Production of a class II two-component lantibiotic of *Streptococcus pneumoniae* using the class I nisin synthetic machinery and leader sequence

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Key words: lantibiotic, peptide engineering, *Streptococcus pneumoniae*, nisin, NisB, chimeric peptides

Running title: Maturation of class II lantibiotics by nisin enzymes
Recent studies showed that the nisin modification machinery can successfully dehydrate serines and threonines and introduce lanthionine rings in small peptides that are fused to the nisin leader sequence. This opens up exciting possibilities to produce and engineer larger antimicrobial peptides in vivo. Here, we demonstrate the exploitation of the class I nisin production machinery to generate, modify and secrete biologically active, previously not yet isolated and characterized class II two-component lantibiotics that have no sequence homology to nisin. The nisin synthesis machinery, composed of the modification enzymes NisB and NisC and the transporter NisT, was used to modify and secrete a putative two-component lantibiotic of Streptococcus pneumoniae. This was achieved by genetically fusing the propeptide-encoding sequences of SPR1765 (PneA1) and SPR1766 (PneA2) to the nisin leader-encoding sequence. The chimeric prepeptides were secreted out of Lactococcus lactis, purified by cation exchange fast protein liquid chromatography and further characterized. Mass spectrometry analyses demonstrated the presence and partial localization of multiple dehydrated serines and/or threonines and (methyl)lanthionines in both peptides. Moreover, after cleavage of the leader peptide from the prepeptides, both modified propeptides displayed antimicrobial activity against Micrococcus flavus. These results demonstrate that the nisin synthetase machinery can be successfully used to modify and produce otherwise difficult to obtain antimicrobially active lantibiotics.
INTRODUCTION

Small antimicrobial peptides produced by Gram-positive bacteria are named bacteriocins. One group of bacteriocins, the non-lantibiotics, comprises peptides that do not require modification for their antimicrobial activity (46). Members of another group, the lantibiotics, require post-translational modifications to acquire biological activity (11, 47). Lantibiotics are produced as inactive prepeptides, consisting of an N-terminal leader peptide and a C-terminal propeptide part. Most of the serine and threonine residues of the propeptide are dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively, by LanB or LanM type enzymes (Lan - is a general abbreviation for proteins involved in lantibiotics biosynthesis). LanC or LanM enzymes can subsequently couple these dehydroresidues to cysteines thus forming a (methyl)-lanthionine ring. After the leader peptide is removed from the prepeptide by the extracellular LanP or transmembrane LanT proteins, the active lantibiotic is released. The immunity against the produced lantibiotics is provided by the LanI and/or LanFEG proteins (3, 21, 41). Three classes of lantibiotics are distinguished (47). Class I lantibiotics are modified by two enzymes, LanB and LanC. In class II lantibiotics, dehydration and cyclization are performed by a single enzyme called LanM. The C-terminal sequences of LanM type enzymes share homology with the LanC proteins. LanM enzymes share no homology with the LanB proteins (36, 45). Class II also includes two-component lantibiotics, of which antimicrobial activity mainly depends on synergistic action of both peptides (33, 34). Each of the peptides of the two-component lantibiotics, except cytolysin, possesses its own dedicated modification LanM enzyme. Class III consists of lantibiotics with other functions than antimicrobial activity (17, 48).

Due to an increasing resistance of bacteria to available antibiotics, there is an urgent need to search for substances active against multi-drug resistant pathogens. Since some lantibiotics exhibit a stable activity at nanomolar (nM) concentrations against antibiotic
resistant pathogens, it is currently of great interest to apply lantibiotics (7, 32, 40). It has already been shown in a mouse model that mersacidin is active against methicillin-resistant *Staphylococcus aureus* strains (MRSA) (18). Another lantibiotic, lacticin 3147, is a successful antimicrobial agent against MRSA *S. aureus*, vancomycin-resistant *Enterococcus faecalis*, penicillin-resistant *Streptococcus pneumoniae*, *Propionibacterium acne* and *Streptococcus mutans* (8).

One of the most studied lantibiotics is nisin (9, 10, 22, 27), a Class I lantibiotic, produced by certain *Lactococcus lactis* strains. It has a long record of safe industrial usage as a food preservative (7). Due to the broad activity spectrum against Gram-positive pathogens including *S. pneumoniae*, nisin has good potential for a number of other applications (9). Recently, it was shown that designed hexapeptides and non-lantibiotic peptides fused to the leader peptide of nisin could be successfully modified by NisB and NisC and exported ot of *L.lactis* via NisT (16, 20, 38). The discovery that the lantibiotic modification enzymes LanB, LanC and LanM possess rather low substrate specificities, brings a new opportunity to use them as a tool to improve stability and activity of peptides potentially valuable for medical applications (2, 16, 39).

