  F  E  E  N  T  S  I  K  V  L  R  I  A  S  I    241
       ATGTGCAGGATATCACGGACATTTTAGTCGTTGAAAAACAACTCAAAATTTTCTATCAAAGAGATAATATAATGGATTACCATATTATTGGTAATCTTTATATCCTTAATATTATTTTCA  1320
PepT V  R  G  I  T  G  T  F  D  A  V  K  Q  Q  T  K  F  S  L  K  E  I  I  N  G  L  P  I  I  V  M  L  F  I  P  I  I  I  F  T    201
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PepT L  L  T  M  K  W  D  M  I  F  L  M  T  I  S  G  I  L  T  I  F  S  P  I  I  A  P  L  K  Q  S  I  F  N  N  I  L  S  T  D    161
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PepT D  T  I  R  S  I  L  Q  G  S  E  N  K  D  F  F  S  T  N  L  F  L  I  H  K  W  L  I  S  R  I  S  Y  I  I  K  E  G  V  K    121
       CTAATTATTTTATTCGGATTAGGTGATTTTTTATCGTAGATCATTTTTGTAATCTTAATTTTTATGATACTTAAAATTAAGATTACAAACAAATTGATCATCTAGATAATTCAATGGGCA  1680
PepT I  L  L  F  L  G  L  G  S  F  I  A  D  L  L  F  M  L  I  L  F  V  I  F  K  L  E  L  T  Q  K  V  L  L  D  I  L  N  G  T    81
       ATCTTCTCCCTGATACTAAAGTCACTATGACGGCTACCTTCTTTATTAATAACGTTATTATTTATTGCTGAATCCGGTAAATTAAAAACTCTTATTTTTTTTATCACCCAAAAGAAAAAA  1800
PepT L  L  P  V  I  I  E  T  I  S  G  I  S  S  I  I  I  A  I  I  F  L  S  K  P  W  K  I  K  S  F  L  F  F  L  P  N  E  K  K    41
       5'-AATAAAACCTTTCATAACTAATATTTATTTTCTATCAGGTATTAAAAAAAATTTTACAAAAACAGAGAAAATATTATACGATGCTAATAAAAGTATAACATGGTAAATAATATCATAA  1920
                RBS
       GTATTATTTTGGAAAGTATTGATTATAAATAAAAGATAGT-5'
PepT M                                                                                                                          1

                                    p-35                    p-10        ↓ transcription start    RBS
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PepI                                                                                          M  N  I  Y                         4
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PepI L  K  V  I  L  T  S  L  F  F  A  L  I  I  F  I  V  T  Y  I  T  T  K  Q  W  G  T  S  L  G  F  S  S  L  S  F  I  G  N  F    44
                                                                       p-35              p-10          ↓transcription start
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PepI I  I  Y  D  Y  S  T  K  L  S  D  K  K  Y  E  K  R  I  N  S  N  K  K  D  K  L  *                                             69

