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Pseudomycoicidin, a Class II Lantibiotic from *Bacillus pseudomycoides*

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Lantibiotics are ribosomally synthesized antimicrobial peptides with substantial posttranslational modifications. They are characterized by the unique amino acids lanthionine and methyllanthionine, which are introduced by dehydration of Ser/Thr residues and linkage of the resulting dehydrated amino acids with Cys residues. BLAST searches using the mersacidin biosynthetic enzyme (MrsM) in the NCBI database revealed a new class II lantibiotic gene cluster in *Bacillus pseudomycoides* DSM 12442. Production of an antimicrobial substance with activity against Gram-positive bacteria was detectable in a cell wash extract of this strain. The substance was partially purified, and mass spectrometric analysis predicted a peptide of 2,786 Da in the active fraction. In order to characterize the putative lantibiotic further, heterologous expression of the predicted biosynthetic genes was performed in *Escherichia coli*. Coexpression of the prepeptide (PseA) along with the corresponding modification enzyme (PseM) resulted in the production of a modified peptide with the corresponding mass, carrying four out of eight possible dehydrations and supporting the presence of four thioether and one disulfide bridge. After the proteolytic removal of the leader, the core peptide exhibited antimicrobial activity. In conclusion, pseudomycoicidin is a novel lantibiotic with antimicrobial activity that was heterologously produced in *E. coli*.

Lantibiotics are ribosomally synthesized peptides that have antimicrobial activity (1, 2). They are characterized by the presence of the thioether cross-linked amino acids lanthionine (Lan) and methyllanthionine (MeLan), which are formed by intramolecular addition of cysteine thiols to dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues, respectively (3, 4), in a precursor peptide (LanA). The C-terminal part of the precursor is called the core peptide and is converted into the mature lantibiotic, whereas the N-terminal part, the leader, is removed in the final step of maturation (1, 2).

The core peptides are modified by LanB and LanC enzymes (class I lantibiotics) or by a single LanM enzyme (class II lantibiotics). LanB is required for the selective dehydration of Ser or Thr residues in the LanA core peptide to Dha or Dhb (5). LanC is responsible for the formation of lanthionine rings by linking Cys residues to Dha and Dhb to yield Lan and MeLan, respectively (6). LanM is a bifunctional modification enzyme that carries out both dehydration and cyclization (7). Secretion and processing are performed by the exporter LanT. In addition, the gene clusters are comprised of genes coding for immunity proteins (*lanEFG*, *lanI*, and *lanH*), e.g., the ABC transporter (LanEFG), which protects the producer strains against the antimicrobial effects of their lantibiotics. Regulatory genes (*lanRK*) are present in some, but not all, biosynthetic gene clusters (for reviews, see references 8 and 9).

Lantibiotics are active mainly against Gram-positive bacteria. Some peptides show broad antibacterial activity. For instance, nisin, a food preservative, exerts potent activity against Gram-positive bacteria, including spoilage and pathogenic bacteria, such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus*, *Streptococcus*, and *Enterococcus* (10, 11). Many lantibiotics kill susceptible cells by inhibiting cell wall biosynthesis via binding to the cell wall precursor lipid II and/or by formation of pores in cell membranes, leading to efflux of small molecules and dissipation of the membrane potential (12). However, other lanthionine-containing peptides (lanthipeptides [2]), such as SapB and the labyrinthopeptins, do not have antibacterial activity and are modified by alternative thioether-introducing enzymes (13, 14).

In recent years, much attention has been given to the lantibiotics because of their potential antimicrobial activities against multiresistant human and veterinary pathogens. With the availability of abundant genomic sequences in public databases, several lantibiotics and lanthipeptides, such as haloduracin (15), lichenicidin (16, 17), Bsa (18), venezuelin (19), and the prochlorosins (20), have been identified based on genomic data mining.

Besides *in vitro* modification assays, the heterologous expression of complete gene clusters was successfully performed for several lantibiotics, including production of gallidermin by *Lactococcus lactis* using NisB, NisC, and GdmD (21), of lacticin 3147 by *Enterococcus faecalis* (22), or even of lichenicidin in *Escherichia coli* (23). Recent studies have described another novel methodology for conducting posttranslational modifications that generates lanthipeptides in the heterologous host *E. coli*, yielding fully modified prepeptides by coexpression of His-tagged LanA with LanM. Thereafter, the modified prepeptides can be obtained by a one-step purification; thus, this strategy is a simple and powerful tool for *in vivo* production of lanthipeptides (24, 25, 26). In the present study, we performed a database search for new lantibiotic gene clusters using the biosynthetic enzyme of the class II lantibiotic mersacidin MrsM (27). This search identified a putative lantibi-

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otic biosynthetic gene cluster in *Bacillus pseudomycoides* DSM 12442 (28) with a core peptide of a very intriguing structure, resembling that of a lantibiotic in its N-terminal part and reminiscent of a putative heterocycloanthracin, a recently identified subgroup of bacteriocins, in its C-terminal domain (29, 30). In this study, we report the successful heterologous production and characterization of the novel lantibiotic, which we have termed pseudomycoicidin, in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The producer strain *Bacillus pseudomycoides* DSM 12442 or NRRL B-617 (T) (31) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). *B. pseudomycoides* was grown aerobically in nutrient broth (NB) or on nutrient agar (Oxoid, Hampshire, Great Britain).

Detection of antimicrobial activity. For the detection of antibiotic production, the producer was cultured in Trypticase soy broth (TSB; Difco, Detroit, MI, USA), Luria-Bertani (LB) (32), Mueller-Hinton broth (MHB; Oxoid, Basingstoke, United Kingdom), brain heart infusion (BHI; Oxoid), or nutrient broth at different incubation temperatures (28°C, 30°C, 37°C, and 40°C) for 24, 45, 48, 68, and 71 h, respectively. For antibiotic activity tests, the following indicator strains were used: *Bacillus cereus* DSM 31, *Bacillus halodurans* DSM 18197, *Bacillus licheniformis* MW3, *Bacillus megaterium* KM3 (ATCC 13632), *Bacillus subtilis* 168 (DSM 402), *Bacillus amyloliquefaciens* FH 1856, *Bacillus subtilis* TMB 016, *Bacillus subtilis* BIS 2470, *Enterococcus faecium* L4001, *Enterococcus faecalis* 017940, *Lactococcus sake* 790 E2, *Lactococcus lactis* NCTC 497, *Listeria monocytogenes* DSM 20649, *Micrococcus flavus* DSM 1790, *Micrococcus luteus* ATCC 4698 (used as a standard indicator strain), *Staphylococcus aureus* 825/96 (methicillin-resistant *S. aureus* [MRSA]), *S. aureus* 1450/99 (northern German epidemic strain; German Reference Centre for Staphylococci, Wernigerode, Germany), *S. aureus* Cowan (ATCC 12598), *S. aureus* Newman (NCTC 8178), *S. aureus* SG511 (33), *S. aureus* Wood 46 (ATCC 10832), *S. aureus* COL, *S. aureus* Mu50, *Staphylococcus carnosus* TM300 (34), *Staphylococcus epidermidis* 5, *Staphylococcus saprophyticus* DSM 20229, *Staphylococcus simulans* 22 (35), and the following clinical isolates: *S. aureus* LT740/09 (community-acquired MRSA), *S. aureus* 1000/93 (MRSA), *S. aureus* LT769 (MRSA), *S. aureus* 905/99 (MRSA), *S. aureus* 635/93 (MRSA), *S. aureus* 2757/97 (MRSA), *Streptococcus* sp. strain G-3645-10, and *Streptococcus pyogenes* O-19310. Antimicrobial activity was examined by agar diffusion assays on Mueller-Hinton agar II plates (Difco, Detroit, MI, USA) seeded with the indicator strain prior to the addition of 50 μl extracts into wells. After incubation at 37°C overnight, the inhibition zones were measured. The activity was expressed as diameter of the inhibition zones formed around the wells.