Here, we present the successful application of the nisin expression/modification system to produce, modify and secrete entirely unrelated putative lantibiotics that, based on bioinformatics’ predictions, belong to the class II lantibiotics. The produced peptides were dehydrated multiple times, as shown by MALDI-TOF mass spectrometry. Importantly, the modified peptides showed antimicrobial activity against *Micrococcus flavus*. Our study demonstrates that the nisin production/modification machinery can be used to produce and posttranslationally modify silent lantibiotics, i.e. those for which production conditions are not known, and that otherwise would be difficult to obtain from their natural sources.
MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Strains were stored in 10% glycerol (v/v) at -80 °C. *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Streptococcus mitis* strains were grown at 37 °C in standing M17 (Difco) broth supplemented with 0.5% (w/v) glucose (GM17) and, when appropriate, 2 µg/ml chloramphenicol. *Lactococcus lactis* and *Micrococcus flavus* were grown at 30 °C in GM17 or minimal medium (39) supplemented with 5 µg/ml chloramphenicol and/or 5 µg/ml erythromycin when appropriate.

Construction of chimeric peptides. Standard genetic manipulations were essentially performed as described by Sambrook *et al.* (43). Plasmid pIL3BTC encoding the nisin modification machinery (39) and plasmid pNZnisA-E3 (19) were used to produce and modify chimeric peptides. Briefly, two open reading frames spr1765 and spr1766 (pneA1 and pneA2, respectively) were amplified by PCR from genomic DNA of *S. pneumoniae* R6 and cloned into pNG8048E resulting in a plasmid, named pNGspr1765-1766, for which *L. lactis* NZ9000 (39) was used as a host. All the subsequent genetic cloning procedures were performed in this organism. This plasmid, pNGspr1765-1766, served as a template to amplify separately the genes spr1765 and spr1766. Subsequently, each of the amplified products of genes spr1765 and spr1766 was subcloned to pNZnisA-E3 expression plasmid. This resulted into two new plasmids, pNZE3-nis-spr1765 and pNZE3-nis-spr1766, which carried the nisin structural gene and a lantibiotic gene next to one another. To construct genetic fusions of the nisin leader sequence and the structural leaderless sequence of the spr1765 and spr1766 (pneA1 and pneA2, respectively) genes in frame, the round PCR method with 5’ phosphorylated primers was used as described earlier (39) using Phusion DNA polymerase (Finnzymes). The final chimeric peptide expression plasmids pNZE3-spr1765 and pNZE3-spr1766 were thus constructed. These plasmids were used separately in combination with a plasmid pIL3BTC, to
produce and secrete modified chimeric peptides. Plasmid isolation was performed by means of the Plasmid DNA Isolation Kit (Roche Applied Science). Restriction analysis was performed with restriction enzymes from Fermentas. DNA ligation was performed with T4 DNA ligase (Fermentas).

**Peptides.** Peptides encoding the sequence of leaderless PneA1 and PneA2 were purchased from Pepscan Lelystad NL. Peptides were purified to homogeneity by HPLC on a C12 Jupiter 4µ Proteo 90Å 250 x 4.6 mm column with an acetonitril gradient.

Expression and purification of microbially produced peptides was performed as follows. Overnight cultures of *L. lactis* NZ9000 containing pIL3BTC and a chimeric peptide expression plasmid, namely pNZnisA-E3 or pNZE3-spr1765, or pNZE3-spr1766, in GM17 were diluted 1:50 in minimal medium containing appropriate antibiotics and for induction 0.5 ng/ml nisin (Sigma). Cultures were grown for 24h at 30 °C. Subsequently, supernatants were separated from cells by centrifugation. Next, supernatants were filtered through a 0.2 µm filter (Millipore). Prior to purification on a 5 ml HiTrap SP cation exchange column (GE Healthcare) using fast protein liquid chromatography (FPLC on Akta purifier; Amersham Bioscience), supernatants were diluted 1:1 with a 100 mM lactic acid solution and filtered through 0.2 µm filters. After passage of supernatant through a column, unbound compounds were washed away with 100 mM lactic acid. Elution was performed with 1 M NaCl in 50 mM lactic acid solution. The fractions containing prepeptides were concentrated and desalted with 50 mM Tris-HCl of pH 5.5 on Microcon columns (Milipore). Intact prepeptides and peptides without leader sequence were analyzed with a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and used for screening of antimicrobial activity.

**N-terminal sequence removal.** The N-terminal sequence from the FPLC-purified prepeptides prePneA1 and prePneA2 was removed by trypsin. Prepeptides were incubated for 2h at 37 °C with 20 µg/ml trypsin in 100 mM Tris-HCl buffer pH 8 containing 10 mM CaCl₂.
Alternatively to remove the leader from prePneA2, 180 µl of the prepeptide was incubated for 30 min at 37 °C with 20 µl of 0.5 M phosphate buffer pH 7.4 and with 10 µl of leucine aminopeptidase (Sigma; suspension in 3.5 M ammonium sulphate).