                     RBS
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PepA T  A  G  P  A  I  R  A  S  V  K  Q  C  Q  K  T  L  K  A  T  R  L  F  T  V  S  C  K  G  K  N  G  C  K  *                    60
                           RBS
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PepP                                  M  K  S  N  H  T  Y  I  K  Q  T  I  T  D  S  I  L  F  I  D  S  G  C  D  F               25
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PepP Y  I  S  G  F  L  P  N  I  N  I  V  L  Y  K  V  T  N  F  Y  G  K  S  K  A  I  D  I  Y  K  A  L  K  I  G  I  K  N  N  F    105
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PepP K  V  I  N  I  S  F  S  G  E  I  Y  D  K  L  M  K  K  F  Q  S  I  I  Y  E  A  Y  K  K  N  I  V  I  C  W  S  S  M  N    145
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PepP N  L  Q  K  S  A  N  H  G  N  K  N  M  V  P  N  Q  L  E  K  V  F  K  I  G  D  L  N  Y  Y  N  S  V  D  F  V  A  P  G  G    185
       GAAACAATAAATGGAAATGAATTAGAGGAAATTACAACAATGATAGTTGCTAACACTAGATTAGTACAAAAAATTTCAGATCACTATATGGGACTTCCTATTGGATATACTTTAAATATG  3240
PepP E  T  I  N  G  N  E  L  E  I  T  T  N  I  V  A  N  T  L  V  Q  K  I  S  D  H  Y  M  G  L  P  I  G  Y  T  L  N  M    225
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PepP G  N  S  I  A  T  S  Y  A  S  G  C  F  M  L  I  I  S  T  F  K  N  K  N  K  R  Y  P  S  I  N  E  I  I  S  L  I  S  K  Y    265
                                                                   RBS
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PepP D  D  K  E  R  N  L  I  E  I  T  K  R  V  I  E  D  E  I  V  *                                                              285
PepB                                                                               M  K  L  F  D  W  Y  M  V  R  E  P  I  L  N  I  N  D  L  K  I  I  K  K   24
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PepB N  N  L  N  D  D  E  Y  L  N  M  L  I  T  F  I  F  D  K  G  L  D  V  C  L  C  H  A  N  I  E  L  F  S  I  I  K  N  Y  Q    64
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PepB L  D  T  T  N  P  I  W  N  N  Y  A  K  K  Y  N  L  E  I  D  S  R  L  L  Y  P  L  I  N  K  L  N  I  V  V  K  N  E  L  Y    144
       TCTAAAAAAAATTCTAATGTTTACGAAGATACAACTCATTTTTATATCCCATATCAAATGCAATTAGAAGGAACAAATACAGTACCTAATAATATTAGTGTGAAAAAGAATGAACTGTC  3960
PepB L  K  K  N  S  N  V  Y  E  D  T  T  H  F  Y  I  P  Y  Q  M  Q  L  E  G  T  N  T  V  P  N  N  I  S  V  K  K  N  E  L  S    184
       TAAATTCATATTTACTAAATTAAATAATATGTTGTACAAAGATCTAGTAAAATTAGTTGAAGATGAATTCGACGCAGATACTATCATGGTTGAAACATACTTAGATAAATTGATAGAGGA  4080
PepB K  F  I  F  T  K  L  N  N  M  L  Y  K  V  E  D  E  F  D  A  D  T  I  M  V  E  T  I  V  L  D  K  L  I  E  E    224
       AAATTTTATAGTTACTGAACTAGAAGATGCTATCAGTCATAAAGATCGTGACATATTCTTGATAAAAAAATTAGAACAAAAAGGGTTTTCTAATAGTTATTATTATAAGGCTTTAAAAGA  4200
PepB N  F  I  V  T  E  L  E  D  A  I  S  H  K  D  R  D  I  F  L  I  K  K  L  E  Q  K  G  F  S  N  S  Y  Y  Y  K  A  L  K  E    264
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PepB E  Y  K  N  K  F  L  E  I  Y  G  Q  D  E  Y  V  N  I  L  K  L  L  N  K  N  T  G  I  Q  Y  P  I  N  I  Y  N  N  K  A  F    384
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PepB Q  Y  N  N  E  K  V  Y  S  L  L  T  E  W  Q  Y  K  A  I  R  D  K  T  D  I  I  L  N  E  E  R  I  E  L  L  K  R  V  G  K    424
       AAAACCTATTGATAAATCGTTTGATATGGTTTTTACTGTTACAAAGATGGATTTAACAATAATATTTATCATTTAAACGATAATGTTACGTCTTTAAAGGCTACTAATATATATGGGCG  4800
PepB K  P  I  D  K  S  F  D  M  V  F  T  V  T  K  D  G  F  N  N  N  I  Y  H  L  N  D  N  V  T  S  L  K  A  T  N  I  Y  G  R    464
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PepB F  R  Y  L  N  T  K  Y  F  E  E  Q  L  N  K  F  P  D  S  V  G  V  P  S  D  N  L  N  Y  H  H  P  N  P  I  I  D  N  V  K    504
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Fig. 2. Nucleotide sequence of the 7.9-kbp fragment of pED503 shown in Fig. 1 and deduced amino acid sequences of PepT (opposite orientation), PepI, PepA, PepP, PepB and PepC. Putative ribosome-binding sites (RBS) are boxed, the putative promoters (p-35, p-10) of *pepI* and *pepA* are marked above the sequence; the transcription start sites are indicated by arrows, the inverted repeats between *pepA* and *pepP* and downstream of *pepC* are marked with lines.

```
     ATTTAAAAGTATATTTTTTGATAAGCAGACAAGCTTTACAGATATTTTTGTTTTTTTAGGAGAAGATTTAAATTTTTATTTAAAAAATTTAAAAAATGGTGAAACTTTCATACCAAAAAT 5040
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PepB S  D  M  H  N  T  Q  Y  S  P  N  L  I  D  F  L  S  K  V  E  N  Q  F  I  Q  E  Y  W  N  I  E  H  Y  L  S  D  Y  T  F  L  584
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PepB P  R  I  V  Y  K  N  I  I  S  P  R  K  W  K  I  N  F  L  E  K  I  E  N  Y  S  Q  F  I  K  I  F  L  A  K  K  D  E  L  624
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     TGAAAATTTTTCAGATATAAAATACTTTATTAGATATGCAGATGAAAAAGAACATATTAGACTAAGAATGAAATTAGAGTTTGATCAAGTAAAATCTGCATTACATCTTTTCAAAGAAAT 5760
PepB E  N  F  S  D  I  K  Y  F  I  R  Y  A  D  E  K  E  H  I  R  L  R  M  K  L  E  F  D  Q  V  K  S  A  L  H  L  F  K  E  M  784
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PepC                                                               M  N  R  F  L  N  T  Y  I  K  Q  V  T  N  I  D  N  17
PepB H  E  E  L  V  M  E  F  A  S  R  T  L  I  D  Y  Q  N  M  R  K  Y  L  *                                                     967
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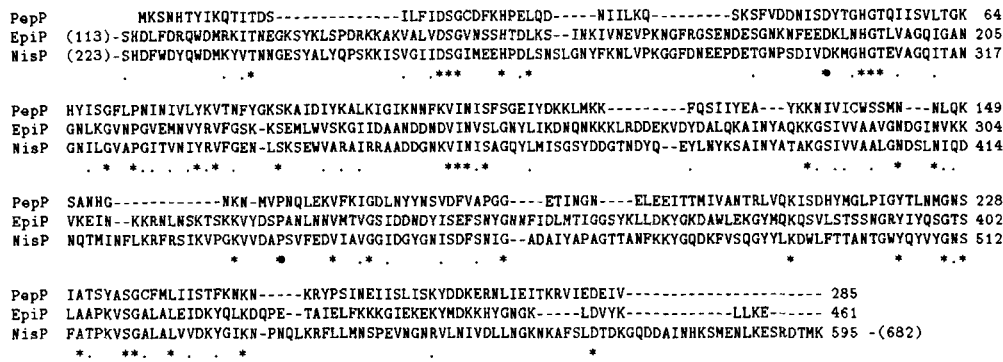
Fig. 2. (continued).
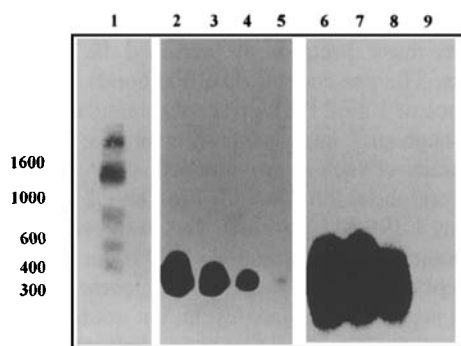
Fig. 3. Northern blot analysis of pepI and pepA transcription. Lane 1, size standard (nucleotides); lanes 2—5, hybridization with a pepI-specific probe; RNA was prepared from cells taken after 1 min (lane 3), 2 min (4) and 5 min (5) of incubation in the presence of rifampicin; lane 2, control without rifamipicin; lanes 6—9, detection with a pepA-specific probe; lane 6, 0 min; lane 7, 10 min; lane 8, 20 min; lane 9, 40-min incubation in the presence of rifampicin.

RXXWCYG motif around position 300 and a consensus CHG some 48 residues further towards the C-terminus (Fig. 7).

Role of PepC in Pep5 biosynthesis. In order to demonstrate the necessity of pepC for Pep5 biosynthesis, the 6.8-kbp fragment of pED503 was shortened to 5.7 kbp with SpeI (Fig. 1), thereby deleting the 231 C-terminal amino acids of pepC. The corresponding clone S. epidermidis 5 Pep5⁻ pCM7 was not able to

inhibit our standard indicator strain S. simulans 22 but produced a small inhibition zone when tested against S. carnosus TM300. This indicated that, just as with the protease-deficient clone, incompletely modified or fragmented Pep5 may be produced, although only intracellular accumulation of pre-Pep5 was visible (Fig. 5B). The culture supernatant of the PepC-deficient clone was subjected to the routine Pep5 purification protocol. Five major peaks and a number of smaller peaks and peak shoulders eluted from the HPLC reverse-phase column (Fig. 8). The five peaks were lyophilized, treated with dithioerythritol and rechromatographed; each of these five peaks split into several further peaks indicative of disulfide bond formation between different peptides during purification. After rechromatography we identified three major peptides (designated peptides 1, 3 and 5) as the dominant species in peaks I, III, and V, which also occurred in smaller amounts in peaks II and IV (Fig. 8). Peak II also contained peptide 3, possibly linked to peptide 1 by intermolecular disulfide bonds, whereas peak IV contained peptide 5 as well as peptide 5 with a surplus water molecule. The molecular masses, amino acid composition data and N-terminal amino acid sequences of the three major components clearly identified them as degradation products of incompletely modified Pep5 (Table 2).

Hydrolysates of these fragments did not contain Thr and Ser; only one of the Pep5 fragments which was found in peak IV contained one Ser residue; this most likely corresponds to the incompletely dehydrated Pep5 peptides observed with our Pep5 expression system (Bierbaum et al., 1994). Lan and MeLan also were not detectable except for one residue in the major peptide