The inhibitory activity of heterologously expressed lanthipeptides was assayed by the spot-on-the-lawn method. To this end, a Mueller-Hinton agar plate was seeded with the indicator strain *M. luteus* ATCC 4698. Ten microliters of the factor Xa cleaved sample was applied directly onto the plates, which were incubated overnight at 37°C, and the activity was judged by the appearance of an inhibition zone.

Detection of protease activity. Protease activity of cell-free *B. pseudomycoides* culture supernatant was detected on skim milk agar plates according to reference 16. Fifty microliters of fresh, filter-sterilized supernatant was added into wells that had been introduced onto the agar surface with a sterile cork borer. Protease activity was determined by the appearance of clear zones around the wells after incubation at 37°C overnight.

Production and peptide preparation. For lantibiotic production, *B. pseudomycoides* DSM 12442 was cultured in 50 ml TSB in a 500-ml flask at 30°C with agitation. After 24 h of incubation, the cells were pelleted by centrifugation (10,000 × *g*; 4°C; 30 min). For further analysis, the culture supernatant was sterilized by filtration and stored at −20°C. The cell pellet was resuspended in 35 ml 70% isopropanol (adjusted to pH 2 with HCl) and incubated at 4°C for 4 h under stirring. The cells were removed by centrifugation (10,000 × *g*; 4°C; 30 min), and the supernatant was sterilized by filtration and stored at −20°C.

For high-performance liquid chromatography (HPLC) analysis, the isopropanol was removed by rotary evaporation (Rotavapor R-3; Büchi, Essen, Germany). Two milliliters of the extract containing 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, Taufkirchen, Germany) was applied to a Poros column (10 R2; 100 by 4.6 mm; Perseptive Biosystems, Freiburg, Germany) and eluted in a gradient of 20% to 100% acetonitrile (ACN) (containing 0.1% TFA). The peaks were detected by measuring the absorbance at 266 and 230 nm. The fractions were collected and assayed for antimicrobial activity against *M. luteus* and analyzed by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS). Active fractions were collected and, after evaporation of acetonitrile, applied to a C_{18} column (RP C_{18}; 5 μm; 250 by 4.6 mm; Schambeck SFD GmbH, Bad Honnef, Germany) for rechromatography. The peaks were detected by measuring the absorbance at 220 nm and 266 nm. Fractions were collected and assayed for the antimicrobial activity against *M. luteus* as well as being analyzed by MALDI-TOF MS.

MS analysis. The mass spectrometry analysis of the peptide preparations was performed using a MALDI-TOF mass spectrometer (Bruker Biflex; Bruker Daltonics, Bremen, Germany). Aliquots of 1 μl of the isopropanol cell wash extracts were mixed with 2 μl matrix (α-cyano-4-hydroxycinnamic acid in acetonitrile–0.1% TFA in water, 1:3). For MALDI-TOF analysis of active HPLC fractions, 20 μl of each fraction was concentrated 1:10 using a rotational vacuum concentrator (RVC 2-18; Christ, Osterode, Germany). The samples were spotted onto the MALDI target and air dried. Mass spectra were measured in positive ion mode in the range of 1,000 to 5,000 Da and 5,000 to 20,000 Da and analyzed by flexAnalysis 2.0 (Bruker Daltonics).

Growth of the test microorganism in the presence of the antimicrobial agent. *M. luteus* ATCC 4698 and *S. aureus* SG511 were grown in Mueller-Hinton (MH) broth. After 5 h of growth (exponential phase), 500 μl of the partially purified compound was added to 20 ml of culture, and the culture was incubated further. As a control, 20 ml of culture was incubated without the addition of the antimicrobial compound. Aliquots were taken at 1-h intervals, and the optical density at 600 nm (OD_{600}) was measured.

Bioinformatic tools. Information on the lantibiotic gene cluster of *Bacillus pseudomycoides* was accessed via the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) under the accession numbers CM000745 and ACMX01000000 (28). Subcellular localizations of the proteins were predicted using the online tool CELLOv.2.5 (subCELlular LOcalization predictor; http://cello.life.nctu.edu.tw/). The potential trypsin cleavage sites in PseA were detected using the ExPASy PeptideCutter tool (http://www.expasy.ch/tools/peptidecutter/). Transmembrane sequences were identified employing the TMHMM web server v. 2.0. (http://www.cbs.dtu.dk/services/TMHMM/). The molecular weight (MW) of the peptide was calculated using the ExPASy ProtParam tool (http://web.expasy.org/protparam/).

DNA and plasmid preparation. Genomic DNA was prepared using the PrestoSpinD bug kit (Molzym, Bremen, Germany) according to the recommendations of the supplier. Plasmid DNA was isolated using the GeneJET plasmid miniprep kit (Fermentas, St. Leon-Rot, Germany). PCR products were purified using the GeneJET PCR purification kit (Fermentas).

Molecular cloning of *pseA* and *pseM* genes. The structural gene *pseA* (*bpmyx0001_45460*) was PCR amplified with the primer pair PseAforI and PseArevI (Table 1) using Phusion polymerase (NEB, Frankfurt/Main, Germany) by following the manufacturer's instructions. The insert DNA fragment and the pET28b vector (Novagen, Merck Chemicals, Darmstadt, Germany) were double digested with NdeI and XhoI (fast digest; Fermentas) according to the manufacturer's instructions.

The gene *pseM* (*bpmyx0001_45470*) was amplified by PCR using

TABLE 1 Primers used in this study[a]