**Mass spectrometry analysis.** To investigate whether chimeric peptides possess free cysteine residues, reactions with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) were performed. To obtain higher mass spectra resolutions with MALDI-TOF both prepeptides and propeptides, prior to CDAP treatment, were purified on a Hewlett Packard 1050 HPLC apparatus using a C12 Jupiter 4µ Proteo 90Å 250 x 4.6 mm column. Reverse phase purification was used with a gradient of 10% - 40% acetonitril in purified water. All buffers contained 0.1% TFA (trifluoroacetic acid). The reactions with CDAP were performed as described before (16, 35). Briefly, the pH of vacuum-dried trypsinated, non-trypsinated peptides and a control peptide, termed NisB2, (H-CRYTDPKPHIRLRK-OH) resuspended in 16 µl MilliQ was adjusted to 2-3 with 0.1% TFA. Prior to treatment with CDAP, 1 µl of 100 mg/ml of the reducing agent, triscarboxyethyl phosphine (TCEP), was added to each mixture and a reaction was carried out for 5 min at room temperature. Subsequently, 2 µl of 100 mg/ml CDAP was added to the mixtures, followed by incubation for 15 min at room temperature.

Analysis was essentially performed as described before (19). Briefly, ZipTips (C18 ZipTip, Millipore) were wetted with 100% acetonitrile and washed with 0.1% TFA. Subsequently, the supernatant containing the peptides was mixed with 0.1% TFA and applied to a ZipTip. Peptides that were bound to the column were washed with 0.2% TFA and eluted with 50% acetonitrile and 0.1% TFA. The eluent was mixed in a ratio of 1:1 with 10 mg/ml α-cyano-4-hydroxycinnamic acid (matrix). 1.5 µl of such prepared mixture was spotted on the target and allowed to dry. Mass spectra were recorded with a Voyager-DE Pro (Applied
Biosystems) MALDI-TOF. In order to increase the sensitivity external calibration was applied with six different peptides (Protein MALDI-MS Calibration Kit, Sigma).

**Amino acid sequence alignment.** Amino acid sequence alignment of nisin with pneumococcin A1 and A2 (SPR1765 and SPR1766, respectively) was performed with Clustal W (25), a program for multiple sequence alignments. The peptide sequences were derived from the NCBI database.

**Gel Electrophoresis.** Prepeptides and mature peptides were analyzed on Tris-tricine gels (44) and stained with Coomassie (Fermentas).

**Peptide concentration determination.** Peptide concentrations were determined using the DC protein assay of Bio-Rad. HPLC-purified nisin was used as standard.

**Minimum Inhibitory Concentration (MIC) determination.** MICs for *M. flavus*, *S. pneumoniae*, *E. faecalis*, *S. aureus* and *S. mitis* were performed in 96-well microtiterplates in GM17. The assay was performed as follows. Overnight cultures of the above mentioned strains were diluted 1:50 and growth was continued to OD$_{600}$ of 0.2. Subsequently, 150 µl cultures were mixed with 50 µl of appropriate medium and various concentrations of peptides. The microtiterplates were incubated in a GENios (TECAN Benelux) at a suitable temperature for overnight growth of the strain and the OD$_{600}$ was measured every 30 min. The MIC values were determined at the time when the cells without antimicrobial substance reached half of the maximal optical density. MICs were calculated from the lowest concentration of the antimicrobial substance that was able to inhibit the growth of the tested strain. All the susceptibility assays were performed in triplicate at least.

**RESULTS**

*In silico* analysis of the putative two-peptide lantibiotic-like cluster from *S. pneumoniae*. After *in silico* analysis of 11 putative bacteriocin genes, identified by BAGEL
(5), of S. pneumoniae R6 and their adjacent ORFs, we selected two of them, i.e. spr1765 and spr1766. *In silico* analysis of these two genes, as well as their nine adjacent ORFs, which most likely constitute a single cluster (Fig. 1), indicated that SPR1765 and SPR1766 belong to the class II of two-component like lantibiotics. We here propose to name SPR1765 and SPR1766, pneumococcin A1 and A2 (PneA1 and PneA2), respectively. So far there are no experimental data indicating that these bacteriocin-like peptides are expressed under laboratory growth conditions or under any other growth media or conditions we have tried (data not shown). *In silico* analysis showed that the nine adjacent genes (Fig.1), are likely involved in pneumococcin A1 and A2 modification, transport, processing and immunity. Gene spr1767 encodes a protein with amino acid sequence similarity to a classic bifunctional LanM-like modification enzyme. The spr1768 gene encodes a putative FAD-dependent flavoprotein that could catalyze the oxidative decarboxylation of C-terminal residues. The functions for SPR1764 and SPR1769, and SPR1774 are unknown. SPR1770 protein is a predicted ABC transporter containing a putative N-terminal double-glycine peptidase activity (peptidase of C39 family) and is most likely responsible for transport of modified bacteriocins and for prepeptide processing. The SPR1771 protein shares 48% identity with NisP, the nisin leaderpeptidase. The last two genes of the regulon, spr1772 and spr1773, encode a putative immunity protein and a putative ABC transporter, respectively. This analysis clearly shows that PneA1 and PneA2 very likely belong to the class II two-component lantibiotics.

