Chemistry & Biology
Article

Biosynthesis of the Antimicrobial Peptide Epilancin 15X and Its N-Terminal Lactate

Juan E. Velásquez,[1,2] Xingang Zhang,[1] and Wilfred A. van der Donk[1,2,3,*]
[1]Department of Chemistry
[2]Institute for Genomic Biology
[3]Howard Hughes Medical Institute
University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, IL 61801, USA
*Correspondence: vddonk@illinois.edu
DOI 10.1016/j.chembiol.2011.05.007

SUMMARY

Lantibiotics are ribosomally synthesized and post-translationally modified antimicrobial peptides. The recently discovered lantibiotic epilancin 15X produced by *Staphylococcus epidermidis* 15X154 contains an unusual N-terminal lactate group. To understand its biosynthesis, the epilancin 15X biosynthetic gene cluster was identified. The N-terminal lactate is produced by dehydration of a serine residue in the first position of the core peptide by ElxB, followed by proteolytic removal of the leader peptide by ElxP and hydrolysis of the resulting new N-terminal dehydroalanine. The pyruvate group thus formed is reduced to lactate by an NADPH-dependent oxidoreductase designated ElxO. The enzymatic activity of ElxB, ElxP, and ElxO were investigated in vitro or in vivo and the importance of the N-terminal modification for peptide stability against bacterial aminopeptidases was assessed.

INTRODUCTION

Bacterial resistance to known classes of antibacterial agents has been rising at an alarming rate. Currently, 60%–70% of all *Staphylococcus aureus* strains isolated in hospitals are multidrug resistant (Taubes, 2008). In order to prevent potential epidemic outbreaks of infectious diseases, new antibacterial drugs not affected by existing resistance mechanisms are much needed. Lantibiotics are ribosomally synthesized and posttranslationally modified polycyclic peptides that contain thioether crosslinks and that demonstrate promising activity against pathogenic bacteria (Willey and van der Donk, 2007). Nisin (Figure 1), the most studied lantibiotic, has been used as a food preservative during the last 40 years in more than 80 countries without the development of stable resistance, possibly as a consequence of its multiple modes of action (Breukink and de Kruijff, 2006). The N-terminal portion of nisin recognizes the membrane-bound cell wall precursor lipid II, inhibiting peptidoglycan biosynthesis (Breukink et al., 1999; Brötz et al., 1998). Once the molecule is docked to the membrane, the C-terminal portion generates stable pores that result in membrane damage and depolarization (Wiedemann et al., 2001).

The lantibiotic epilancin 15X (Figure 1) was isolated from *Staphylococcus epidermidis* 15X154 and is active against several pathogenic bacteria, including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococci*, with minimal inhibitory concentration (MIC) values among the lowest reported for lantibiotics (Ekkelenkamp et al., 2005; Verhoef et al., 2005). Epilancin 15X contains one lanthionine (Lan) and two 3-methyllanthionine (MeLan) bridges, one 2,3-dehydroalanine (Dha) residue, three (*Z*)-2,3-dehydrobutyrine (Dhb) residues, and an unusual N-terminal 2-hydroxypropionyl group (lactate; Lac). The C-terminal B and C rings of epilancin 15X are structurally similar to the D and E rings of nisin A (Figure 1) that are believed to be involved in pore formation (Wiedemann et al., 2001). However, formation of pores would not explain by itself the low MIC values of epilancin 15X. In the case of nisin, the potent antibacterial activity is a consequence of docking onto lipid II by the A and B rings (Hsu et al., 2004), which greatly enhances the pore-forming ability (Breukink et al., 1999). However, epilancin 15X does not contain the A and B rings found in nisin, and lipid II does not appear to be a target of epilancin K7 (Figure 1), a structurally closely related analog of epilancin 15X (van de Kamp et al., 1995a, 1995b). Thus, the N-terminal portion of epilancin 15X and the unusual lactate group may be involved in a currently unknown alternative mechanism. Indeed, additional posttranslational modifications beyond the Lan or MeLan rings are often important for biological activity of lantibiotics. In the case of cinnamycin, a β-hydroxylated aspartate residue and a lysinoalanine ring are important for recognition of its target (Hosoda et al., 1996). In another example, the lantibiotic microbisporicin, which has a very similar ring topology as epidermin, is two orders of magnitude more potent than the latter compound against several strains of *S. aureus*, presumably because of hydroxylated proline and chlorinated tryptophan residues that are absent in epidermin (Castiglione et al., 2008). Thus, the N-terminal lactate group might also be important for the antimicrobial activity of epilancin 15X.

An N-terminal lactate is also present in epicidin 280 and epilancin K7 (van de Kamp et al., 1995b), but its biosynthetic origin has not been determined. In the case of epicidin 280, the putative oxidoreductase EciO may be involved, but this hypothesis has not been confirmed experimentally (Heidrich et al., 1998). Herein we report the gene cluster involved in the biosynthesis of epilancin 15X. Additionally, we demonstrate that ElxO is an