Primer	Template	Primer sequence (5′–3′)
PseAforI	PseA	AAA**CATATG**AATGATAAAATTATCCAATACTGGAA
PseArevI		AAA**CTCGAG**TTAGCAAGACCAGCTCCAACAA
PseMfor	PseM	AAA**CCATGG**GGATGCTTGCAAATCAAGCCTTAAAA
PseMrev		AAA**CTCGAG**TTTTAGTGCTGTTATAGACTCCAA
PseMfor3	Internal PseM primers for sequencing PseM	GGACGAGGCCTCAACATAAG
PseMrev3		GAGCCAGCTGAACCGTCTAT
PseAXafor	pET28bPseA	GCTTTCAGACGCTGATCTGGATAAAATAGAGGGTCGTGGTGATTGCGGT
PseAXarev		ACCGCAATCACCACGACCCTCTATTTTATCCAGATCAGCGTCTGAAAGC
LanAXaC52Afor	pET28bPseAXa	TAAAATAGAGGGTCGTGGTGATGCCGGTGGTACTTGTACA
LanAXaC52Arev		TGTACAAGTACCACCGGCATCACCACGACCCTCTATTTTA
LanAXaC56Afor	pET28bPseAXa	TGGTGATTGCGGTGGTACTGCTACATGGACAAAAGATTGC
LanAXaC56Arev		GCAATCTTTTGTCCATGTAGCAGTACCACCGCAATCACCA
C62Afor	pET28bPseAXa	TGGTACTTGTACATGGACAAAAGATGCCTCAATTTGTCCATCATGG
C62Arev		CCATGATGGACAAATTGAGGCATCTTTTGTCCATGTACAAGTACCA
C65Afor	pET28bPseAXa	CTTGTACATGGACAAAAGATTGCTCAATTGCTCCATCATGGTCTTGT
C65Arev		ACAAGACCATGATGGAGCAATTGAGCAATCTTTTGTCCATGTACAAG
C70Afor	pET28bPseAXa	GTTAGCAAGACCAGCTCCAAGCAGACCATGATGGACAAATTG
C70Arev		CTTGTTGGAGCTGGTCTGCCTAACTCGAGCACCACC
C75Afor	pET28bPseAXa	CTTGTTGGAGCTGGTCTGCCTAACTCGAGCACCACC
C75Arev		GGTGGTGCTCGAGTTAGGCAGACCAGCTCCAACAAG
forPseL	PseA	AATA**GGATCC**AATGATAAAATTATCCAATACTGG
revPseL		TTTATCCAGATCAGCGTCTGA
forAnaA_XA_ prepeptide	AnaA	ATAGAAGGTAGAGGTACTCCA
revAnaA_XA_ prepeptide		TTA**GCGGCCGC**TTACCTCATTCTGCAGCTTCT

[a] Restriction enzyme sites are in boldface and mutations are underlined.

PseMfor and PseMrev as primers (Table 1). The insert DNA fragment and pET22bΔpelb vector (36) were double digested with NcoI and XhoI (fast digest; Fermentas). DNA products were ligated using T4 DNA ligase (0.7 U/μl; Fermentas). *E. coli* JM109 cells were transformed with 6 μl of the ligation mixture by chemocompetence transformation, and cells were plated on LB agar plates containing the appropriate selection marker. The sequences of the resulting recombinant vectors were confirmed by DNA sequencing (Sequiserve, Vaterstetten, Germany). Finally, recombinant plasmids were transferred into the expression host *E. coli* C43 for the heterologous expression of the His-tagged proteins and peptides.

Site-directed mutagenesis. A site-directed mutagenesis kit (QuikChange Lightning site-directed mutagenesis kit; Agilent, Waldbronn, Germany) was used to install a factor Xa cleavage site directly between the leader and core peptide of PseA. The primers were designed (https://www.genomics.agilent.com) to contain nucleotide sequences necessary to encode the amino acids IEGR in place of four wild-type peptide residues (VVGA). The plasmid pET28b_lanAXa was PCR amplified according to the manufacturer's instructions using the primer pair PseAXafor and PseAXarev (Table 1) and pET28b_lanA as the template. According to the supplier's protocol, the recombinant plasmids were transferred into *E. coli* XL10 Gold ultracompetent cells. The sequences of the resulting plasmid products were confirmed by DNA sequencing (Sequiserve). Finally, the recombinant plasmids were transferred into the expression host, *E. coli* C43, for heterologous expression of the His-tagged proteins and peptides. A series of PseAXa analogs was made in *E. coli* C41 in which each cysteine residue was replaced by an alanine residue (PseAXaC52A, PseAXaC56A, PseAXaC62A, PseAXaC65A, PseAXaC70A, and PseAXaC75A) by using the corresponding primer pairs (Table 1).

Overexpression and purification of PseM, PseA, and PseA mutants. The overexpression and purification of His-tagged PseM and PseAXa and its mutants was performed according to McClerren et al. (15), with modifications. Expression was induced by the addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the culture was incubated at 37°C for an additional 4 h for PseAXa and its mutants and 20 h for PseM. Cells were harvested by centrifugation at 6,500 × *g* for 20 min at 4°C. DNase and RNase then were added to the sample, vortexed carefully, and incubated on ice for 30 min. The sample was centrifuged at 15,000 × *g* for 20 min at 4°C. The cell-free supernatant was filtered through 0.45-μm filters (Whatman, Dassel, Germany). The peptides were purified by IMAC (immobilized metal ion affinity chromatography) using a 2-ml Ni^{2+} nitrilotriacetic acid (NTA) column (Qiagen, Hilden, Germany). After the sample had been applied, the column was washed with two column volumes of wash buffers 1, 2, and 3 for PseM and wash buffers 1 and 2 for PseA and its mutants, respectively (15). The protein was eluted with two column volumes of elution buffers 1 and 2.

Cotransformation, coexpression, and purification. Equal amounts of pET28b_lanAXa and pET22bΔpelb_lanM were used to cotransform chemocompetent cells of *E. coli* BL21 or C43. Expression and purification of PseAXa and its mutants were performed as mentioned above. Two milliliters of culture was used for a plasmid extraction in order to check that both plasmids had been maintained after induction. After IMAC purification, the fractions containing the lantibiotic precursor were pooled and desalted via dialysis. Dialysis was performed using a dialysis cassette (2,000-MW cutoff) (Thermo Scientific, Rockford, IL, USA) in which the peptide sample buffer was exchanged for 0.05% HCl. Finally, the lantibiotic precursors were analyzed by MALDI-TOF MS.

Factor Xa cleavage of peptide leader sequence. After IMAC purification, the protein sample buffer was exchanged for 20 mM Tris-HCl buffer (pH 8), 100 mM NaCl, and 2 mM $CaCl_2$ by dialysis. Factor Xa (New England BioLabs, Frankfurt am Main, Germany) was used to remove the leader sequence from PseAXa and its mutant peptides at a final concentration of 0.075 mg/ml. The samples were incubated at 4°C overnight to digest the peptide completely. An aliquot of the reaction mixture was stopped with 0.1% TFA to yield pH 2, desalted by $ZipTip_{C18}$ (Millipore, Billerica, MA, USA), and analyzed by MALDI-TOF MS. Trypsin (Serva, Heidelberg, Germany) digests were performed at pH 7.6 and 37°C for 1 h.

Stability assays and antimicrobial spectrum. To determine the sensitivity of heterologously expressed pseudomycoicidin to different temperatures (20°C, 37°C, 60°C, and 100°C) and pH (2, 4, 7, and 10), stability assays were performed for 1 h and 4 h. The residual antimicrobial activi-