Additionally, the gene cluster of pneumococcins contains a putative flavoprotein (spr1768). This type of enzyme is also found in the gene clusters of epidermin and mersacidin. The modifications made by the flavoproteins are required for full activity of these peptides (24, 29). Whereas NisT displays rather broad transport specificity of peptides fused to the leader peptide of nisin, NisP has been shown to only process fully modified prenisin (19). Figure 2 shows the amino acid sequence alignment of pneumococcin A1 and A2 with
the nisin structural peptide. There is a low similarity between the peptides both in the part of
the leader sequence and in the part of the propeptide. On the basis of lantibiotic leader
sequences (Fig. 2), the predicted pneumococcin A1 and A2 peptides possess three candidate
cleavage sites, one behind GlyGly / GlyAla (after which SPR1770 might cleave ?), one
behind a shared GlyAla and one behind ProArg. Strikingly, prenisin, prePneA1 and prePneA2
share the sequence GaxPRxT, the x being a variable residue, which sequence comprises in the
middle the site behind ProArg. These data together with the shared 48% identity of the
putative leaderpeptidase, SPR1771, with NisP indicate that the Pne-leaderpeptides end with
ProArg.

**Production, secretion and purification of the chimeric peptides.** To investigate the
production and secretion of chimeric peptides, pNZE3-spr1765 and pNZE3-spr1766 were
introduced into *L. lactis* NZ9000 containing pIL3BTC. Cultures of *L. lactis* NZ9000 plasmid-
containing derivatives induced with nisin were grown for 24 hours in minimal medium.
Subsequently, the supernatants were collected and the prepeptides were purified on a HiTrap
SP cation exchange column. The same procedure was applied for prenisin, a positive control.
All prepeptides were produced and secreted as visualized by Tris-tricine electrophoresis (Fig.
3). Prepeptide concentrations were determined with HPLC-purified nisin of known
concentration, as reference. Without nisin induction, no secreted peptides were observed (data
not shown). The production levels of both prePneA1 and prePneA2 were approximately 50%
lower, than that of prenisin.

**Streptococcal chimeric peptides are modified by nisin synthetase enzymes.** To test
whether the purified chimeras prePneA1 and prePneA2 were modified, they were analyzed by
MALDI-TOF spectrometry. Table 2 presents a summary of the obtained masses.
Interestingly, analysis of prePneA1 and prePneA2 showed that both prepeptides were
modified multiple fold. PrePneA1 showed four- and three-fold dehydration and prePneA2
four, three and two-fold. Chimeric prepeptides were processed by trypsin or leucine aminopeptidase and further characterized. Since the leader peptide keeps the prepeptide inactive, its removal allows to assess the antimicrobial activity of the mature peptides. Trypsin cleaves a peptide bond behind lysine or arginine, with arginine being preferred over lysine allowing arginine-specific cleavage under controlled conditions (Table 2).

The prePnA1 was processed by trypsin and only the nisin leader sequence was clipped off, leaving the mature peptide with multiple dehydrations (Table 2). However, prolonged digestion at higher concentration of trypsin resulted in two additional mass peaks corresponding to two fragments (Table 2). The N-terminal part of PnA1 (Table 2) was cleaved off and showed no dehydrations, likely due to protection against dehydration of Ser/Thr by their directly flanking residues (39). The identity of this N-terminal fragment was confirmed by sequence data obtained by postsource decay (data not shown). The other lysine residue in the mature peptide (…SSK…) appears protected against proteolysis, likely due to posttranslational modifications in its vicinity (26). The five dehydrations that were observed in the mature PnA1 are therefore located in the C-terminal part of the peptide, in which six serines are present. Since one of those serines is located next to the lysine that is cleaved by trypsin it is most likely that the observed dehydrations are all within the last 25 amino acids of PnA1, except for the first serine at SSK.