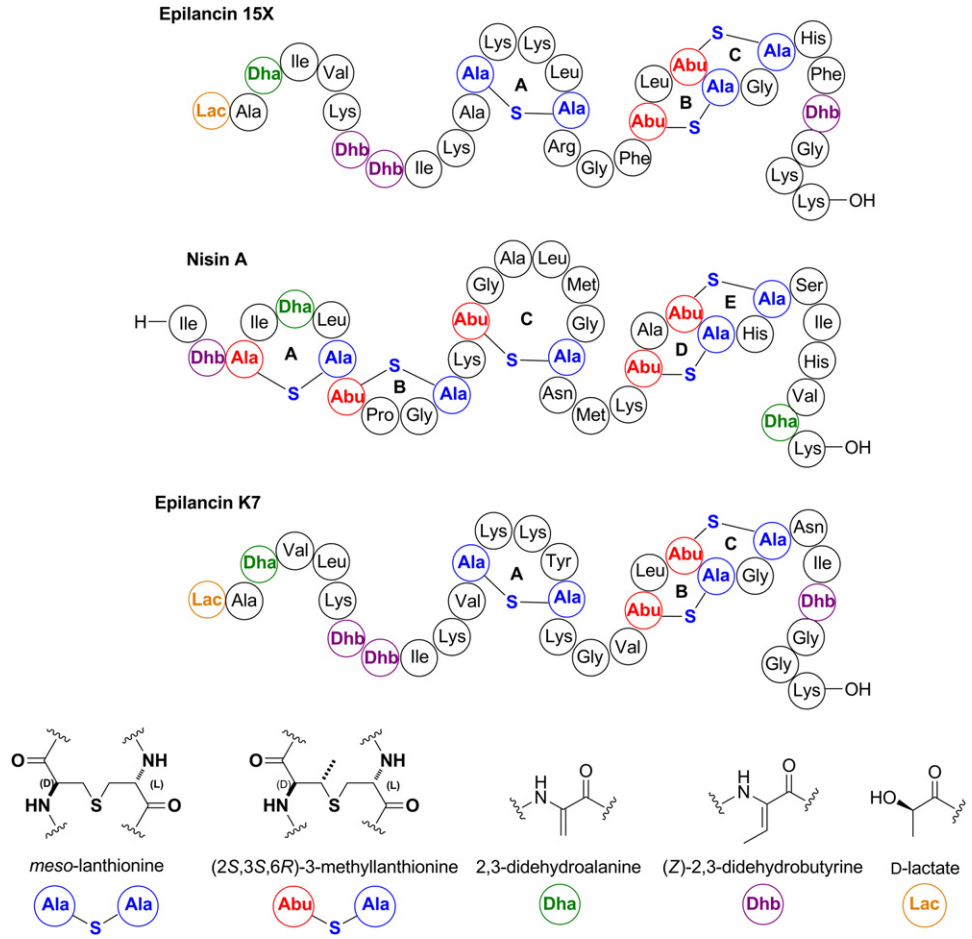

Figure 1. Structures of the Lantibiotics Epilancin 15X, Nisin A, and Epilancin K7

A previously reported shorthand notation (Chatterjee et al., 2005b) is used. The stereochemical configuration of the N-terminal lactate group in epilancin 15X was determined in this study.

NADP(H)-dependent alcohol dehydrogenase that catalyzes the conversion of an N-terminal pyruvate to lactate and that the lactate group plays a protective role against proteolytic degradation. We also report the in vitro reconstitution of the enzymatic activity of ElxP, to the best of our knowledge the first such example for a member of the serine-type lantibiotic proteases.

RESULTS

Cloning and Sequencing of the Epilancin 15X Biosynthetic Gene Cluster

To identify and sequence the epilancin 15X biosynthetic gene cluster, a fosmid library of *S. epidermidis* 15X154 genomic DNA was constructed in *Escherichia coli*. The fosmid library was screened by PCR using degenerate primers to amplify a fragment of *elxA* (the gene encoding the precursor peptide) and *elxC* (the gene encoding a lanthionine cyclase). The primers were designed based on the amino acid sequence of epilancin 15X and conserved nucleotide sequences in the genes encoding the cyclase enzymes. Two positive clones containing nonoverlapping DNA fragments were isolated and the fosmids were sequenced using transposon insertions. Specific primers annealing with regions of *S. epidermidis* 15X154 genomic DNA were then used to amplify by PCR a bridging 1.2 kb DNA fragment that was sequenced to obtain the biosynthetic gene cluster (Figure 2). The open reading frames (ORFs) were analyzed with the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), and six ORFs encoding putative proteins with high sequence identity to enzymes involved in the production and transport of lantibiotics were identified: *elxA*, *elxB*, *elxC*, *elxP*, *elxT*, and *elxO* (Table 1 and Figure 2; see Figure S1 available online). Additionally, three genes with no homology to characterized lantibiotic genes and presumably involved in immunity were identified and designated as *elxI1*, *elxI2*, and *elxI3*. With the exception of *elxO*, the role of the other genes in epilancin 15X biosynthesis can be predicted based on homology to previously characterized lantibiotic genes. The gene encoding the precursor peptide *elxA* encodes a serine residue at the first position of the core peptide (the region of the precursor peptide that is modified to the mature lantibiotic) (Oman and van der Donk, 2010), which corresponds to lactate in epilancin 15X. Thus, Ser1 must be posttranslationally modified by an undetermined enzyme.

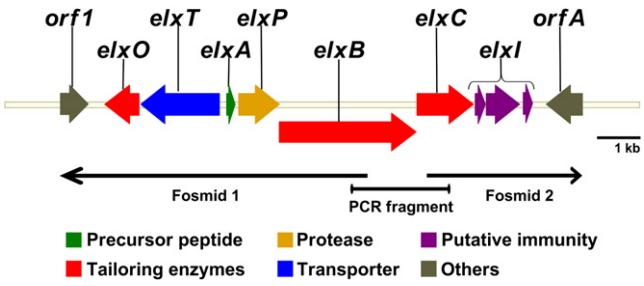

Figure 2. Epilancin 15X Gene Cluster
For amino acid sequences, see Figure S1.

Cloning and Overexpression of *elxO*

To study the role of ElxO, the corresponding gene was cloned into a pET28b vector to generate pHis$_6$-ElxO, which encodes an N-terminal hexahistidine fusion protein (His$_6$-ElxO). His$_6$-ElxO was heterologously produced in *E. coli* Rosetta 2 cells and the enzyme was purified by immobilized metal ion-affinity chromatography (IMAC) with Ni^{2+}, resulting in 60 mg of purified protein per liter of cell culture. The enzyme migrated as a protein of approximately 30 kDa by SDS-PAGE analysis, close to the predicted monomeric molecular weight of His$_6$-ElxO (29.7 kDa). Native molecular weight analysis using gel-filtration chromatography showed that His$_6$-ElxO exists as a dimer (59 kDa, observed).