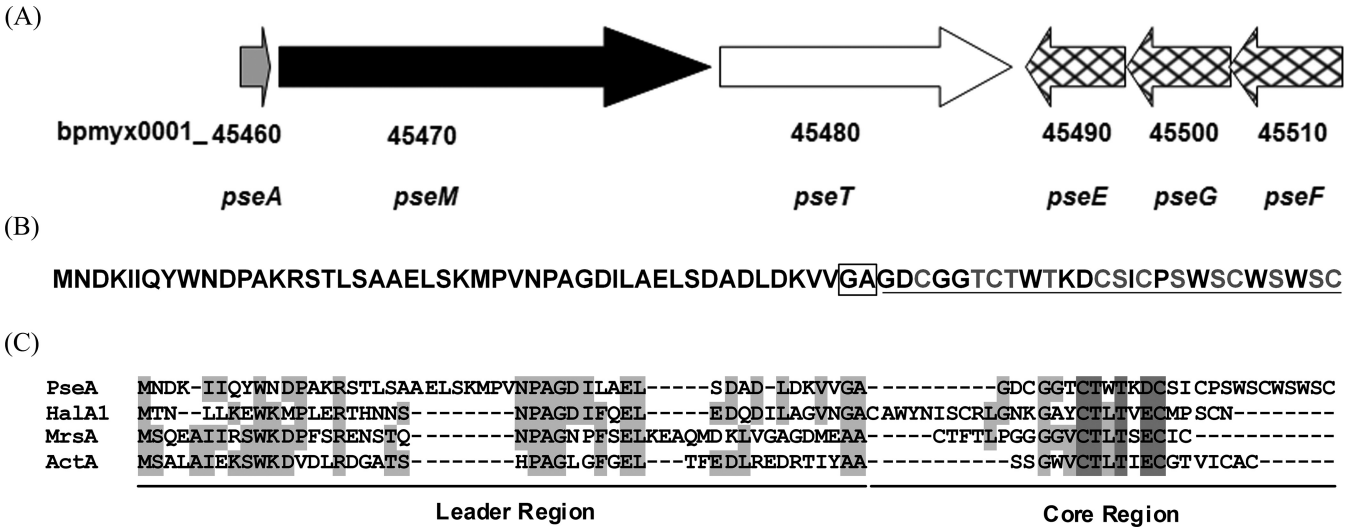

FIG 1 (A) Putative lantibiotic gene cluster in *Bacillus pseudomycoides* consists of the precursor gene *pseA* (gray), modification gene *pseM* (black), the transporter *pseT* (white), and three immunity genes, *pseEFG* (diamond pattern; *bpmyx0001_45490*, *pseE*; *bpmyx0001_45500*, *pseG*; and *bpmyx0001_45510*, *pseF*). The numbers indicate the locus tags of the genes. The arrows indicate the relative direction of transcription. (B) Amino acid sequence of PseA. The cysteine residues and possible dehydration sites are shown in gray. The cleavage site is indicated by the box. The predicted core peptide region is underlined. (C) Amino acid sequence alignment of PseA with class II lantibiotics: haloduracin (HalA1), mersacidin (MrsA), and actagardine (ActA). Amino acid identities are highlighted in light gray, while the lipid II binding motif (TxS/TxE/DC) is highlighted in dark gray.

ties were determined by the spot-on-the-lawn method with *M. luteus* as the indicator strain. The activity screen was performed by the same method.

Determination of free cysteines and free dehydrated amino acid residues in the modified peptide. To detect the presence of free cysteine thiols in the heterologously produced prepeptides, an iodoacetamide (IAA) assay was performed (15). The modified prepeptide was mixed with 10 mM IAA (Sigma-Aldrich), 1 mM tris(2-carboxyethyl)phosphine (TCEP; Sigma-Aldrich), and 50 mM Tris, pH 8.3. The reaction mixture was incubated at 25°C for 45 min in the dark. Subsequently, the samples were purified by ZipTip$_{C18}$ (Millipore) and subjected to MALDI-TOF MS.

To determine the presence of free Dha or Dhb residues in the modified peptide, β-mercaptoethanol (ME; Sigma-Aldrich) was employed (37). The modified peptide was incubated at 30°C for 2 h with 5 mM β-ME at pH 8.5 and subjected to MALDI-TOF MS.

Coexpression of AnaA with PseM. The leader of PseA was fused to the core peptide of AnaA using blunt-end PCR products (primers are listed in Table 1), and the chimeric gene was cloned into pCDF_Duet1 (Merck, Darmstadt, Germany) by employing the NotI and BamHI restriction sites. The plasmid then was transformed into *E. coli* BL21 containing pET22b_PseM. The culture was grown to an OD$_{600}$ of 0.7 and induced using 0.8 mM IPTG. After 3 h, the culture was harvested and the LanA peptide was purified by IMAC and analyzed by MALDI-TOF MS as described above.

RESULTS

A lantibiotic gene cluster in *Bacillus pseudomycoides*. During a bioinformatic search for new lantibiotics, a putative class II gene cluster was identified in *Bacillus pseudomycoides* DSM 12442 (GenBank accession no. NZ_CM000745 and NZ_ACMX01000000) (Fig. 1A and Table 2) (28) on contig00045 (accession no. ACMX01000081). As demonstrated below, this gene cluster results in the production of a novel lantibiotic that we named pseudomycoicidin. Applying the common nomenclature for lantibiotic biosynthetic genes, the genes are referred to as *pse*. *pseA* encodes a peptide of 75 amino acid residues, with a 48-residue leader sequence separated by a conserved Gly-Ala cleavage site from a 27-residue core peptide (Fig. 1B). It is rich in Ser (5 residues), Thr (3 residues), and Cys (6 residues), and the leader sequence shows highest (37%) similarity to the leader sequence of mersacidin (38). The N terminus of the core peptide contains the conserved lipid II binding motif of class II lantibiotics (TxS/TxE/DC) and, interestingly, the C terminus of the core peptide contains two SWSC motifs (Fig. 1B). Such motifs also are found in the heterocycloanthracins, a new subgroup of thiazole-containing bacteriocins (30), and have not yet been observed in lantibiotics; the first member of the heterocycloanthracin group, sonorensin from *Bacillus sonorensis* MT93, contains five repeated SCWSC motifs and has been purified recently (29).

Just upstream of *pseA*, a peptide might be encoded that harbors a stretch with similarity to the *pseA* leader sequence. The genome of the producer strain has been deposited in NCBI as a scaffold of 305 contigs (28). According to the NCBI entry, this sequence is located right at the 5′ end of contig00045, which is separated by a gap from contig00074 (accession no. ACMX01000082), and the start codon of this second peptide gene presumably is located in the gap sequence. The adjacent gene on contig00074 is annotated as a thioredoxin reductase gene (*bpmyx0001_45890*). However, attempts to close the gap in the genomic sequence using primers located in contig00045 and contig00074 as well as reverse PCR did not yield any products.

The downstream open reading frame (ORF) was predicted to encode PseM, the enzyme that performs the posttranslational modification resulting in thioether formation. It contains the conserved Zn-binding motifs GXXHGXXG, WCXG, and CHG/CCG (39). The ABC exporter PseT is located immediately downstream of *pseA* and *pseM* and harbors an N-terminal double-glycine peptidase domain and the ATP binding site. It may be responsible for export of the modified peptide and the removal of the leader peptide. In contrast, the three ORFs located even farther downstream were predicted to form a transporter that is involved in self-im-

TABLE 2 Overview of proteins encoded by the pseudomycoicidin gene cluster and genes in its vicinity[a]