```
PepP        MKSNHTYIKQTITDS-------------ILFIDSGCDFKHPELQD-----NIILKQ--------SKSFVDDNISDYTGHGTQIISVLTGK  64
EpiP   (113)-SHDLFDRQWDMRKITNEGKSYKLSPDRKKAKVALVDSGVNSSHTDLKS--INKIVNEVPKNGFRGSENDESGNKNFEEDKLNHGTLVAGQIGAN 205
NisP   (223)-SHDFWDYQWDMKYVTNNGESYALYQPSKKISVGIIDSGIMEEHPDLSNSLGNYFKNLVPKGGFDNEEPDETGNPSDIVDKMGHGTEVAGQITAN 317
                   .    . .*        .    .***   *  .*                    .        *  .***  .    .

PepP   HYISGFLPNINIVLYKVTNFYGKSKAIDIYKALKIGIKNNFKVINISFSGEIYDKKLMKK---------FQSIIYEA---YKKNIVICWSSMN---NLQK 149
EpiP   GNLKGVNPGVEMNVYRVFGSK-KSEMLWVSKGIIDAANDDNDVINVSLGNYLIKDNQNKKKLRDDEKVDYDALQKAINYAQKKGSIVVAAVGNDGINVKK 304
NisP   GNILGVAPGITVNIYRVFGEN-LSKSEWVARAIRRAADDGNKVINISAGQYLMISGSYDDGTNDYQ--EYLNYKSAINYATAKGSIVVAALGNDSLNIQD 414
             . *  *...  .*.*  .        *       .      ***..*            *  .      *    .  *  . *  *..

PepP   SANHG-----------NKN-MVPNQLEKVFKIGDLNYYNSVDFVAPGG----ETINGN----ELEEITTMIVANTRLVQKISDHYMGLPIGYTLNMGNS 228
EpiP   VKEIN--KKRNLNSKTSKKVYDSPANLNHVMTVGSIDDNDYISEFSNYGNNFIDLMTIGGSYKLLDKYGKDAWLEKGYMQKQSVLSTSSNGRYIYQSGTS 402
NisP   NQTMINFLKRFRSIKVPGKVVDAPSVFEDVIAVGGIDGYGNISDFSNIG--ADAIYAPAGTTANPKKYGQDKFVSQGYYLKDWLFTTANTGWYQYVYGNS 512
                   *   *   * .*.    .   . *                            *                 *        *   *.*

PepP   IATSYASGCFMLIISTFKNKN-----KRYPSINEIISLISKYDDKERNLIEITKRVIEDEIV-------------------- 285
EpiP   LAAPKVSGALALEIDKYQLKDQPE--TAIELFKKKGIEKEKYMDKKHYGNGK-----LDVYK-----------LLKE------ 461
NisP   FATPKVSGALALVVDKYGIKN-PNQLKRFLLMNSPEVNGNRVLNIVDLLNGKNKAFSLDTDKGQDDAINHKSMENLKESRDTMK 595  -(682)
       *.  **. *. .  *            .                       *
```

Fig. 4. Partial sequence alignment of PepP, EpiP and NisP. Sequences were compared with the Clustal W software. Identical amino acids are marked by asterisks and conservatively exchanged amino acids are marked by dots.

Table 2. Peptides produced by the pepC-deficient mutant S. epidermidis 5 Pep5⁻ pCM7.

Peptide	N-terminal sequence	Molecular mass measured (theoretical)	Pep5 fragment	Lan/MeLan found	Amino acids found (present in the prepeptide)		
					Ser	Thr	Cys
		Da		mol/mol peptide			
1	blocked	2766.0 ± 0.1 (2767)	9−34	1	0 (2)	0 (3)	2 (3)
3	I-R-A-blocked	2575.5 ± 0.5 (2577)	6−29	0	0 (2)	0 (3)	2 (2)
5	blocked	2955.6 ± 0.6 (2959)	1−29	0	0 (2)	0 (3)	2 (2)
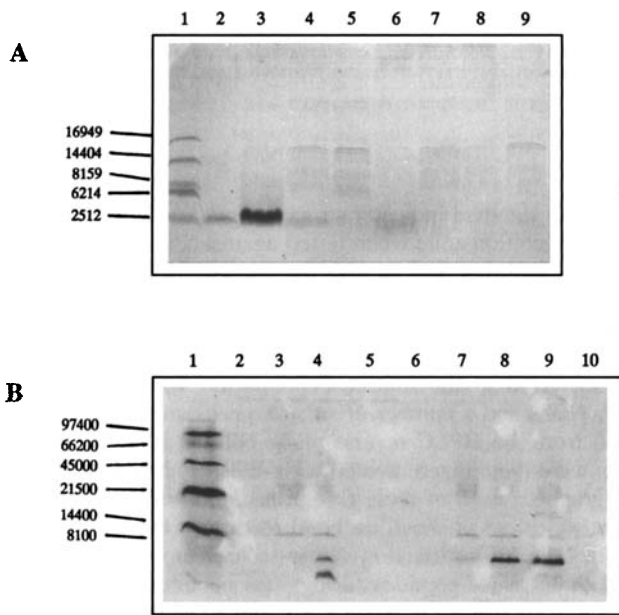
Fig. 5. (A) SDS/PAGE separation of Pep5 peptides partially purified from the supernatant of S. epidermidis 5 and of its indicated variants. (B) Western blot of total cell extracts of the respective strains immunoblotted with anti-Pep5 leader peptide antiserum for detection of Pep5 prepeptides. (A) Lane 1, molecular mass standard (Da); lane 2, purified Pep5, 2.5 µg; lane 3, wild-type S. epidermidis 5; lane 4, S. epidermidis 5 Pep5⁻ pGB10R; lane 5, S. epidermidis 5 Pep5⁻pGRO1; lane 6, S. epidermidis 5 Pep5 pCM7; lane 7, S. epidermidis 5 Pep5⁻ pMR4; lane 8, S. epidermidis 5 Pep5⁻ pMR7; lane 9, S. epidermidis 5 Pep5⁻. (B) Lane 1, biotinylated molecular mass standard (Da); lane 2, wild-type S. epidermidis 5; lane 3, S. epidermidis 5 Pep5⁻ pGB10R; lane 4, S. epidermidis 5 Pep5⁻ pGRO1; lanes 5, 6, S. epidermidis 5 Pep5 pGRO1 (culture supernatant); lane 7, S. epidermidis 5 Pep5⁻ pCM7; lane 8, S. epidermidis 5 Pep5⁻ pMR4; lane 9, S. epidermidis 5 Pep5⁻ pMR7; lane 10, S. epidermidis 5 Pep5⁻.

fragment 1 (Table 2). We analyzed the major peptides for the presence of free Cys residues using Ellman's reagent and subsequent mass spectrometry. With peptide 1 (Table 2) addition of two Ellman reagent molecules was possible (Fig. 9) indicative of two free Cys residues. Only traces of addition products of peptides 3 and 5 were detectable despite dithioerythritol treatment before chromatography, indicative of rapid intramolecular disulfide bond formation under oxidative conditions. Dithioerythritol treatment and addition of excess Ellman's reagent shortly before mass spectroscopy increased the amount of addition products. The presence of disulfide bonds is also indicated by a difference of $1.5-2$ Da between the calculated and detected masses, although such interpretations must take into account the limited accuracy of such a measurement.

We interpret the results compiled in Table 2 as follows. Peptide fragment 1 ($9-34$) has three Cys residues, one of which forms a thioether. Bridge formation could occur anywhere in the peptide except with Dha9, because lanthionine formation with Dha9 would just lead to a blank cycle, but not to a block during N-terminal sequencing. Also Cys33 is likely to be part of this thioether because peptide 1, unlike peptides 3 and 5, is not fragmented at position 29; as a result of the removal of ring C by site-directed mutagenesis, we found fragmentation at Gly29 to occur rather rapidly (Bierbaum et al., unpublished). The lanthionine ring in peptide 1 could impose conformational constraints on the peptide which does not allow for the remaining two Cys residues to form a disulfide bond; in contrast, this can occur rapidly with peptides 3 (fragment $6-29$) and 5 (fragment $1-29$) which just contain two Cys residues each.

These Pep5 fragments had no detectable antimicrobial activity as determined as inhibition zones in a lawn of S. carnosus TM300. Some activity eluted close to peak IV (Fig. 8) and was directly detectable without concentrating the fraction; obviously, a small amount of a peptide with a higher specific activity is also produced which, however, could not be separated and purified for further characterization.