In Vitro Reconstitution of the Enzymatic Activity of His$_6$-ElxO and Determination of the Stereochemistry of the Reaction

On the basis of its amino acid sequence (Figure S1D), ElxO is a member of the short-chain dehydrogenase/reductase (SDR) superfamily that catalyzes the interconversion of alcohols to aldehydes or ketones using NAD(P)(H) as a cofactor. Dehydroepilancin 15X, containing an N-terminal pyruvyl group, could therefore be the substrate for ElxO. Because dehydroepilancin 15X was not available, the small peptide AAIVK was synthesized by Fmoc-based solid-phase peptide synthesis (SPPS) followed by coupling of pyruvic acid to produce the ketone containing substrate Pyr-AAIVK. This peptide resembles the N-terminal portion of dehydroepilancin 15X, with Dha at position 3 replaced by Ala for simplicity. Incubation of Pyr-AAIVK with ElxO resulted in a decrease in absorbance at 340 nm over time with NADPH but not NADH. The reaction sample was also analyzed by liquid chromatography mass spectrometry (LC-MS), and a new peak with slightly shorter retention time and with *m/z* 573.4, corresponding to Lac-AAIVK, was observed (Figure 3).

To determine the stereochemical configuration of the N-terminal Lac, the two possible reaction products (D-Lac-AAIVK and L-Lac-AAIVK) were synthesized by Fmoc-based SPPS using D- or L-lactic acid during the last coupling step. The enzymatic product of Pyr-AAIVK, after incubation with His$_6$-ElxO and NADPH, was combined with D-Lac-AAIVK or L-Lac-AAIVK and analyzed by HPLC (Figures S2A–S2D). The enzymatic product of His$_6$-ElxO coeluted with D-Lac-AAIVK but not with L-Lac-AAIVK, demonstrating that ElxO catalyzes the formation of an N-terminal D-lactate ([*R*]-2-hydroxypropionate).

Production of Epilancin 15X and Stereochemical Characterization of the N-Terminal Lac Group

The production of lanthionine-containing polypeptide antibiotics by staphylococci is highly dependent on the composition of the media (Horner et al., 1989, 1990). To identify optimal conditions for the production of epilancin 15X, a set of cultures was grown in which the concentrations of meat extract, NaCl, and NH_4Cl were systematically varied. Analysis of culture supernatants for bioactivity using an agar-diffusion assay with *Staphylococcus carnosus* TM300 as indicator strain demonstrated that a medium containing 10% Lab-Lemco meat extract, 2% NaCl, 20 mM NH_4Cl, 3% malt extract, and 0.4% $Ca(OH)_2$ produced the highest concentration of epilancin 15X. Purification yielded about 3.0 mg of bacteriocin per liter of culture, compared with a yield of 0.5 mg/l reported previously (Ekkelenkamp et al., 2005).

To determine the stereochemical configuration of the N-terminal Lac in epilancin 15X, a sample of purified lantibiotic was treated with trypsin, generating Lac-ADhaIVK among other peptide fragments. The resulting peptide mixture and synthetic samples of D-Lac-ADhaIVK and L-Lac-ADhaIVK, produced by SPPS, were analyzed by LC-MS (Figures S2E–S2H). The N-terminal proteolytic fragment of epilancin 15X coeluted with D-Lac-ADhaIVK, confirming the stereochemical configuration of the N-terminal Lac in epilancin 15X.

Cloning and Overexpression of *elxP*

The gene *elxP* was cloned initially into a pET28b vector to generate an N-terminal hexahistidine fusion of ElxP (His$_6$-ElxP). However, attempts to overexpress the protein in *E. coli* were unsuccessful, because the rate of growth of the host was greatly reduced after induction with isopropyl β-D-1-thiogalactopyranoside (IPTG), suggesting that ElxP is toxic to the heterologous host. To overcome toxicity problems and improve solubility, *elxP* was cloned into pHis$_6$-MBP-ElxP which encodes for a fusion protein containing an N-terminal pelB signal, followed by a hexahistidine tag for purification and a maltose-binding protein (MBP) tag for solubility, separated from ElxP by a tobacco etch virus (TEV) protease cleavage site (Figure S3A). Heterologous expression trials in *E. coli* Rosetta 2 cells using the plating method (Suter-Crazzolara and Unsicker, 1995) afforded successful production of His$_6$-MBP-ElxP. The enzyme was purified by IMAC with Ni^{2+}, resulting in about 9 mg of purified protein per liter of cell culture. After treatment of His$_6$-MBP-ElxP with TEV protease and SDS-PAGE analysis, protein bands at about 34 kDa and 45 kDa, corresponding to ElxP (predicted mass of 34.3 kDa) and His$_6$-MBP (predicted mass of 45.5 kDa), respectively, were observed.

In Vitro Reconstitution of ElxP

Based on its amino acid sequence (Figure S1C), ElxP is a serine protease that may cleave the leader peptide from the fully cyclized precursor peptide. Because modified ElxA was not available, linear His$_6$-ElxA was tested as substrate, obtained by expression from a pET28b vector in *E. coli* Rosetta 2 cells and purification by IMAC with Ni^{2+} and subsequent HPLC. Linear His$_6$-ElxA was incubated with ElxP or His$_6$-MBP-ElxP at pH 7.5. The reaction products were analyzed by MALDI-TOF MS, confirming the formation of two proteolytic products in both cases (Figure 4). The peak at *m/z* = 4865 corresponds to the

Table 1. Open Reading Frame Analysis of the Epilancin 15X Gene Cluster Using BLASTp at the NCBI Website

Predicted ORF	Number of Amino Acids	Protein Homology (GenBank Accession Number)[a]	Identity in the Aligned Region	Expectation Value
elxA	55	ElkA, epilancin K7 precursor peptide, *S. epidermidis* K17, (AAA79236) (55 aa)	38/55 (69%)	7e-15
elxB	986	PepB, Pep5 dehydratase, *S. epidermidis* 5 (CAA90025) (967 aa)	327/994 (32%)	7e-104
elxC	402	PepC, Pep5 cyclase, *S. epidermidis* 5 (CAA90026) (398 aa)	142/379 (37%)	8e-56
elxO	248	EciO, oxidoreductase, *S. epidermidis* BN280 (CAA74346) (247 aa)	(126/248) 50%	8e-64
elxP	297	EciP, epicidin 280 protease, *S. epidermidis* 5 (CAA74349) (300 aa)	123/286 (43%)	3e-48
elxT	573	PepT, Pep5 ABC transporter, *S. epidermidis* 5 (CAA90021) (571 aa)	354/571 (61%)	0.0
elxI1	72	Hypothetical protein SE2390, *S. epidermidis* ATCC 12228 (NP_765945) (76 aa)	43/65 (66%)	7e-18
elxI2	241	CAAX amino protease, *S. epidermidis* M23864:W1 (ZP_04817536) (248 aa)	70/178 (39%)	8e-24
elxI3	71	Hypothetical protein, *S. aureus* subsp. *aureus* USA300_TCH959 (ZP_04865952) (75 aa)	43/71 (60%)	6e-17
orf1	177	Recombinase, *S. aureus* subsp. *aureus* TCH70 (ACZ58811) (182 aa)	154/176 (87%)	4e-85
orfA	261	Membrane-spanning protein, *Staphylococcus hominis* SK119 (ZP_04060547) (257 aa)	249/257 (96%)	1e-110