Locus tag and category	Gene designation	Function	Similar protein	Identical aa (%)	Reference(s)
Pseudomycoicidin gene cluster					
bpmyx0001_45460	*pseA*	Lantibiotic prepeptide	MrsA, mersacidin, of *B. amyloliquefaciens* HIL-8554728	40	38
bpmyx0001_45470	*pseM*	Lantibiotic modifying enzyme	MrsM	36	27
			LicM1 (BLi04128) of *B. licheniformis* DSM13	35	16, 17
			HalM1 (BH0455) of *B. halodurans* C-125	37	15
bpmyx0001_45480	*pseT*	Lantibiotic ABC transporter	MrsT	42	27
bpmyx0001_45490	*pseE*	Immunity transporter, membrane domain	MrsE	33	27
bpmyx0001_45500	*pseG*	Immunity transporter, membrane domain	MrsG	29	27
bpmyx0001_45510	*pseF*	Immunity transporter, ATP binding domain	MrsF	59	27
Genes located farther downstream					
bpmyx0001_45520		Hypothetical GPP 34 superfamily protein	BH0832 of *B. halodurans* C-125	27	64
	pbuG		BLi00688 of *B. licheniformis* DSM13	28	65
bpmyx0001_45530		Hypothetical small multidrug export protein	BH1312 of *B. halodurans* C-125	49	64
			BLi01407 of *B. licheniformis* DSM13	44	65
bpmyx0001_45540		Putative lipoprotein	YgaO (BLi00945) of *B. licheniformis* DSM13	45	65
bpmyx0001_45550		Helix-turn-helix XRE family regulator protein	YgzD (Bli00946) of *B. licheniformis* DSM13	62	65

[a] Also shown are the percentages of identical amino acids compared to proteins of the mersacidin gene cluster of *B. amyloliquefaciens* HIL Y-85,54728 (63) and proteins encoded by the genomes of *B. licheniformis* DSM13 and *B. halodurans* C-125.

munity of the producer strain. The four ORFs following farther downstream do not seem to be part of the lantibiotic gene cluster. As shown in Table 2, similar genes can be found in *B. halodurans* and *B. licheniformis*, but these have not been associated with lantibiotic production and are not located in the haloduracin or lichenicidin gene clusters (15). In conclusion, the biosynthetic gene cluster represents at least six ORFs, comprising one lantibiotic structural gene (*pseA*), one modification enzyme (*pseM*), an exporter (*pseT*), and immunity genes (*pseFEG*).

***B. pseudomycoides* produces antimicrobial activity.** Cell-free culture supernatant and an isopropanol wash extract of the cell pellet of *B. pseudomycoides* DSM 12442 were tested for antimicrobial activity against the indicator strain *M. luteus*. Cell-bound antimicrobial activity was present when the producer strain had been grown in 50 ml TSB, LB, and NB in a 500-ml flask at 30°C or 37°C with agitation for 21, 24, 45, and 48 h, although the maximum production was observed in TSB at 30°C for 24 h (see Fig. S1 in the supplemental material), but only the cell wash extract was antimicrobially active (see Fig. 3A, inset; also see Fig. S2). When tested against various indicator strains, the extract was found to be active against most of the tested Gram-positive bacteria, such as *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus simulans*, *Bacillus* spp., *Enterococcus* spp., *Lactococcus* spp., *Streptococcus* spp., etc., but not against Gram-negative bacteria and *Candida* (Fig. 2A). Analysis of the crude cell wash extract and HPLC-purified active fractions by MALDI-TOF MS revealed one dominant mass signal at *m/z* 2,786.03 (Fig. 3A) that, assuming the presence of the conserved cleavage site, corresponded, with a mass difference of about 2 Da, to the calculated mass of the predicted PseA core peptide (unmodified, 2,860.1 Da) with four dehydrations (2,788.1 Da).

Remarkably, the antimicrobial activity was present exclusively in the cell wash extract. In order to check the ability of the producer strain to excrete proteases during antibiotic production, which might inactivate the antimicrobial activity after secretion into the culture supernatant, cell wash extract and the culture supernatant were mixed and incubated for 2 h. The culture supernatant was verified to contain protease activity on skim milk agar plates. However, the activity of the cell wash extract against *M. luteus* was not diminished after incubation (see Fig. S2 in the supplemental material). This indicates that the antibacterial activity was resistant against any proteases excreted by the producer.

Addition of the partially purified antimicrobial activity to cultures of *M. luteus* and *S. aureus* SG511 at late exponential phase led to a loss of optical density compared to that of the untreated control strains (Fig. 2B). This result suggests that the antimicrobial activity in the cell wash extract had a lytic effect on sensitive indicator strains.

Expression of PseA and PseM in *E. coli* results in an active lantibiotic. Since *Bacillus* strains often are able to excrete several different antibacterial compounds, a further search for proteins that might be involved in antibiotic biosynthesis was performed and indicated the presence of several ORFs with similarity to parts of nonribosomal peptide synthetases encoded by, e.g., *bpmyx0001_50260* to *bpmyx0001_50280* on contig00057 (accession no. ACMX01000094). Contig00076 (accession no. ACMX01000261) and contig00431 (accession no. ACMX01000147) also represent parts of nonribosomal peptide biosynthesis operons. Furthermore,