```
PepB   --MKLFDWYMVREPILNINDLKIIKKNNLNDDEYLNMLITFIFDKGLDVCLCHANIELFSIIKNYQNIN--LSKKKKKNLSLSLYNYITRMILRSTPFGF    96
EpiB   GEIELDNIFVPSNIYMVRTPIFSIELYNQFLKSDNIDYDLILQNDIFKESIMTTTYNLYQSIGKIDWE---KDNKKTRNVKESLLKYLIRMSTRSTPYGM   97
NisB   ----MIKSSFKAQPFLVRNTILCPNDK-RSFTEYTQVIETVSKNKVFLEQLLLANPKLYDVMQKYNAG--LLKKKRVKKLFESIYKYYKRSYLRSTPFGL   93
SpaB   ----MKSLYTPTDYYMIRVPLVHQDLKNENSQDIDQLLHDLCNDSLFREQILVSSRTLYETIHTFLQAPDKLKGKKKRNFQQAILKYATRRATRTTPFGL   96
                .        .          *. .             *. . .. * * *.**.*

PepB   MSSINIKALDTTNPIWNNY--AKKYNLEIDSRLLYPLINKLNIVVK-NELYLKK-NSNVYEDTTHFYIPYQMQLEGTNTVPNNISVKKNELSKFIFTKLN   192
EpiB   LSGVALGEFSENNNIKIKDSSFHKKDVKIDGQWLYKLVHYLESDYTYYKDSFVIWNQQNYIYNNRLYLDNNSSITENKRNDV-LSVKYNSILVFIHENSK   196
NisB   FSETSIGVFSKSSQYKLMG--KTTKGIRLDTQWLIRLVHKMEVDFS-KKLSFTR-NNANYKFGDRVFQVYTINSSELEE----VNIKYTNVYQIISEFCE   185
SpaB   FSSVGIGSFSDKNHLSFNQH-SFYKKARVDFEWLYQLIRKLENEYT-DRLSFTL-NSACYIKGDRAYLLYSTDG-KSEE----VSVRATSVFYLINELCG   188
       *        .       .* * *..     * *   .           ..... *

PepB   --NMLYKDLVKLVEDEFDADT-IMVETYLDKLIEENFIVTELEDAISHKDRDIFLIKKLEQKGFSNSYYYKALKEIYDIRQIMEKGNIHDDILYRIIEVK   289
EpiB   -KNITYEELVQLISSKYSIENKEEVKVFVQELINKEIIFSDLRPTLENKNPLDYIINSLNPK---NSLVGTLINISNEITKYSKMPLG--KGEYKYLDIV   290
NisB   NDYQKYEDICETVTLCYGDEYRELSEQYLGSLIVNHYLISNLQKDLLSDFSWNTFLTKVEAIDEDKKYIIPLKKVQKFIQEYSEIEIG--EGIEKLKEIY   283
SpaB   -ESAAYQDIIRCLIDNYPNTPINKINQYVADLIDKEFLISNLRPPMTVSDQFQYLIDQAESRHIPNELIQACKNIQYQIDAYNRITIG--EGEHQYLNLI   285
        * ...   .   .      .. **   . .*    .           .                    *           .  .