See also Figure S5.
[a] Results are from a BLASTp search of the GenBank protein database in January 2010.

N-terminal hexahistidine-tagged leader peptide (residues −1 to −43) resulting from amide bond hydrolysis at the predicted Gln(−1) Ser(1) cleavage site, whereas the peak at *m/z* = 3316 corresponds to the C-terminal unmodified core peptide.

Cloning and Coexpression of *elxA* and *elxB*

Based on bioinformatic analysis (Table 1), ElxB catalyzes the dehydration of the precursor peptide ElxA. To confirm the role of ElxB in epilancin 15X biosynthesis, the gene *elxA* and a synthetic codon-optimized version of the gene *elxB* were cloned into a pRSFDuet-1 vector, as previously described for nisin (Shi et al., 2011), to generate pHis$_6$-ElxA.ElxB, which encodes for an N-terminal hexahistidine fusion of ElxA (His$_6$-ElxA) and for untagged ElxB. Upon coexpression in *E. coli* BL21(DE3) cells, followed by peptide purification and cleavage of the leader region with His$_6$-MBP-ElxP, a mixture of partially dehydrated peptides was observed by MALDI-TOF MS (Figure S3B). Attempts to obtain fully dehydrated peptide or to reconstitute the enzymatic activity of ElxB in vitro were not successful.

Incubation of Peptides with an Aminopeptidase

A sample of epilancin 15X was incubated with a commercially available aminopeptidase from *Aeromonas proteolytica*. Interestingly, no proteolysis products were observed by MALDI-TOF MS, suggesting that the lactate group protects the lantibiotic against degradation (Figure S4). To further evaluate the possibility of the lactate group conferring stability against aminopeptidases, the peptide Lac-AAIVKBBIKA (where B stands for L-2-aminobutyric acid) mimicking the N-terminal portion of dehydroepilancin 15X and the corresponding peptide without the Lac group were generated. The two peptides were incubated with the aminopeptidase from *A. proteolytica*, followed by analysis by electrospray ionization mass spectrometry (ESI-MS) (Figure 5A). Whereas the peptide lacking the lactate group was completely degraded, the peptide containing the N-terminal lactate was detected intact.

In contrast, when lacticin 481 (Figure 5B), a lantibiotic lacking N-terminal modifications, was incubated with the aminopeptidase under similar conditions, a peptide missing the first seven N-terminal residues was obtained as the main product (Figure 5C). Thus, the aminopeptidase was able to remove N-terminal unmodified amino acids with the exception of the His preceding the first MeLan ring that protects the lantibiotic from additional proteolysis. Lacticin 481(2–27) and lacticin 481(3–27) were also observed, suggesting that the GG motif at position 2 is processed at a lower rate and may partially protect the bacteriocin against the action of aminopeptidases. The proteolyzed lacticin 481 and control samples were tested by agar-diffusion assays using the indicator strain *Lactococcus lactis* subsp. *cremoris* HP (Figure 5D), demonstrating a decrease in antibacterial activity of the proteolyzed peptides. These results are consistent with previous reports that have shown that the linear N-terminal portion of lacticin 481 is important for bioactivity (Levengood et al., 2009; Uguen et al., 2005).

DISCUSSION

Lantibiotics are produced from ribosomally synthesized linear precursor peptides that consist of an N-terminal leader region and a C-terminal core peptide. The mature lantibiotic is generated from the core peptide after several posttranslational modifications. The reactions involved in the formation of the characteristic Lan and MeLan rings have been investigated (Goto et al., 2010; Li et al., 2006; Xie et al., 2004), but only a few enzymes introducing other posttranslational modifications have been studied (Kupke et al., 1994; Majer et al., 2002; Müller et al., 2010). This investigation focused on the mechanism of formation of N-terminal lactate groups and on the enzymatic cleavage of the leader peptide.

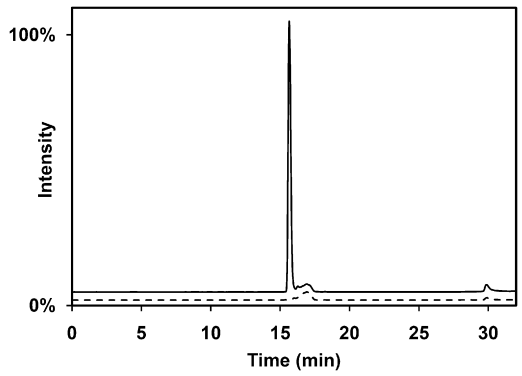

Figure 3. Enzymatic Assay of His_6-ElxO with Pyr-AAIVK

Shown are single-ion chromatograms at *m/z* = 573.4, corresponding to the expected $[M+H]^+$ ion for Lac-AAIVK, for the reaction mixture (solid line) and a control sample (dashed line). The peak at 16 min observed in the reaction sample but not in the control confirms the enzymatic reduction of the peptide. The peak at 17 min in the control sample is derived from higher-molecular weight isotopologues of the substrate (monoisotopic *m/z* = 571.4). See also Figure S2.