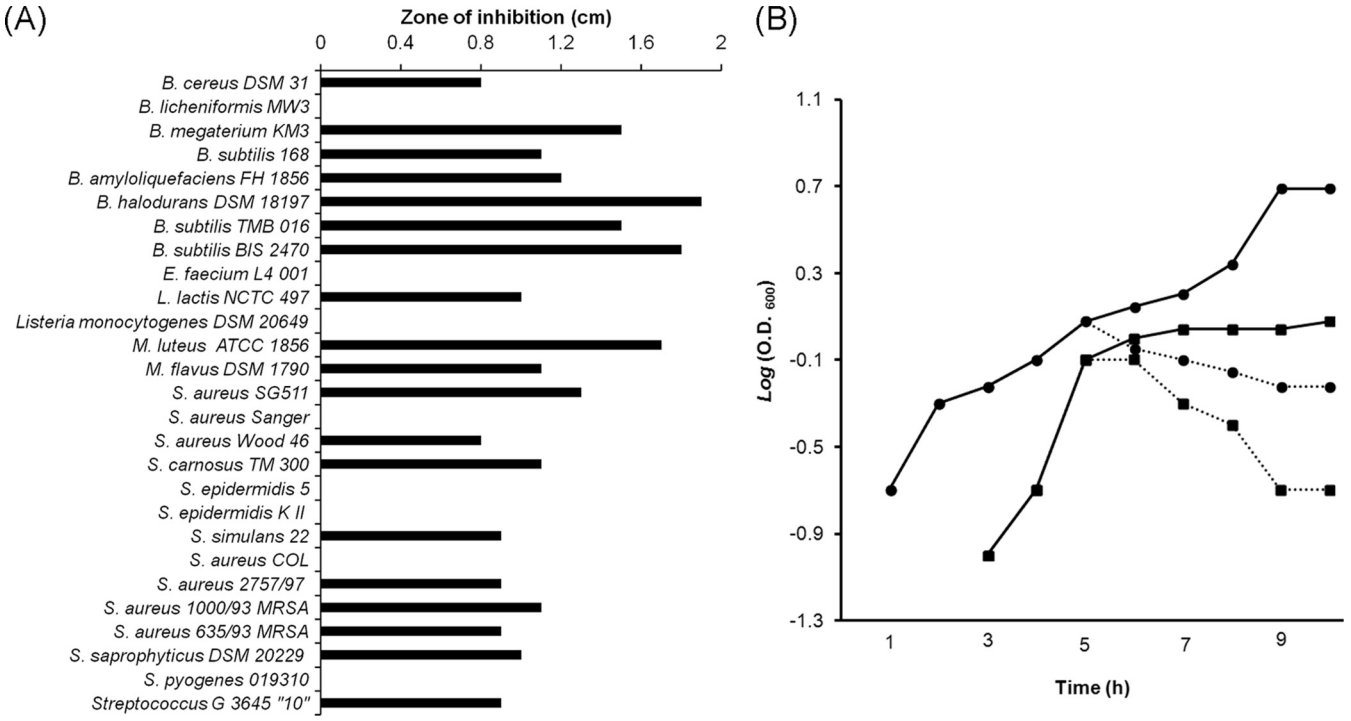

FIG 2 (A) Antibacterial spectrum of the *B. pseudomycoides* cell wash extract. The cell extract showed activity against most Gram-positive bacteria. (B) Effect of the antimicrobial compound on the growth of *M. luteus* (■) and *S. aureus* SG511 (●). The bacterial cultures treated with the antimicrobial substance are indicated by a dashed line, while the untreated controls are indicated by a solid line. The antimicrobial substance was added after 5 h of growth.

BLAST searches identified three proteins with high similarity to the putative heterocycloanthracin biosynthesis genes described by Haft (30) on contig00260 (accession no. ACMX01000028) and comprise a protein carrying the conserved ocin_THiF_like domain (*bpmyx0001_11050*), a SagD/YcaO-like cyclodehydratase (*bpmyx0001_11040*), and an MbcC-like oxidoreductase (*bpmyx0001_11030*). In contrast to *B. licheniformis* ATCC 14580/DSM13, no precursor peptide is encoded upstream of these genes, but *bpmyx0001_22790* on contig00122 (accession no. ACMX01000049) shows similarity to the heterocycloanthracin precursor genes described in reference 30. As a consequence, it could not be excluded that the antibacterial activity in the cell wash of *B. pseudomycoides* extract represents another substance. In order to test whether the antimicrobial activity was associated with the putative lantibiotic gene cluster, the C-terminally His-tagged PseM and N-terminally His-tagged PseAXa (containing a factor Xa cleavage site; described see below) constructs were overexpressed in *E. coli* C43. To investigate the biological activity of a lantibiotic, the leader peptide must be removed. Therefore, the predicted leader cleavage site and two upstream residues (VVGA)

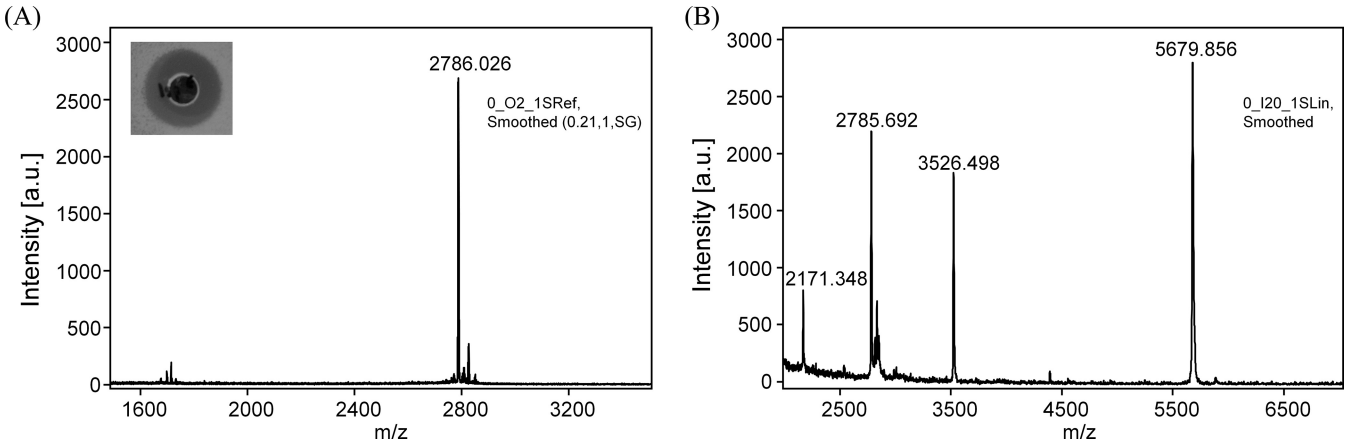

FIG 3 (A) MALDI-TOF mass spectrum of the HPLC-purified isopropanol cell wash extract of *B. pseudomycoides* with a mass signal of *m/z* 2,786.0. (Inset) Zone of inhibition produced by the HPLC fraction of cell wash extract against *M. luteus*. (B) Heterologously expressed PseAXa after cleavage of the leader by factor Xa with a mass signal of *m/z* 2,785.7 for the core peptide and three mass signals (*m/z* 2,171.3, 3,526.5, and 5,679.8) representing the leader fragments. a.u., arbitrary units.