PepB   KFLKEKFNLDVHNIFKVDTIDTNIEIDLSKSEINNIKKVIDMLLYLPSDYDNSL---QEYKNKFLEIYGQDEYVNILKLLNKNTGIQYPIN-------IY   379
EpiB   NLMSQLFVSK--NYLQIDTYIDYSRNELKQSLADNISEAAYILWLLSPNHFGTKT-IRNYHEFFMDKYGFEQLVNLKQLLSDINGFGYPK------KDSY   381
NisB   QEMSQILEND--NYIQIDLIS-DSEINFDVKQKQQLEHLAEFLGNTTKSVRRTY--LDDYKDKFIEKYGVDQEVQITELFDSTFGIGAPYNYNHPRNDFY   378
SpaB   ETMNKLIKAS--SPLQVDAGAGDSSIQLDNETSLAISELASMFTYMASPSANTLDHLEKYKNVFLERYGYEREVPLLEMLCSSTGIGAPATYTNPANEFF   383
              ..*         .          *. *.. **. * .      * *

PepB   NNKAFQYNN-EKVYSLLTEWQYKAIRDKTDIILNEERIELLKRVG--KKPIDKSFDMVFTVTKDGFNNNIYHLNDNVTSLKATNIYGRFR------YLNT   470
EpiB   -SFSNNIAFLKEKYLLAIQNNSHIEITENDVKNLEKNNTVSKINAPVSTEIYS--EIYFGNSIKGYE-DFAVISPILGSFNAGATFGRFTGNFNIKKKNQ   477
NisB   ESEPSTLYYSEEEREKYLSMYVEAVKNHNVINLDDLESHYQKMDLEKKSELQG-LELFLNLAKEYEK-DIFILGDIVGNNNLGGASGRFS---ALSPELT   473
SpaB   EETSFGEQFSPEMKQFFMRKYFESVRKKAPIQLDDETFHRICNSEIADEEIPLSFELNFFVKLRNGR-VKLYLGPNVGSTRAGKTFGRFS---HMSDSIS   479
                                                           .. .           .  .     ***

PepB   KYFEEQLN--KFPDSVG-VPSDNLNYHHPNPIIDNVKFKSIFFDKQTSFTD-----------IFVFLGEDLN--FYLKNLKNGETFIPKISDMHNTQYS   553
EpiB   LQKEIVHHYNNYNMENG-LEISQLNEGPLNSRNVNILNNNRIYNTCLNLNL--PKSD---IDINDIFIGATFN-KLYLYSEKHDSRIVFVSNSMFNYEFG   570
NisB   SYHRTIVDSVERENENKEITSCEIVFLPENIRHANVMHTSIMRRKVLPFFT-STSHN--EVLLTNIYIGIDEKEKFYARDISTQEVLKFYITSMYNKTLF   570
SpaB   EIIKTLHNKEKELTECN-TKVCELSIVPNQTRSGNVTRNVSYREKEMSLFTNSALHLNDSVKAEDILIGINKDHIFYARHKTTGEILSFESNHMFNPLLM   578
                   *.                                    ..*        *        * *

PepB   PNLIDFLSKVENQFIQ--EYWNIENYLSDYTFLPRIVYKNIIISPRKWKINFLEKIENYS-----QFIKIFLAKKDELLIPNRFYIQQADLKIYIDLENE   646
EpiB   SELYKFLREISFEKTKFIQPITEEGIDS-LPFCPRIIYKNIILKPATWKINSEMFSETEN------WLNRFATIREKWHIPKDVIIAFGDNRLLLNLLND   663
NisB   SNELRFLYEISLDDK--FGNLPWELIYRDFDYIPRLVFDEIVISPAKWKIWGRDVNS---------KMTIRELIQS-KEIPKEFYIVNGDNKVYLSQKNP   658
SpaB   TNAVRLLLEISRDGKRKWNDFPWFSIYSDFKYIPEIKYKEITLSCEQWLIYKNDLSMHSNASLEEIKSAFFEFHRT-YELPQTFYIVNADNRLLIDIEND   677
       *     .           . * .. *.  . **            .   . .*   *   *... *

PepB   RCMEILFKESKKNISII-----IVEIEKNLLQNKKVKEAVFSIKSDLHQEINYNYMMKKDHIPN---LSKEILYIDDGWISVNLYYNDNNINKFIGEFFL   738
EpiB   KHLIILKKELKKHGRIR-------ILES--FINESNNERMLEIVTPLYKKTSLKE--QSFIIPK---NRNKHFNNLKDWFSIHLSIPKTYQDNFIQDYLL   749
NisB   LDMEILESAIKKSSKRKDFIELQEYFEDENIINKGEKGRVADVVVPFIRTRALGNEGRAFIREKRVSVERREKLPFNEWLYLKLYISINRQNEFLLSYLP   758
SpaB   CTLDVFFWELKKTNHNQPLQLVAVEHDADALMDRNQNDYSGEIVVPLLRKQPVKPLYLPVLNAIEGSGSDRIKMPFEDWLFIKLYCKQTREEELIAFEIA   777
        . .     **        .        .   .                             .   .*   *   *...  *

PepB   TNIFESEN--FSDIKYFIRYADEKEHIRLRM--KLE--FDQVKSALHLFKEMVDLNYIIDYKIVPYYRETYRYGGKEVIEIVEACFEKDSEIVSKFYAY-   831
EpiB   PFITELKVNNFINKFFYIKFKEDEDFIKLRLLREDEDYSQIYSFIKNWKDYCLLKSELYDYSIVDYVPEVYRYGGPHVIEDIENFFMYDSLLSINIIQ--   847
NisB   DIQKIVAN--LGGNLFFLRYTDPKPHIRLRI--KCSDLFLAYGSILEILKRSRKNRIMSTFDISIYDQEVERYGGFDTLELSEAIFCADSKIIPNLLTLI   854
SpaB   DFYNQISDQ-YPVRHFFMRYRDPKPHIRLRFNGKAEVLYSLFPQLLNWLKSLREKGLVSESVITQYEREIERYGGLSLMEAAEQLFCEDSKVVEMMIRMH   876
       ...... .  *.**             .       .  *  *  *  ****  .*  *  *  **.

PepB   --SNISSKEKLYFNILNIFDILSYFSESINDKITFFKDINHKVQNKKIYRKYKDNLLKTLNGSYEENYNSYLSIRRKEYQKLKE------ALKYEEVNYK   923
EpiB   --SEFKIP-KEFIVAISIDFLLDYLEINKSEKEEILINNAEDLYRSNDIREYKNLLAKLTNPKN--DYEILKKEFPNLHEFLFNKISILENLKKTLQKSL   942
NisB   KDTNNDWKVDDVSILVNYLYLKCFFQNDNKKILNFLN-LVSTKKVKENVNEKIEHYLKLLKVNNLGDQIFYDKNFKELKHAIKN------LFLKMIAQDF   947
SpaB   RMKDITIS-KEIAGMVSVIQFLEQFELTFEEQLTFLERNSLQNEYRTEFKKDREMYIEICNSDRDWDNLKKTSDGGMLYETLKTRKMAAAHYAFLIKKAF   975