The epilancin 15X gene cluster contains five genes involved in biosynthesis (*elxABCOP*), one gene involved in the export of the mature peptide (*elxT*), and three genes potentially involved in immunity (*elxI1*, *elxI2*, and *elxI3*) (Table 1; Figure 2). The cluster organization resembles that of the lantibiotics Pep5 (Meyer et al., 1995) and epicidin 280 (Heidrich et al., 1998) produced by different strains of *S. epidermidis*, suggesting that these clusters have evolved from a common ancestor. The predicted peptide ElxA has high amino acid sequence similarity to the epilancin K7 precursor peptide ElkA (van de Kamp et al., 1995b) (Figure S1A). ElxA contains an N-terminal leader sequence of 24 amino acids and a C-terminal core peptide region composed of 31 amino acids, as predicted from the chemical structure of epilancin 15X. The leader region also contains the conserved motif F-(N/D)-L-(N/D/E) and a Pro at position −2 that are characteristic of class I lantibiotics (Figure S1A) (van der Meer et al., 1994).

Downstream of *elxA*, an ORF designated *elxP* was identified. The encoded protein ElxP possesses high amino acid sequence similarity to EciP, the protease involved in the biosynthesis of epicidin 280 (Heidrich et al., 1998), and to other subtilisin-like serine proteases. Importantly, the residues of the predicted catalytic triad and oxyanion hole (Asp27, His62, Ser240, and Asn154) are conserved in ElxP (Figure S1C). The lack of an N-terminal sec-signal sequence and a C-terminal cell wall anchor sequence (LPXTG) suggests that ElxP is localized inside the cytoplasm, possibly as part of a membrane-bound biosynthetic complex. Thus, ElxP likely removes the leader peptide before the mature peptide is transported outside the cell, in contrast to other class I lantibiotic proteases, such as NisP or EpiP, that are located extracellularly and remove the leader region once the peptide has been secreted (Figure S1C).

In a previous attempt to study LanP proteases, an *E. coli* host carrying a plasmid encoding for NisP was able to express the protease at low concentrations based on SDS-PAGE analysis using [^{35}S]Met (van der Meer et al., 1993). Although NisP was not purified, the *E. coli* cell extracts cleaved the nisin A cyclized precursor peptide, producing a biologically active compound (van der Meer et al., 1993). Additional in vivo studies indicated that NisP is able to cleave the leader region only from fully processed precursor peptide but not from uncyclized dehydrated or unmodified NisA (Kuipers et al., 2004). Similar results were obtained from in vivo studies of the lantibiotic Pep5 (Meyer et al., 1995). In contrast, culture supernatants of *S. carnosus* TM300 expressing EpiP processed unmodified EpiA to the expected proteolytic products (Geissler et al., 1996). In the present study, ElxP was successfully expressed in *E. coli* and its enzymatic activity was reconstituted in vitro. The protease was able to process unmodified His_6-ElxA, indicating that neither the Lan/MeLan ring nor the dehydrated residues, including Dha at position 1, are strictly required for enzyme recognition and proteolytic processing.

Downstream of *elxP*, an ORF designated *elxB* encodes a protein with homology to PepB, the enzyme that catalyzes the dehydration of Ser or Thr residues in the Pep5 precursor

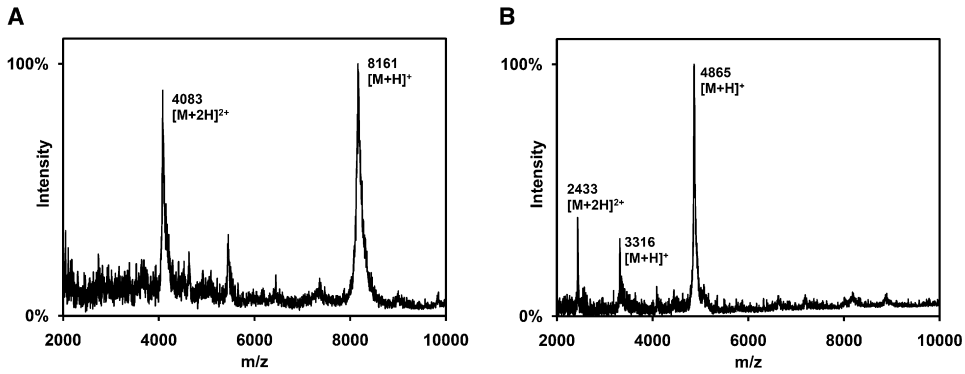

Figure 4. MALDI-TOF MS Analysis of Proteolytic Digestion of His_6-ElxA by His_6-MBP-ElxP

Similar results were obtained when ElxP was used.

(A) Analysis of His_6-ElxA (calculated mass = 8162.3 Da) after incubation without enzyme in reaction buffer.

(B) Analysis of peptide mixture after incubation with enzyme under the same conditions. Peaks corresponding to the leader peptide (calculated mass = 4864.3 Da) and to the core peptide (calculated mass = 3316.0 Da) are observed.

See also Figure S3.