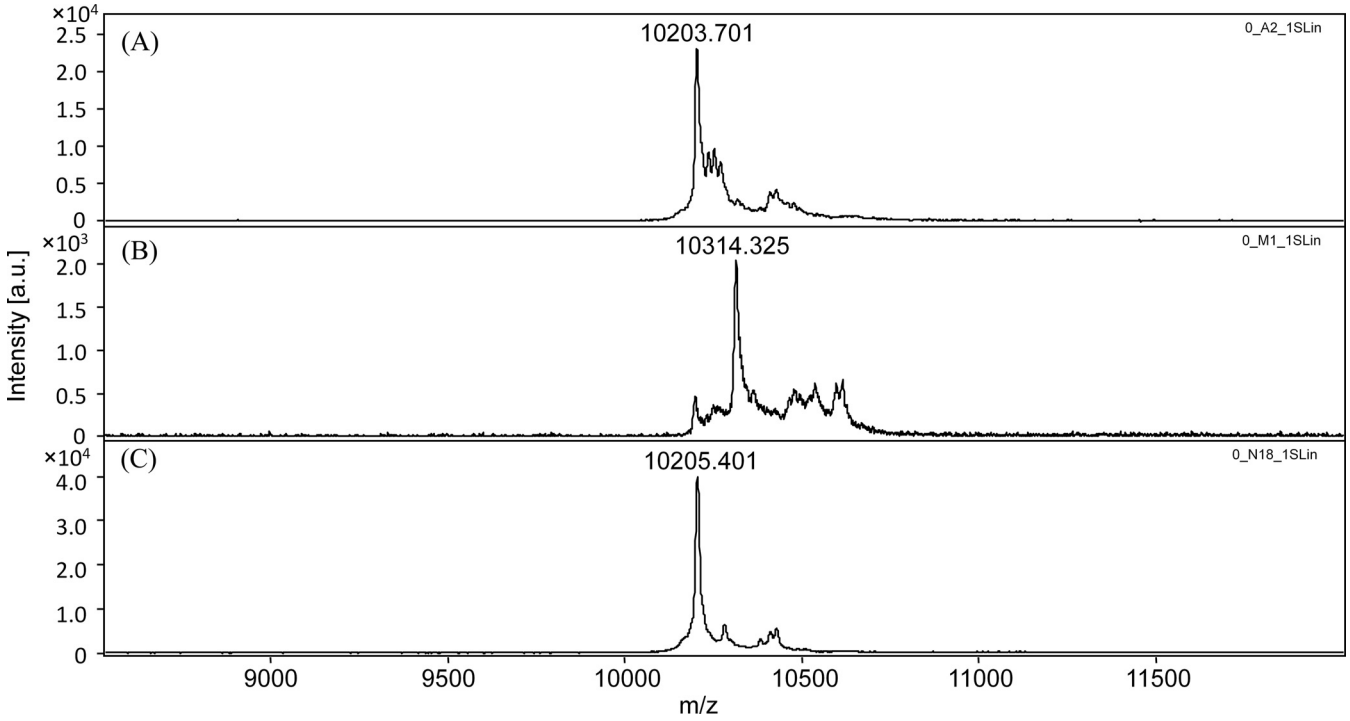

FIG 4 (A) MALDI-TOF mass spectrum of heterologously produced PseAXa with a mass signal of *m/z* 10,203.7. (B) Peptide after IAA treatment in the presence of TCEP, with an observed mass of 10,314.3 Da and expected mass of 10,318.6 Da (m − $4H_2O$ + 2 IAA adducts). (C) PseA after β-ME treatment; it did not show the addition of β-ME (observed mass, 10,205.4 Da), but the mass difference of 2 Da indicates reduction of the disulfide bridge.

had been replaced before by the factor Xa cleavage site (IEGR) for *in vitro* processing of the heterologously expressed PseAXa peptide. The successful production of His-tagged PseM was determined by SDS-PAGE analysis after Ni-NTA purification. However, expression of the lantibiotic precursor PseAXa alone was not successful, as judged by SDS-PAGE.

In contrast, coexpression of PseAXa together with PseM resulted in the production of the modified His-tagged pseudomycoicidin precursor. MALDI-TOF MS analysis of the heterologously expressed prepeptide showed a mass signal at *m/z* 10,203 for the protonated ion (Fig. 4 A). Thus, the observed MW of the protonated peptide was 74 Da smaller than the calculated MW of the ion of the unmodified propeptide (*m/z* 10,277.4). This difference in MW could be explained by the loss of four water molecules and two hydrogens (see below) during the posttranslational modification. In conclusion, the peptide obtained by coexpression was the modified product of the bioinformatically predicted structural gene (*pseA*).

Subsequently, MALDI-TOF MS analysis confirmed that the proteolytic cleavage of the leader yielded the modified lantibiotic core peptide with a mass of 2,785.7 Da. MALDI-TOF MS also revealed minor mass signals representing traces of the 1-, 2-, and 3-fold dehydrated core peptides, as well as three other signals which might be attributed to different fragments of the leader peptide (*m/z* 2,171.3, *m/z* 3,526.4, and *m/z* 5,679.8) (Fig. 3B). The mass signal of the processed and modified core peptide was consistent with the mass obtained from the antimicrobially active compound produced by the producer strain *B. pseudomycoides*. Furthermore, the heterologously produced peptide showed antimicrobial activity after proteolytic removal of the leader sequence from the modified lantibiotic precursor (Fig. 5A).

Stability assays. For further characterization of the peptide expressed in *E. coli*, heat and pH stability were determined by the spot-on-the-lawn method. Here, the appearance of an inhibition zone was judged as a positive reaction. The compound was stable

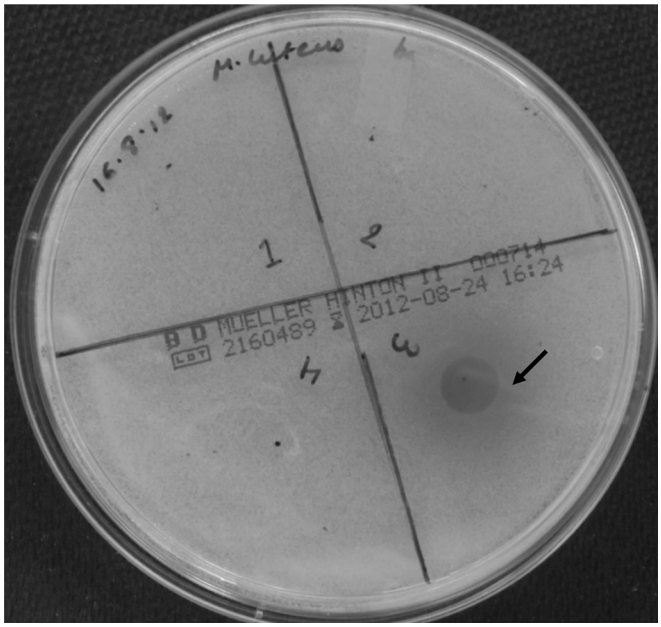

FIG 5 Bioactivity assay against the indicator strain *M. luteus*. Spot 1, Tris buffer; spot 2, heterologously produced PseAXa before treatment with factor Xa; spot 3, modified PseAXa after treatment with factor Xa (the zone of inhibition is shown by an arrow). Spot 4 was not used.

at pH 2, pH 4, and pH 7 for 1 h. After 4 h of incubation, residual activity was found only at pH 2. In contrast, at pH 10, the inhibition zone was completely lost after 1 h. The antimicrobial activity of the peptide was fully stable between 20°C and 100°C at pH 2. An indicator screen using the heterologously expressed peptide yielded inhibition zones for *Bacillus subtilis* strains (TMB016, TMB299, and TMB588), *Streptococcus* sp. strain G-3643-10, all tested *S. aureus* strains (*S. aureus* SG511, *S. aureus* 1000/93, and *S. aureus* 635/93), *Lactococcus lactis* NCTC497, and *Micrococcus luteus*.

Pseudomycoicidin contains two Cys residues that are not involved in thioether formation. To verify the presence of thioethers in PseA, the heterologously produced peptide was monitored for the presence of free cysteines by alkylation with IAA in the presence of the reducing agent TCEP. A reaction of PseAXa with IAA should result in the addition of a carbamidomethyl group to each free cysteine, increasing its mass by 57 Da (15). After treatment with IAA in the presence of TCEP, the mass of PseAXa increased by approximately 110 Da. This increase in mass indicates the alkylation of two Cys residues in the core peptide (Fig. 4 B), indicating that two cysteine thiol groups are free under reducing conditions and that the remaining four Cys residues should be involved in the formation of Lan or MeLan rings. This result also is consistent with the observation that up to four dehydrations were observed for the modified peptide. In contrast, the treatment of the peptide with IAA in the absence of TCEP did not detect any free Cys residues. A mass shift of 2 Da was caused by treatment with TCEP only, indicating that the free Cys residues form a disulfide bridge. In addition, the incubation of modified peptide with β-ME did not result in addition of β-ME but, again, in an addition of 2 Da, demonstrating the absence of free dehydrated amino acid residues and reduction of a disulfide bridge in the active peptide (Fig. 4C).