PepB   K------SIILSIVHMFNNRLSGINREHEELVMEFASRTLIDYQNMRKYL-----   967
EpiB   YTS--RSRIIGSFIHMRCNRIFGINPEKEKFVLSIFNEITKTKKYWDGCD-----   990
NisB   ELQ-KVYSIIDSIIHVHNNRLIGIERDKEKLIYYTLQRLFVSEEYMK--------   993
SpaB   DNKDEVYSRIGSIIHLHCNRLFGTDRELENKILTLCRHSLYAQRYQKMNGSLAWK  1030
         * * .*.  **.*   . *   .
```

Fig. 6. Sequence alignment of PepB, EpiB, NisB and SpaB as described for Fig. 4.

A transporter gene, *pepT*, is located upstream of *pepA*. Another open reading frame in reverse orientation was found upstream of *pepI* which codes for a putative protein of 571 amino acids with a molecular mass of 64.8 kDa. Data base searches revealed similarities to the LanT proteins of the biosynthetic gene clusters for epidermin (EpiT'/EpiT''), subtilin (SpaT) and nisin (NisT). The highest degree of similarity with 30% identical and 24% conservatively exchanged amino acids was found between PepT and EpiT'/EpiT''. PepT shows the typical features of the ABC transporter protein family; the N-terminal domain covering the first 300 amino acids is predicted to contain six membrane-spanning segments while the C-terminal domain is hydrophilic and includes the ATP-binding site formed by the conserved Walker motifs A (amino acids 362−373) and B (amino acids 470−482) (Fig. 2).

Western blot and SDS/PAGE analysis of Pep5 production.
Complementation of *S. epidermidis* 5 Pep5⁻ with *pepIAPBC* resulted in a clone (pGB10R; Bierbaum et al., 1994) which produced approximately 10% of the amount of Pep5 as compared to the wild-type producer (Fig. 5A, lane 4). After deletion of the C-terminal part of *pepC* from this construct the amount of peptide excreted increased (lane 6), while after inactivation of *pepP* hardly any peptide was detectable (lane 5). Neither the clones pMR4 and pMR7, harbouring only *pepIAP* and *pepA*, respectively, excreted any peptide in the size range of Pep5 (lanes 7, 8) nor did the host control *S. epidermidis* 5 Pep5⁻ (lane 9).

Western blot analysis of SDS cell extracts from the same clones using antiserum raised against Pep5 leader peptide indicated that, in particular, clones pMR4 and pMR7 accumulated prepeptides inside the cells (Fig. 5B, lanes 8, 9). Also the extract of the protease-deficient clone pGRO1 contained considerable amounts of prepeptide (lane 4) in addition to the incorrectly processed leader peptide. In contrast, no prepeptide was detectable in the wild-type producer because of rapid modification and processing (Weil et al., 1990). Only small amounts of prepeptide can be found in clones pGB10R and pCM7 (lanes 3, 7).

```
PepC   --------------MNR-------------FLNTYIK-QVTWIDNVDSYINNLYGPEP-IYKASLIT--------------GYPGIAISLFAIYKETNNF    57
EpiC   LAVLYTCVVIEYSVLILKKKNLFYLFLMKLQKLKNIGMVVININNIKKILENKITFLSDIEKATYIIENQSEYWDPYTLSHGYPGIILFLSASEKVFHKD   100
NisC   ----------MRIMMNK------------KNIKRNVEKIIAQWDERTR--KNKENFDF--GELTLST--------------GLPGIILMLAELKNKDNSK    60
SpaC   ---------MERGTVSR----------IEVEIVKEMARQISNYDKVLEIVNQKDNFRS-IGEVPLIP------WKSTALSHGIPGICMLYGELHAHFPEE    74
                                                                     *  ***  .

PepC   EYYELCNKYLEKTIELINDTPHYSTSLFEGAFGTIFSLLVCSDSGSNYSWIIKNLLFEYKKISKNEIDRLRTKLKNNNIQFYEFDIISGCAGTLSLLLLA   157
EpiC   -LEKVIHQYIRKLG-PYLESGIDGFSLFSGLSGIGFALDIASDKQYSYQSILEQIDNLLVQYVFDFLNN-----DALEVTPTNYDIIQGFSGIGRYLLNR   193
NisC   IYQKKIDNYIEYIVSKLSTYGLLTGSLYSGAAGIALSILHLREDDEKYKNLLDSLNRYIEYFVREKIEGF----NLENITPPDYDVIEGLSGILSYLLLI   156
SpaC   GWDDIGHQYLSILVNEIKEKGLHTPSMFSGAAGIGLAAICLSQRFTYYNGLISSINEYLAETVPQLLTEF----DQRQVCMSDYDVIEGVSGIANYLLLF   170
              *.        . *..* *   .         *  .. .            .       .       .*.* * .*   **

PepC   ----------TDIFPELSELLVDEIVQIT---SILTELVIKFNN-DDYLLDTILSNLGYAHGIPGIINTLCNSYKRGYGIIKTKKILEQSIFTLLQNLKL   243
EpiC   ---ISYNYNAKKALKHILNYFKTIHYSKD---NWLVSNEHQFLDIDKQNFPSGNINLGLAHGILGPLSLTALSKMNGIEIEGHEEFLQDFTSFLLKPEFK   287
NisC   N-DEQYDDLKILIINFLSNLTKENNGLIS----LYIKSENQMSQSESEMYPLGCLNMGLAHGLAGVGCILAYAHIKGYSNEASLSALQKIIFIYEKFELE   251
SpaC   QEDKAMGDLLIDILKYLVRLTEDIIVDGEKVPGWHIPSQHQFTDIEKKAYPYGNFNMGLAHGIPGPICVLSSALIQGIKVKGQERAIEKMANFLLEFSEK   270
                                                      *.* ***. *    . .  *     .

PepC   ENG----------TIYIPNDIESPND-YRDAWCYGLPSVAYTIFNVSSTLKNKSLIELSESLLHQVFLRSDNATKLISPTLCHGFSGVVMISLLMN---N   329
EpiC   NNN--EW-----FDRYDILENYIPNYSVRNGWCYGDTGIMNTLLLSGKALNNEGLIKMSKNILINIIDK--NNDDLISPTFCHGLASHLTIIHQANKFFN   378
NisC   RKKQFLWKDGLVADELKKEKVIREASFIRDAWCYGGPGISLLYLYGGLALDNDYFVDKAEKILESAMQ---RKLGIDSYMICHGYSGLIEICSLFKRLLN   348
SpaC   EQDSLFWKGIISFEEYQYGSPPNAVNFSRDAWCYGRPGVCLALVKAGKALQNTELINIGVQNLRYTIS---DIRGIFSPTICHGYSGIGQILLAVNLLTG   367
              *  ****    .       .* *     *          .  *   *** .    *        .