PnA2 contains a number of residues that are substrates for trypsin and some of them are not protected by modified residues (Fig. 2). Therefore, we initially obtained a smaller fragment (PnA2 fragment 2, Table 2), which lacked the C-terminal extension but contained multiple dehydrated residues. Additionally, in HPLC-purified chimeric prePnA2, there was also a clear fraction containing a peptide fragment consisting of part of the nisin leader sequence and the N-terminal part of PnA2 peptide (prePnA2 fragment 1, Table 2). Interestingly, this peptide fragment contained up to four dehydrations, which clearly shows
that four out of five dehydrated residues are located in the first 11 amino acids of the mature peptide.

Subsequently, removal of the N-terminal leader peptide from chimeric peptides by NisP overexpressed in *L. lactis* was also investigated. However, neither the release of the leader peptide nor antimicrobial activity of PneA1 or PneA2 was detected (data not shown). Intact PneA2, was obtained using leucine aminopeptidase and appeared to be 3- to 6-fold dehydrated (Table 2). Importantly the activity of the leucine aminopeptidase apparently had stopped after the last Arg. This indicates the presence of a thioether bridge starting at either Ser1 or Thr2. Studies by Rink et al indicated that flanking hydrophilic residues on both sides of a Ser or Thr would not favor dehydration. Furthermore serines are less readily dehydrated than threonines (39). Therefore a large extent of dehydration of Ser1 flanked by Arg and Thr is not likely. Taken together this indicates that Thr2 is fully dehydrated and thioether crosslinked to Cys5.

To investigate whether thioether rings were formed, CDAP, which reacts only with free cysteines, was used. The control NisB2 peptide that contains one free cysteine was used as a positive control (not shown) for CDAP modification. CDAP modification converts a free thiol group of cysteine into an isothiocyanate, yielding a mass increase of 25 Da. The five-fold dehydrated PneA1 showed hardly any CDAP modification indicating that there were no free cysteine residues and thus the formation of two thioether rings. The three-fold dehydrated PneA1 peptide showed single and double CDAP modifications indicating the formation of either one or no thioether rings (Fig. 4). Similar studies on CDAP modification of PrePneA2 indicated the presence of two thioether rings in the extensively dehydrated peptides (not shown). These data demonstrate that the putative lantibiotics, which are entirely unrelated to nisin, can be successfully produced, modified, and secreted by the nisin synthetase machinery.
The produced and modified peptides have significant antimicrobial activity. To investigate the antimicrobial activity of the modified peptides, the chimeric prepeptides were incubated with trypsin or leucine aminopeptidase to remove the N-terminal leader sequence. Various dilutions of trypsin-treated peptides, namely prenisin (positive control), pneumococcin A1 and A2 were tested for antimicrobial activity in the Minimum Inhibitory Concentration (MIC) assay (Table 3). Of all microorganisms tested, i.e. *M. flavus*, *S. pneumoniae*, *E. faecalis*, *S. aureus* and *S. mitis*, only *M. flavus* was susceptible to the tested peptides, i.e. PneA1 and PneA2 (Table 3). In control experiments no significant inhibition was found with either buffer or BSA-treated with trypsin or with empty samples, i.e. fractions from prepeptide purifications that did not contain peptides (Table 3). Additionally, undigested chimeric prepeptides did not show significant antimicrobial activity against the indicator strain (Table 3). Unmodified PneA1 (MIC > 50 µM) and PneA2 (MIC > 1.5 mM) propeptides obtained by chemical synthesis were respectively at least 30-fold or 170-fold less active than the HPLC-purified active fraction of the corresponding NisBC-modified peptides, without leader peptide. This proves that NisBC induced modifications are required for lantibiotic activity. PneA1 inhibited growth of *M. flavus* at a peptide concentration of 0.6 µM. PneA2, from which the leader sequence was removed either by trypsin or leucine aminopeptidase, inhibited growth at approximately 10 or 8.5 µM, respectively (Table 3). Combination of both modified chimeric peptides, PneA1 and PneA2, did not act synergistically (Table 3). Thus, the data demonstrate that it is possible to utilize the nisin synthetase machinery for the production of antimicrobially active peptides unrelated to nisin.