Generation of LanA analogs by replacing Cys with Ala and tandem mass spectrometry analysis previously has been used to determine the ring topology of other lanthionine-containing peptides (19, 23). Therefore, PseAXa analogs were created by replacing each of 6 Cys residues with Ala, one at a time, so that only one thioether or disulfide bridge should be disrupted each time. The MALDI-TOF analyses of all six mutated peptides after coexpression with PseM in *E. coli* showed mixtures of mass signals indicating the presence of incompletely dehydrated peptides (see Fig. S4 in the supplemental material), and the PseAXa mutants were inactive against *M. luteus* after removal of the leader by factor Xa in the spot-on-the-lawn assay. After incubation with trypsin, the signal of the wild-type peptide still was visible in MALDI-TOF MS (see Fig. S3). In contrast, digests with trypsin led to loss of the mass signals of the core peptide for PseAXaC62A, PseAXaC65A, PseAXaC75A, and most of PseXaC52A. The trypsin cleavage site ($\downarrow$) is located in the core of the conserved lipid II binding motif (TC_{56}**TWTK** $\downarrow$ **DC**$_{62}$$SIC_{65}PSWSC_{70}W$; conserved residues are in boldface) in the wild-type peptide. The susceptibility of the mutant peptides to tryptic digest and incomplete dehydration indicated that the rings had not been formed correctly, because the Cys residues that participate in the ring structures had been removed by mutagenesis and/or because of incorrect modification. On the other hand, the ring structures originating from Cys70 and Cys56, which are not present in PseAXaC56A and PseAXaC70A, did not seem to stabilize the trypsin cleavage site, since these peptides were stable when digested with trypsin.

Coexpression of AnaA and PseM. In order to further probe the substrate specificity of PseM, we also tried heterologous expression of another lantibiotic structural gene. *Caldicellulosiruptor bescii* DSM 6725 (formerly *Anaerocellum thermophilum*) has been completely sequenced (40). It encodes a putative lantibiotic gene cluster and the structural gene that we called *anaA*, from the ORF Athe_1106. The leader of PseA was fused to the core peptide of AnaA in order to ensure activation of PseM, and a factor XA cleavage site was included, yielding PseL-AnaA_XA. PseL-AnaA_XA and PseM then were coexpressed in *E. coli* BL21. The results showed that the coexpression of PseM and PseA did not result in any loss of water molecules from PseL-AnaA_XA, indicating that the peptide had not been modified by PseM (see Fig. S5 in the supplemental material).

DISCUSSION

The intensive use and misuse of antibiotics have led to a constant rise in multidrug-resistant microorganisms, which has increased the need to investigate and develop new antibacterial compounds. In recent years, lantibiotics have become promising candidates for future antibiotics, since they have a high potency to inhibit diverse pathogenic bacteria (10, 41, 42) or selectively target problematic nosocomial agents like *Clostridium difficile* (43). The possibility that *B. pseudomycoides* DSM 12442 produces a class II lantibiotic was recognized during a database search for new lantibiotic gene clusters in different bacteria. An SWSCWSWSC motif that is unusual for lantibiotics and resembles motifs found in the thiazole-containing heterocycloanthracins (30) constitutes the C terminus of PseA and makes it an attractive compound for investigations. The interesting question was whether this peptide would be a lantibiotic or whether additional formation of thiazole/oxazole residues would be needed for activity.

The production assays indeed confirmed the expression of an antimicrobially active substance by *B. pseudomycoides*; however, a further database search indicated that other genes that might be involved in synthesis of antimicrobial substances are present in the genome of *B. pseudomycoides*. Additionally, possible thiazole and oxazole modification enzymes are encoded by the genome but not by the lantibiotic gene cluster. It is not unusual for *Bacillus* species to produce an array of different antibiotics, for example, *B. amyloliquefaciens* FZB42 harbors gene clusters that enable the production of 10 different antimicrobial substances (44, 45), and even *B. subtilis* 168 still is able to excrete several antimicrobial compounds (46). Since the transformation of *B. pseudomycoides* did not work in our hands, the structural gene *pseA* and the modification gene *pseM* were coexpressed in *E. coli* and the correctly modified PseA with four (out of eight possible) dehydrations and antibacterial activity was produced, indicating that the active substance in the cell wash extract is indeed a lantibiotic that carries four thioether bridges and one disulfide bridge. Evidence for thiazole or oxazole ring formation could not be found. The fate of ribosomally synthesized, posttranslationally modified peptides seems to be determined by the sequences of their leader peptides, which direct the prepeptides to their respective modification machineries (47). This has been demonstrated for some lantibiotics as well as microcin B17 and streptolysin S, which both contain thiazole and oxazole residues. Here, the leader sequence of pseudomycoicidin shows high similarity to that of MrsA and ActA; therefore, it directs the peptide to the LanM enzyme (Fig. 1).

Interestingly, the N-terminal part of the core peptide harbors

an amino acid sequence (CTxTxDC) which resembles the conserved mersacidin-like lipid II binding motif (CTxT/SxE/DC) that also was found in other class II lantibiotics (48). The C-terminal part of the core peptide contains three Trp residues, and another Trp is present in the N terminus. This relatively high proportion of Trp residues might contribute to the insertion of the peptide into the membrane. Such a mode of action has been reported already for Trp-rich nonlantibiotic antimicrobial peptides, such as tritrpticin, indolicidin, puroindoline, and the antibacterial fragments of lactoferrin and lactoferricine (49, 50). In contrast to the abovementioned eukaryotic peptides, the Trp residues stabilize the conformation of pediocin-like bacteriocins (51).

Nisin and Pep5 (52) are inactivated by chymotrypsin, which is an essential prerequisite for nisin's use as a preservative in food (53), and epidermin (54) is subject to trypsin cleavage. The abovementioned lantibiotics are elongated peptides that possess a hinge region, which is essential for pore formation and which is susceptible to the activity of proteases like trypsin. In contrast, pseudomycoicidin was found to be resistant to trypsin, suggesting that the trypsin cleavage site (TCTWTK ↓ DCSICPSW; conserved residues are underlined), which is located in the conserved lipid II binding motif, is protected by the presence of at least one thioether ring structure. This was confirmed by the experiments with the site-directed mutant peptides; in these peptides, the removal of Cys residues resulted in protease sensitivity. We also observed a disulfide bridge within the active pseudomycoicidin, a posttranslational modification that is present in only a few lantibiotic peptides. So far, only haloduracin (55), plantaricin W (56), enterocin W (57), bovicin HJ50, and similar peptides (58, 59), as well as the labyrinthopeptins (14), have been described to possess disulfide bonds. In the alpha peptide of enterocin W, haloduracin, and plantaricin W, the two N-terminally located Cys residues form the disulfide. On the other hand, for the single peptide lantibiotic bovicin HJ50 and related peptides, the cystine was experimentally confirmed to be located in the C-terminal part of the peptide (58, 59).

In the experiments with the mutant peptides, the modification by PseM was disturbed by the removal of Cys residues, indicating that the substrate specificity of PseM is much narrower than that of NisB (60), LctM (61), or especially ProcM (62). This also was confirmed when we tried to coexpress another predicted lantibiotic structural gene of *Caldicellulosiruptor bescii* (40) fused to the pseudomycoicidin leader along with PseM in *E. coli*, which did not result in modification of the core peptide. For this reason, nuclear magnetic resonance spectroscopy will be needed for analysis of the complete ring topology of pseudomycoicidin, but this will need more pure material than is currently obtainable.

In summary, we were able to heterologously produce the novel lantibiotic pseudomycoicidin in *E. coli* and show that it is a lantibiotic with four thioether rings and a disulfide bond. Future work will concentrate on structure investigation of pseudomycoicidin as well as detailed mode-of-action studies of pseudomycoicidin.

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