PepC   NELSSKYQKKIIQ-----S--YIDQIDGLYFDINDPSN----FSKDIGLLNGNAGILLTLLSYDNNK-LINIRWFDFMIMS   398
EpiC   LSQVSTYIDTIVR---KIISHYSEESSFMFQDIEYSYG-QKIYKNKVGILEGELGVLLALLDYIDTQNQSRKNWKNMFLIT   455
NisC   TKKFDSYMEEFNVNSEQILEEYGDESGTGFLEGISGCILVLSKFEYSINFTYWRQALLLFDDFLKG-------GKRK----   418
SpaC   QEYFKEELQEIKQ---KIMSYYDKDYIFGFHNYESMEGDEAVPLQYVGLLDGAVGVGLGVLNMELG---SKTDWTKALLI-   441
              *        .                *                            *
```

Fig. 7. Sequence alignment of PepC, EpiC, NisC and SpaC as described for Fig. 4.
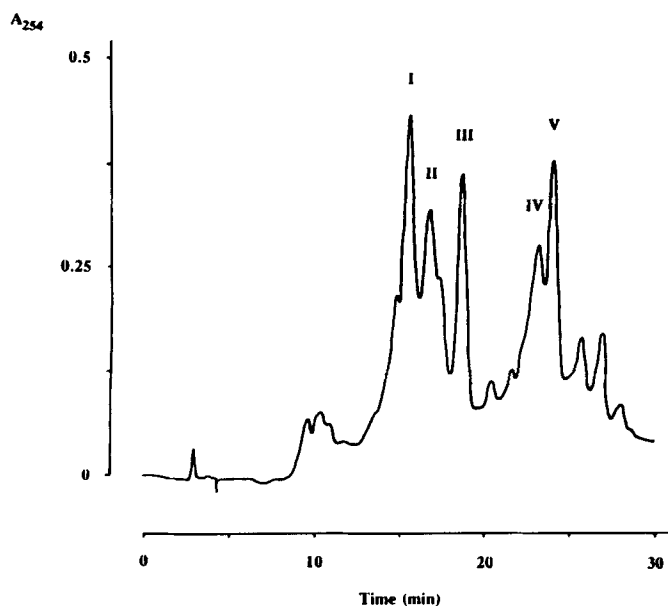
Fig. 8. HPLC elution profile of Pep5 peptides isolated from the culture supernatant of the PepC deficient mutant *S. epidermidis* 5 Pep5⁻ pCM7.

Fig. 9. Electro-spray mass spectroscopy of peptide 1 (purified from peak I of the supernatant of *S. epidermidis* 5 Pep5⁻ pCM7, see Fig. 8 and Table 2) before (A) and after (B) treatment with Ellman's reagent. The figure shows the mass range of the triply charged ion $(m+3H)^{3+}$ resulting in peptides of 2766 Da and 3162 Da, respectively; the calculated mass for peptide 1 after addition of two Ellman's reagent molecules is 3162 Da.

DISCUSSION

The Pep5 gene cluster. Several lantibiotic biosynthetic gene clusters have been sequenced so far and a general picture is emerging on how lantibiotic biosynthesis may proceed and which classes of proteins are involved. Besides several proteins providing general functions such as processing proteases, transporters and two-component regulators, two putative modification enzymes LanB and LanC have been identified. In the case of lactocin S (Skaugen, 1994) and lactococcin DR (Rince et al., 1994) only one enzyme, such as LasM, which in the C-terminal half shares sequence similarity with the LanC proteins (Sahl et al., 1995) is sufficient for the modifications.

The Pep5 biosynthetic gene cluster comprises *pepTIAPBC*, i.e. besides the structural gene and the immunity gene there is information for a translocator, a protease and the modification enzymes PepB and PepC. Genes for regulatory proteins, the reg-

ulator LanR and the corresponding sensor kinase LanK, are not present. Both genes were found in the nisin (*nisR, nisK*; Klein et al., 1993) and subtilin (*spaR, spaK*; Engelke et al., 1994) gene clusters, whereas in the epidermin cluster only the regulator EpiQ (equivalent to a LanR protein) is encoded (Schnell et al., 1992). The absence of *pepR* and *pepK* from the Pep5 gene cluster does not necessarily imply that Pep5 production is not regulated; rather, the failure of transforming of *S. carnosus* TM300 into a Pep5 producer with the plasmid pGB7 suggests that essential information for the heterologous expression of Pep5 production is missing on this plasmid (Bierbaum et al., 1994). Just as in the case of epidermin, where the location of one gene (*epiK*)

is unknown (Peschel et al., 1993), the information for both of the corresponding Pep5 genes may be located on the chromosome or on one of the other four plasmids of the producer strain (Ersfeld-Dressen et al., 1984).

The location of potential promoters and transcriptional terminators as indicated in Fig. 2 suggests that the structural gene *pepA* together with the genes for the essential modification and processing enzymes *pepPBC* are coordinately transcribed. The promoter in front of *pepA* is functional as indicated by the clone pMR7 which only contains the structural gene and which produces and accumulates pre-Pep5 (Fig. 5B). Downstream of *pepA* there are no further potential promoters for *pepP*, *pepB* and *pepC* detectable. The relatively weak terminator downstream of *pepA* could allow partial readthrough, thus reducing the production of PepP, PepB and PepC and thereby ensuring a high prepeptide/enzyme ratio, while the terminator behind *pepC* is probably strong enough for transcription termination. However, using a *pepA* probe we could not detect any transcript longer than approximately 300 bases indicating that, if a *pepAPBC* transcript exists, it is rapidly processed. Similar observations were reported for nisin (Engelke et al., 1992; Kuipers et al., 1993) and epidermin (Schnell et al., 1992). Although it cannot be excluded that the short *pepI* transcript also derives from processing, it could be transcribed as a monocistronic mRNA. In any case, it seems crucial for the Pep5-producing strain to ensure a balanced production ratio between the lantibiotic and the immunity peptide; it remains to be studied how this is achieved. Previous work has shown that there is no expression of the immunity phenotype when *pepA* is deleted or truncated (Reis et al., 1994) and it appears not inconceivable that the prepeptide in any stage of modification participates in the activation of the *pepI* promoter.

Roles of PepP and PepT in Pep5 biosynthesis. The fact that *pepIAPBC* was sufficient to transform the pED503-cured variant of *S. epidermidis* 5 into a Pep5 producer demonstrates that PepT is not essential for Pep5 in the homologous host (Bierbaum et al., 1994). However, there are indications that the host-provided translocators apparently substituting for PepT are less efficient and that, for high production rates, PepT is indispensable. The strongest argument for such an interpretation is the observation that, in the absence of PepT, Pep5 yields drop to approximately 10% (Fig. 5A). The substituting transporters probably act more slowly because of the thioether-based ring structures; the PepC-deficient strain excretes more processed Pep5 and accumulates less unprocessed prepeptide inside the cells than the PepP-deficient clone; a higher production was also observed with a mutated Pep5 molecule from which the bulky ring C had been deleted by site-directed mutagenesis (Bierbaum et al., unpublished). Our results compare well with the epidermin transporter: *epiT'T''* is a discontinous gene which is not necessary for epidermin production in a heterologous host (Schnell et al., 1992; Augustin et al., 1992).

In contrast, the protease PepP is essential for cleaving off the leader sequence after the propeptide has been modified. Our results show that PepP is the only protease of the producer strain which is able to recognize the specific cleavage site of pre-Pep5; processing by other proteases is possible but yields truncated peptides which show strongly reduced antibacterial activities. The importance of dedicated proteases is also reflected by the fact that, with the exception of subtilin, proteases have been found in all lantibiotic gene clusters sequenced so far. Like PepP, NisP and EpiP belong to the family of the subtilisin-like serine proteases (Siezen et al., 1991; Van der Meer et al., 1993) and both show the features of pre-pro-proteins with a characteristic N-terminal procaryotic secretory signal sequence and a potential