DISCUSSION
To the best of our knowledge we present here for the first time the successful expression, modification, secretion and biological activity of novel class II lantibiotics by the nisin synthetases, which normally produce nisin, a class I lantibiotic. To present a significant challenge as substrate peptides, pneumococcin A1 and A2 from *S. pneumoniae* R6, which presumably belong to class II two-component lantibiotics, were chosen as substrates for the nisin enzymes. The class II two-component lantibiotics require a LanM-type enzyme that performs both dehydratation and cyclization, whereas class I lantibiotics require LanB dehydratases and LanC-cyclases. It has been already shown that LanBC-type enzymes can modify peptides other than nisin which are fused to nisin leader sequence. Kluskens *et al.* and Kuipers *et al.* demonstrated that both medically relevant nonlantibiotic peptides and a truncated lantibiotic, lacticin 3147, fused with the nisin leader sequence, modified by NisB and NisC, were exported via NisT and contained dehydrated amino acids and lanthionine rings (16, 20). The same was proven by Rink *et al.* for various hexapeptides (38, 39). Thus, based on the discovery that the nisin synthetase machinery can accept various peptides as templates for modification, the propeptide part, which is the predicted maturating part of either the PneA1 or the PneA2 peptide, was fused to the nisin leader sequence and introduced in *L. lactis* that overexpresses NisBTC. The produced peptides were multifold dehydrated and contained thioether rings. Some dehydrated residues and one thioether ring could be localized by studying peptide fragments and by applying leucine aminopeptidase.

To be biologically active lantibiotics require prepeptide processing, i.e. removal of the leader sequence. The prepeptide sequences and the homology of the peptidase with NisP indicates that the site behind PR might be the processing site where the propeptide starts. To liberate the mature peptides, we used trypsin or leucine aminopeptidase. Exported, purified and processed peptides were tested for antimicrobial activity and *M. flavus* was found to be highly susceptible to both the PneA1 and PneA2 peptides. Despite the fact that PneA1 and
PneA2 are predicted two-component lantibiotics, we did not observe any significant synergistic effect when these peptides were combined (data not shown). The PneA1 and PneA2 peptides are a mixture of extensively modified and hardly modified peptides. These mixtures probably contain active, less active and inactive peptides. Thus, the determined antimicrobial activity is the mean of those of all these peptides, indicating that the specific activity for a single active peptide might be higher. In this respect a preliminary experiment was performed with processed and NisBC-modified PneA1 and PneA2 peptides which were four and five-fold dehydrated were separated and purified from the total mixture. However, MICs for both peptides were not significantly different from MICs of the unpurified peptides (data not shown). A challenge for future work might be to sort out the active peptide fraction from the inactive fraction in order to get a better picture of which modifications yield antimicrobially active peptides.

The putative cluster of pneumococcins consists of 11 ORFs. In the cluster two genes might be required for proper modification of PneA1 and PneA2. These genes encode a single putative LanM-type modification enzyme and a putative LanD-type flavoprotein. Flavoproteins catalyze the oxidative decarboxylation of a C-terminal cysteine residue involved in ring formation. A FAD-dependent flavoprotein catalyzes this reaction for mersacidin, a lantibiotic produced by Bacillus sp. (29). Another flavoprotein, which is FMN-dependent, catalyzes the same reaction for epidermin, a lantibiotic of S. epidermidis, and this enzyme is essential for formation of a biologically activity peptide (24). It is not known whether the putative LanD-type flavoprotein of PneA1 and PneA2 performs a similar function in this cluster. Because the original cluster of pneumococcins contains LanM and LanD-type modification enzymes, peptides modified by NisB and NisC might not be fully active by lack of the oxidative decarboxylation. Furthermore, we do not know whether the native dehydration and ring pattern is exactly the one installed heterologously by NisB and NisC.
These factors might explain the presumably suboptimal antimicrobial activities and lack of synergism within this putative two-component lantibiotic system. However, both peptides, PneA1 and PneA2 still showed significant antimicrobial activity.

The production of non-lantibiotic or lantibiotic chimeras with a heterologous system has been reported using either closely related or non-lantibiotic peptides. For example, production of chimeric non-lantibiotic bacteriocins: pediocin PA-1 fused to the leader of lactococcin A and/or to enterocin P, or enterocin A fused to the leader of enterococcin P, resulted in the secretion of active peptides (13, 30, 31). These cases of successful production of biologically active bacteriocins concerns non-lantibiotic bacteriocins, which in contrast to lantibiotics do not require posttranslational modifications for antimicrobial activity.

Production of class I lantibiotic chimeras such as nisin/subtilin or subtilin/nisin, with either subtilin or nisin expression machineries, was performed successfully (1, 23). Of the amino acids residues of the leaders and mature peptides of subtilin and nisin 57% is identical (23).

Studies using lacticin 481 synthetase demonstrated its ability to prepare other lantibiotics in the class II lacticin 481 family, including nukacin ISK-1, mutacin II, and ruminococcin A (37).

In contrast, we show here for the first time that it is possible to use the nisin synthetase system to produce, modify and secrete lantibiotics from a very different source and class, which exhibit considerable antimicrobial activity.