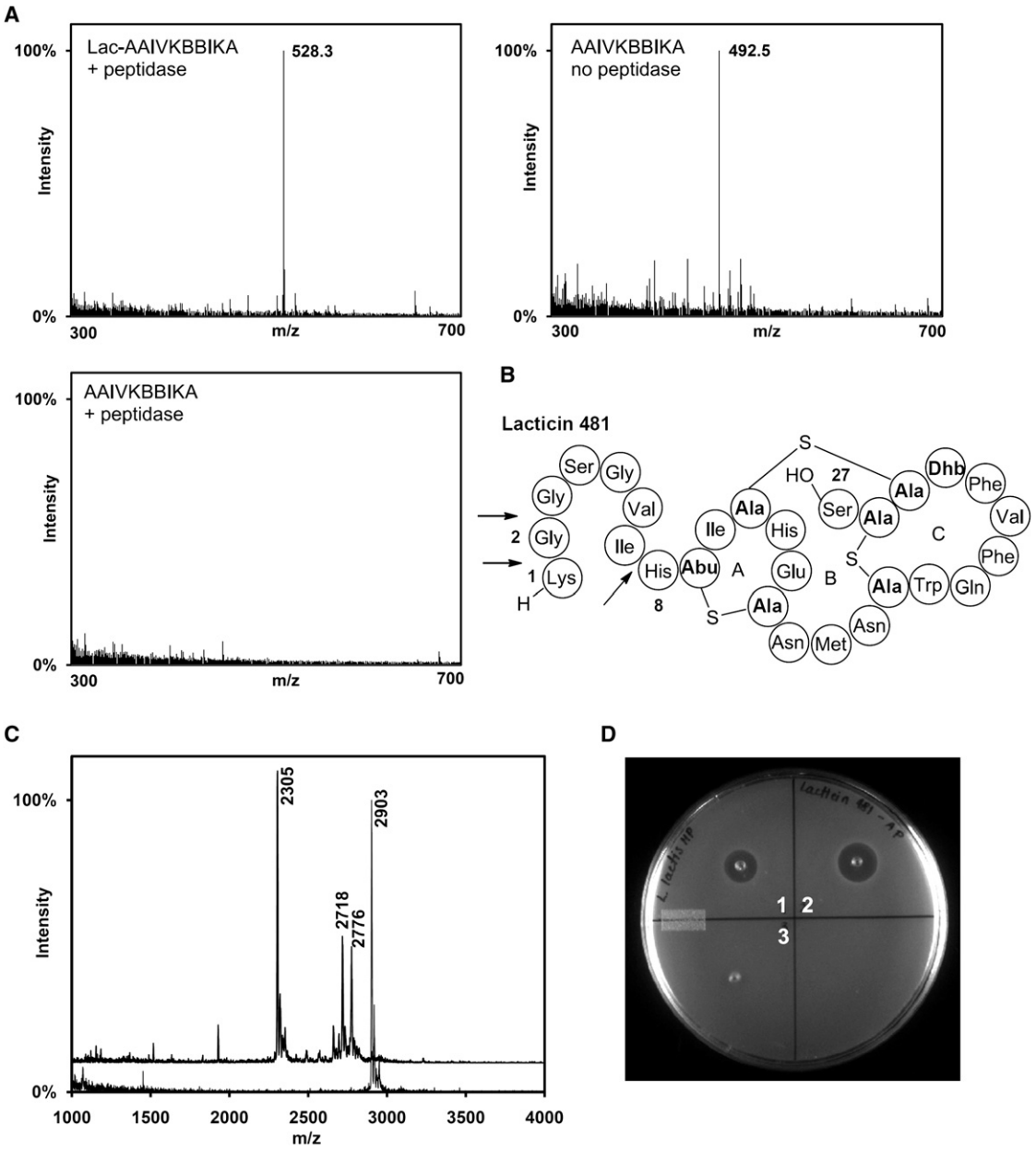

Figure 5. Degradation of Peptides by *A. proteolytica* Aminopeptidase

(A) ESI-MS analysis of the peptides Lac-AAIVKBBIKA (top left) and AAIVKBBIKA (bottom) after incubation with *A. proteolytica* aminopeptidase. Only the peptide lacking the Lac group was completely degraded by the aminopeptidase (bottom) compared with a control sample lacking enzyme (top right). The observed *m/z* peaks correspond to the $[M+2H]^{2+}$ ions.

(B) Structure of the lantibiotic lacticin 481, using the shorthand notation described in Figure 1.

(C) MALDI-TOF MS analysis of lacticin 481 (calculated *m/z* = 2902) after incubation with *A. proteolytica* aminopeptidase (top spectrum) and a control sample lacking peptidase (bottom spectrum). Peaks corresponding to lacticin(8–27) (calculated *m/z* = 2304), lacticin(2–27) (calculated *m/z* = 2774), and lacticin(3–27) (calculated *m/z* = 2717) were observed.

(D) Agar-diffusion bioactivity assay of lacticin 481 after treatment with the peptidase (spot 1) and a control sample without peptidase (spot 2) using *L. lactis* subsp. *cremoris* HP as indicator strain. A control sample containing the peptidase but no lantibiotic was also tested (spot 3).

See also Figure S4.

peptide (Meyer et al., 1995). Analysis of ElxB with SignalP 3.0 (Emanuelsson et al., 2007) suggests that this protein may contain an N-terminal cell membrane anchor signal. The activity of ElxB homologs of class I lantibiotics has never been reconstituted in vitro (Xie et al., 2002), and the cofactors or metals involved in catalysis are currently unknown. In this work, we were also unable to reconstitute ElxB activity in vitro. However, coexpression of His_6-ElxA and ElxB in *E. coli* produced