signal peptidase cleavage site (Siezen et al., 1991; Van der Meer et al., 1993). In addition, the last 30 amino acids of the C-terminus of NisP show a sequence similarity to a consensus membrane anchor sequence which suggests an export across the cell envelope and the attachment to the cellular membrane. PepP does not have a signal sequence and must be located in the cytoplasm because no prepeptide was detected outside the cell after inactivation of PepP, in contrast to the results that were obtained with a clone of *Lactococcus lactis* harbouring *nisABTCIR* without a functional *nisP* (Van der Meer et al., 1993). This also substantiates the interpretation that Pep5 must be processed inside the cell. The putative ElkP protease, that is encoded in the epilancin K7 gene cluster of *S. epidermidis* K7 directly downstream of the *elkA* structural gene (Van de Kamp et al., 1995), resembles PepP by lacking these sequence attributes and is probably also intracellularly located. Epilancin K7 is structurally more closely related to Pep5 than other lantibiotics which obviously is also reflected in increased organizational and functional similarities of the biosynthetic apparatus.

PepC is a thioether-forming enzyme. The absence of any sequence similarity to known proteins connected LanB and LanC proteins with the modification reactions as soon as the respective genes were first described (Schnell et al., 1991). In spite of this, there is no experimental evidence so far as to the exact function of these enzymes. It was assumed that one enzyme would be involved in dehydration of hydroxyamino acids while the other would catalyze the ring formation. However, spontaneous addition of thiol groups at ambient temperature is possible and has been used in the structure determination of lantibiotics and, therefore, was discussed as an alternative (Jung, 1991). Also, it was not clear whether dehydration and ring formation would indeed be separate processes or catalyzed in one step. The construction of a PepC-deficient clone and the characterization of its secretion products now allows us to unambigously asign a function to this protein as well as to deduce the function of the PepB protein.

The PepC-deficient clone secretes completely dehydrated Pep5 peptides, thus excluding the possibility that PepC is itself involved in dehydration. Instead, the majority of the peptides do not contain thioether amino acids, none of the peptides possesses the correct thioether pattern and just a small fraction, an estimated 20% of the total peptides purified, contains one lanthionine molecule out of three. This strongly suggests that PepC is responsible for thioether formation; it seems reasonable to assume that PepC binds to the dehydrated prepeptide and stabilizes a prepeptide conformation which enables the sulfhydryl groups to add to the correct dehydroamino acid. Apparently, in the absence of PepC and consequently of thioether rings, and possibly also in the absence of the correct exporter PepT, which could create a transport bottle neck and prolong the time during which the peptides are exposed to intracellular proteases, unbridged Pep5 peptides are subject to proteolytic destruction; particularly, the Gly29-Lys30 bond is susceptible to hydrolysis, also indicating that in the peptide which contains one lanthionine Cys33 may be part of the ring bridging to one of the N-terminally located dehydroamino acids. The partial formation of this ring could be facilitated by transient stabilization of a peptide structure during some biosynthetic reaction such as processing or transmembrane translocation.

Model for the biosynthesis of Pep5-type lantibiotics. The identification of PepC as the thioether-forming protein makes it very likely that the dehydration of Ser and Thr residues is catalyzed by PepB. This interpretation is supported by the previous isolation of completely unmodified prepeptide from clone *S.*

epidermidis 5⁻ pMR7 (Sahl et al., 1991) which contains only the structural gene but none of the biosynthetic genes (Table 1). This strain accumulates the prepeptide inside the cells and does not secrete any processed peptide (Fig. 5). Similarly, in clone pMR4 which contains *peplAP* but lacks *pepB* and *pepC*, only accumulation but no processing and secretion are detectable (Fig. 5). As there is no reason to assume that the immunity peptide or the protease are involved in dehydration, this function can only be attributed to PepB. Another interesting observation is that, although the protease is present in clone pMR4, obviously processing does not take place; this indicates that PepP may not recognize the cleavage site unless Thr^{+1} is dehydrated to Dhb. It is also remarkable that, at least with the expression *system* used in this study where PepT is missing, Pep5 cannot be transported in the unprocessed form. Secretion of modified but uncleaved nisin in NisP-deficient clones was recently reported by Van der Meer et al. (1993), in which case however the processing protease is located outside the cell.

Based on the present and previously published results (Weil et al., 1990; Sahl et al., 1991), it is possible to draw a rather precise picture of the sequence of events and cellular locations of the reactions leading to mature Pep5.

a) The prepeptide is ribosomally synthesized and rapidly dehydrated in its propeptide part by PepB.

b) The dehydrated prepeptide binds to PepC which folds Pep5 in such a way that correct thioether formation is possible.

c) The completely modified prepeptide is processed by PepP inside the cells.

d) Mature Pep5 is exported from the cells by PepT which can be functionally substituted to some extent by other host-encoded transporters. A somewhat relaxed substrate specificity of ABC transporters is well documented (Fath and Kolter, 1993).

e) PepI protects the producing cells by an as-yet-unknown mechanism (Reis et al., 1994). However, it is interesting to mention that PepI has no equivalent in any other lantibiotic gene cluster and that processing proteases are supposed to be extracellular proteins (Sahl et al., 1995) except in the case of Pep5, lactocin S (Skaugen, 1994) and epilancin K7 (Van de Kamp et al., 1995); it may well be that the intracellular processing of Pep5 requires a particular protection mechanism.

Currently, experiments are in progress to isolate the Pep5 biosynthetic enzymes, in particular PepB and PepC, and to verify their functions *in vitro*.

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