Acknowledgments

We thank Patrick J. Bakkes, Hadi Eskandari and Agnieszka Moskal for their technical help in conducting some experiments presented in this study. Jacek Lubelski was supported by the Dutch Technology Foundation, STW project 06927.
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Figure legends

Fig. 1. Putative pneumococcin A1 and A2 gene cluster. Organization of the chromosomally located biosynthetic gene cluster of pneumococcin A1 and A2 (spr1765 and spr1766, respectively) in *S. pneumoniae*. Open reading frames (ORFs) are represented by thick grey arrows and their numbers, shown in grey arrows, stand for the SPR gene ID. The putative promoters are represented by thin and bent black arrows.

Fig. 2. Amino acid sequence alignment of nisin with pneumococcins A1 and A2. The cleavage site in the peptide sequence is highlighted in grey. Identical amino acids residues for all three sequences are indicated with an asterisk and conserved residues are shown by a colon and semi-conserved amino acids by a point.

Fig. 3. Tris-Tricine gel illustrating trypsin-treated and non trypsin-treated purified chimeric peptides and nisin. Lane M, marker. Lane 1, prePneA1. Lane 1A, trypsin-treated PneA1. Lane 2, prePneA2. Lane 2A, trypsin-treated PneA2. Lane 3, prenisin. Lane 3A, trypsin-treated prenisin.

Fig. 4. Modified PneA1 contains thioether bridges. The SAAASSKVCISAASVSGGLYNSNDCLG fragment 4-fold, 3-fold and 2-fold dehydrated (solid line) is treated with CDAP (dotted line) to detect the presence of modifiable (yielding peptide +25 Da) and non-modifiable thioether-linkage-forming cysteines. The 2643.6 Da peak which is 4-fold dehydrated shows a mild single CDAP addition. The 2661.9 Da (3-fold dehydrated) peak shows single and double CDAP additions as indicated by black arrows. The 2680.5 Da (2-fold dehydrated) peak shows clear single and double CDAP...
additions; 1 CDAP 2705.3 Da (+25 Da) and 2 CDAP 2729.8 Da (+50 Da). The 5-fold dehydration peak is becoming visible after CDAP addition indicating that this peptide has two thioether rings.
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<tr>
<td><strong>S. pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R6</td>
<td>D39 (Δcps2 2538-9862) with increased transformation efficiency</td>
<td>(14)</td>
</tr>
<tr>
<td><strong>L. lactis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZ9000</td>
<td>MG1363 ApepN::nisRK</td>
<td>(15)</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RN6390B</td>
<td></td>
<td>Lab collection</td>
</tr>
<tr>
<td><strong>S. mitis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTCC10712</td>
<td></td>
<td>Lab collection</td>
</tr>
<tr>
<td><strong>E. faecalis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V583</td>
<td></td>
<td>(42)</td>
</tr>
<tr>
<td><strong>M. flavus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIZO B423</td>
<td></td>
<td>NIZO (^{a}) Food Research</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pIL3BTC</td>
<td><em>nisBTC</em>, encoding for nisin modification machinery; Ery(^{R})</td>
<td>(39)</td>
</tr>
<tr>
<td>pNZ8048</td>
<td>nisin-inducible <em>PnisA</em>; Cm(^{R})</td>
<td>(6)</td>
</tr>
<tr>
<td>pNG8048E</td>
<td>nisin-inducible <em>PnisA</em>, pNZ8048 derivative containing Ery(^{R}) gene to facilitate cloning; Cm(^{R}) Ery(^{R})</td>
<td>Lab collection</td>
</tr>
<tr>
<td>pNZnisA-E3</td>
<td><em>nisA</em>, encoding for nisin</td>
<td>(19)</td>
</tr>
<tr>
<td>pNZE3-nis-spr1765</td>
<td>contains <em>nisA</em> gene and SPR1765 gene</td>
<td>This work</td>
</tr>
<tr>
<td>pNZE3-nis-spr1766</td>
<td>contains <em>nisA</em> gene and SPR1766 gene</td>
<td>This work</td>
</tr>
<tr>
<td>pNZE3-spr1765</td>
<td>contains a part of <em>nisA</em> gene which encodes for leader peptide of nisin and leaderless part of SPR1765 gene fused in frame</td>
<td>This work</td>
</tr>
<tr>
<td>pNZE3-spr1766</td>
<td>contains a part of <em>nisA</em> gene which encodes for leader peptide of nisin and leaderless part of SPR1766 gene fused in frame</td>
<td>This work</td>
</tr>
<tr>
<td>pNGspr1765-1766</td>
<td>pNG8048E contains spr1765 and spr1766 gene under own promoter</td>
<td>This work</td>
</tr>
</tbody>
</table>