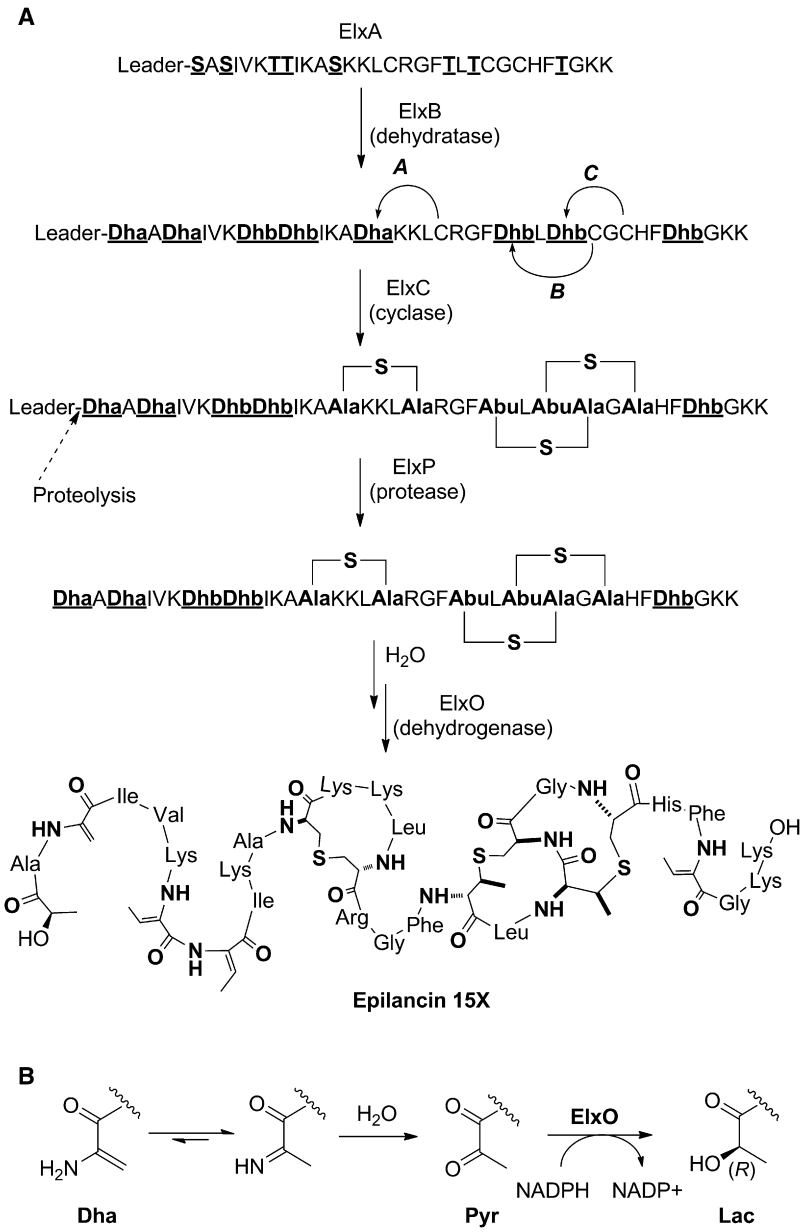

Figure 6. Proposed Biosynthetic Steps Involved in the Production of Epilancin 15X and Its N-Terminal D-Lactate Group

(A) Posttranslational modifications converting ElxA into epilancin 15X. Note that it is not known whether dehydration is completed before cyclization commences.

(B) Conversion of the Dha in position 1 to (*R*)-lactate.

a partially dehydrated peptide, confirming the role of the protein in the biosynthesis of epilancin 15X. Closer inspection of the ElxB sequence indicates that it contains an almost conserved Walker A motif (GXXXXGKT/S: GLLENWKT) and a conserved Walker B motif (hhhhD: IIFPD, where h stands for hydrophobic residue) (Figure S1B). In addition, the three potential binding sites characteristic of GTP-binding proteins are also present (DXXG: DFLG; NKXD: NTID/NDID/NLND/NRND; SAX: SAT) (Kjeldgaard et al., 1996), suggesting that GTP or another nucleotide may be required for dehydration by LanB proteins, similar to class II–IV lanthionine synthetases (Chatterjee et al., 2005a; Goto et al., 2010; Müller et al., 2010; Xie et al., 2004). The ORF *elxC* encodes a protein with high amino acid sequence similarity to PepC, the cyclase responsible for Lan and MeLan ring formation in Pep5 (Meyer et al., 1995). Additionally, ElxC contains the conserved residues comprising a zinc ion binding site (Cys269, Cys318, and His319) and the residues involved in acid-base catalysis (His205 and Asp142) that are characteristic of this family of proteins (Li and van der Donk, 2007; Li et al., 2006).

Based on the experimental results and on bioinformatic analysis, the first steps of epilancin 15X biosynthesis can be postulated (Figure 6A). The precursor peptide ElxA is modified by the dehydratase ElxB and the cyclase ElxC to produce the crosslinked peptide. Then, the leader peptide is removed by the protease ElxP, producing an N-terminal enamine (Dha) present in equilibrium with the corresponding imine that can be hydrolyzed to produce dehydroepilancin 15X (Figure 6B). Although enamine hydrolysis is fast (Sollenberger and Martin, 1970), enzymatic assistance (e.g., by ElxP) cannot be ruled out at present. The reduction of the N-terminal ketone to the respective alcohol will complete the synthesis of mature epilancin 15X.

The ORF *elxO* encodes a protein with homology to EciO, an oxidoreductase hypothesized to be involved in the reduction of pyruvate to lactate in the biosynthesis of epicidin 280 (Heidrich et al., 1998). ElxO contains a predicted N-terminal NAD(P)(H) binding site (GXXGXG: GGFKGIGK) and the catalytic triad residues (Ser139, Tyr152, and Lys156) of the SDR protein superfamily (Figure S1D) (Jörnvall et al., 1995; Tanaka et al., 2001). The absence of the "proximal Asp residue" (Ser33 in ElxO) and the presence of the "proximal basic residues" (Lys12 and Arg34), responsible for cofactor specificity, correctly predicted that ElxO is an NADPH-dependent enzyme and that it belongs to the cP3 subfamily (Kallberg et al., 2002; Tanaka et al., 2001). The stereochemical course of the reaction established here allows assignment of the configuration of the N-terminal lactate group of epilancin 15X as (*R*). Because His_6-ElxO was able to reduce a hexamer peptide, the thioether rings or other structural motifs in dehydroepilancin 15X are not required for enzyme recognition.

The ORF *elxT* encodes a putative protein with homology to PepT (Meyer et al., 1995). The C-terminal domain of ElxT contains an ATP binding site characterized by the conserved Walker A motif (GXXXXGKT/S: GPSGAGKT) or P loop, the Walker B motif (hhhhD: ILLLD), and the C motif or "signature" motif (LSGGQ) specific to ABC transporters (Figure S1E).

Additionally, analysis of ElxT with TMHMM 2.0 (Emanuelsson et al., 2007) indicates that its N-terminal portion contains six transmembrane helices. Thus, ElxT is likely involved in lantibiotic secretion.