2. Ery\(^{R}\), erythromycin resistance; Cm\(^{R}\), chloramphenicol resistance
3. \(^{a}\) Dutch Institute of Dairy Research.
Table 2. NisB-mediated dehydration of chimeric prepeptides and their fragments analyzed by MALDI-TOF mass spectrometry. The average masses are shown.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>Number of observed dehydrations</th>
<th>Mass (M + H+) w/o Met1 (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OBSERVED</td>
</tr>
<tr>
<td>PrePneA1</td>
<td>4</td>
<td>6009.6</td>
</tr>
<tr>
<td>NisinLeader-</td>
<td>3</td>
<td>6026.5</td>
</tr>
<tr>
<td>WTPTPIIKSAASSKVCISAA VSGIGGLVSYNNDCLG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PneA1</td>
<td>6</td>
<td>3655.9</td>
</tr>
<tr>
<td>WTPTPIIKSAASSKVCISAA VSGIGGLVSYNNDCLG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PneA1 fragment 1</td>
<td>0</td>
<td>1068.9</td>
</tr>
<tr>
<td>WTPTPIILK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PneA1 fragment 2</td>
<td>4</td>
<td>2643.6</td>
</tr>
<tr>
<td>SAAASSKVCISAAVSGIGGLVS YNNDCLG</td>
<td></td>
<td>2662.0</td>
</tr>
<tr>
<td>PrePneA2</td>
<td>4</td>
<td>5437.6</td>
</tr>
<tr>
<td>Nisinleader-</td>
<td>3</td>
<td>5454.0</td>
</tr>
<tr>
<td>STIICSATLSFIASYLGSQAQTRC GKDNNKKK</td>
<td></td>
<td>5472.2</td>
</tr>
<tr>
<td>PneA2</td>
<td>6</td>
<td>3086.7</td>
</tr>
<tr>
<td>STIICSATLSFIASYLGSQAQTRC</td>
<td>5</td>
<td>3103.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----</td>
<td>------</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3121.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3139.6</td>
</tr>
<tr>
<td>prePneA2 fragment 1:</td>
<td>4</td>
<td>2085.1</td>
</tr>
<tr>
<td>SKKDSGASPRSTIICSATLSF</td>
<td>3</td>
<td>2103.1</td>
</tr>
<tr>
<td>PneA2 fragment 2:</td>
<td>6</td>
<td>2184.1</td>
</tr>
<tr>
<td>STIICSATLSFIASYLGSARQTR</td>
<td>5</td>
<td>2200.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2218.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2236.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2254.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2273.0</td>
</tr>
</tbody>
</table>
Table 3. Susceptibility of *M. flavus* to NisBC-modified Pne-A1 and Pne-A2 peptides.

MIC values are calculated using the molecular weights of the mature peptides.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Digestion type</th>
<th>MIC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Nisin</td>
<td>None</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td></td>
</tr>
<tr>
<td>Nisinleaderpeptide-PneA1</td>
<td>None</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td></td>
</tr>
<tr>
<td>Nisinleaderpeptide-Pne-A2</td>
<td>None</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>8.5</td>
</tr>
<tr>
<td>NisinLP-PneA1 + nisinLP-PneA2</td>
<td>None</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Trypsin</td>
<td>0.6</td>
</tr>
<tr>
<td>BSA</td>
<td>NI*</td>
<td>NI*</td>
</tr>
<tr>
<td>Buffer</td>
<td>NI*</td>
<td>NI*</td>
</tr>
<tr>
<td>Empty sample</td>
<td>NI*</td>
<td>NI*</td>
</tr>
</tbody>
</table>

* no inhibition
<table>
<thead>
<tr>
<th>PneA1</th>
<th>MTNFNSNEKFCGKSLKSLSADEMMLIYGASDGAEPRWTPTPIILKS---AAASSKVCISA 57</th>
</tr>
</thead>
<tbody>
<tr>
<td>PneA2</td>
<td>------MKNDPVIGKSLKELSEMLEMQLVYGGTDGAPR---STICSA---TLSFIAASYLGS 50</td>
</tr>
<tr>
<td>Nis</td>
<td>---------------MSTKDFNLVLVSILSK-KSASGPR-ITSISLCTPGGTKALMGCNMT 46</td>
</tr>
</tbody>
</table>

```
           *  * . . . : :   .**  : : : : : :  :
```

<table>
<thead>
<tr>
<th>PneA1</th>
<th>AVSGIGGLVSYNNDCLG 74</th>
</tr>
</thead>
<tbody>
<tr>
<td>PneA2</td>
<td>AQTRCG--KDNKKK---- 62</td>
</tr>
<tr>
<td>Nis</td>
<td>ATCHCS--IHVSK---- 57</td>
</tr>
</tbody>
</table>

```
* . . :
```

**Fig. 2**
Mass (m/z) vs. Intensity (%)

2573 2618 2664 2709 2755

100
50
0 2801

5x 4x 3x 2x

2643.6
2661.9
2686.0
2664.4
2705.3 2729.7
2699.0
2680.4

Fig. 4