Epilancin 15X has potent activity against staphylococci, including strains of *S. epidermidis* (Verhoef et al., 2005), suggesting that the producer strain must have an effective self-resistance mechanism. Such immunity is particularly important for epilancin 15X because it is activated by leader peptide removal within the cytoplasm, unlike most lantibiotics, for which leader peptide cleavage occurs after or concomitant with secretion. Downstream of *elxC*, the three ORFs *elxI1*, *elxI2*, and *elxI3* were identified. The genes *elxI1* and *elxI3* potentially encode for 72 and 71 amino acid paralog peptides (47% identity) with no sequence homology to previously characterized proteins. Analysis by TMHMM 2.0 (Emanuelsson et al., 2007) indicates that ElxI1 and ElxI3 contain two highly hydrophobic α-helical domains followed by strongly hydrophilic C-terminal segments (Figure S5). Thus, although ElxI1 and ElxI3 contain no signal peptides, these small proteins may be localized at the cytoplasmic membrane. A similar pattern of helical domains followed by a hydrophilic region is found in small proteins encoded by the gene clusters of the closely related lantibiotics Pep5 (Hoffmann et al., 2004) and epicidin 280 (Heidrich et al., 1998), the structurally unrelated lactosin S (Skaugen et al., 1997), the nonlantibiotic bacteriocin divergicin A (Worobo et al., 1995), and the circular bacteriocins AS-48, acidocin B, butyrivibriocin AR10, and circularin A (Maqueda et al., 2008). In the case of Pep5, the protein was designated PepI and was shown to be a determinant for self-resistance of the producer strain (Reis et al., 1994). Interestingly, in all of the lantibiotics mentioned above, intracellular peptidases remove the leader peptide and the mature bacteriocin is produced inside the cell. PepI has been suggested to bind to a (currently unknown) target molecule, avoiding docking of the lantibiotic Pep5 onto the target (Hoffmann et al., 2004). Despite the absence of significant sequence homology between PepI and ElxI1/3, the topological similarity suggests that these peptides may protect the host organism in a similar fashion (Figure S5).

Finally, the putative immunity protein ElxI2 has high sequence similarity to Abi proteins, membrane-bound metalloproteases that are involved in self-resistance to plantaricin EF and JK, sakacin 23K, or streptolysin S (Datta et al., 2005; Kjos et al., 2010). ElxI2 is predicted to contain seven transmembrane domains, including the final four α helices that form the Abi domain (Figure S1F). Three highly conserved motifs (EEXXXR: EEILYR; FXXXH: FSLIH; and His226) likely constitute the active site of the protease (Pei and Grishin, 2001). Thus, ElxI2 may protect the host against the bacteriocin by direct degradation of the peptide.

Whether *elxI1*, *elxI2*, and *elxI3* are part of the cluster and are involved in the immunity mechanism is at present not certain. Closer analysis of the noncoding sequences upstream of *elxC*, *elxI1*, and *elxI2* suggests the presence of only one relevant inverted repeat between *elxC* and *elxI1*, partially overlapping *elxC*, and with a calculated free energy of −10.2 kcal/mol. This repeat may work as a weak rho-independent transcriptional terminator that allows partial readthrough, indicating that *elxC* and *elxI1–3* may be part of a single operon and the same gene cluster. Downstream of *elxI3*, a noncoding region of 329 bp is followed by the ORF *orfA* in a different operon that encodes a putative ABC transporter with no sequence homology to any known lantibiotic proteins but with significant homology to transporters from staphylococci. Thus, OrfA is not likely to be related to epilancin 15X biosynthesis. Flanking the putative epilancin 15X gene cluster on the other side, an ORF designated *orf1* was identified. Orf1 is a putative recombinase, not likely to be involved in epilancin 15X biosynthesis, transport, or immunity. Thus, the epilancin 15X cluster likely spans a 9.2 kb region in the *S. epidermidis* 15X154 genome and includes the genes from *elxO* to *elxI3* (Figure 2).

The role of the N-terminal Lac in epilancin 15X is currently unknown. However, N-terminal modifications are common in lantibiotics and include (methyl)lanthionines, disulfides, pyruvate and lactate groups, 2-oxobutyrate groups, and acylations. The N-terminal disulfide in Halα (one of the two peptides in haloduracin) was shown not to be important for antimicrobial activity but to protect the peptide from exoproteases (Cooper et al., 2008; McClerren et al., 2006). Similarly, the N-terminal lanthionine in lacticin 3147 A1 is not required for antimicrobial activity (Cotter et al., 2006), but may protect lacticin 3147 A1 from proteolysis. A similar role for the N-terminal lactate group in epilancin 15X is supported in this study.

SIGNIFICANCE

The recently discovered lantibiotic epilancin 15X produced by *S. epidermidis* 15X154 has potent antimicrobial activity against drug-resistant strains of *S. aureus*. Epilancin 15X is structurally simple compared with other lantibiotics and yet is very active. The compound contains an unusual N-terminal D-lactate group that could be essential for biological activity. This study demonstrates that this moiety confers stability against proteolytic degradation by aminopeptidases, a feature that may be applied for the engineering of novel lantibiotics with enhanced antibacterial activities or different spectra of action. Furthermore, the gene cluster for epilancin 15X was determined and the enzymatic activities of the dehydratase, protease, and oxidoreductase involved in the biosynthesis were demonstrated in vitro or in vivo.

EXPERIMENTAL PROCEDURES

Genomic Library Construction, Screening, and DNA Sequencing

For all primer sequences and microorganisms, see Tables S1 and S2. Genomic DNA was obtained as described in Supplemental Experimental Procedures and partially digested with Sau3A1 (New England Biolabs) in the presence of RNase A (Sigma-Aldrich) using serial dilutions of the enzyme. The DNA solutions were analyzed by field inversion gel electrophoresis on a 1% agarose/TBE gel and the fraction containing ~20–60 kb DNA fragments was treated with shrimp alkaline phosphatase (Roche Diagnostics).

Cosmid pJK050 was digested with NheI (New England Biolabs), treated with shrimp alkaline phosphatase, and purified. The cosmid was further treated with BamHI (Invitrogen). Digested pJK050 and genomic DNA were ligated with T4 DNA ligase (New England Biolabs) and cosmid constructs were packaged into lambda phage with a MaxPlax Lambda Packaging Extract kit (Epicenter) according to the manufacturer's protocol, followed by transfection of *E. coli* WM4489. Library clones were screened by PCR for the *elxA* gene using forward primer elxA-F1 and reverse primer elxA-R2, or for the *elxC*

gene with forward primer elxC-596F and reverse primer elxC-781R, *Taq* polymerase (Invitrogen), and 1× PCR premix A (Epicenter).

Plasmid pAE5 was digested with BglII, and the 1084 bp fragment to be used as a transposon was gel purified. For each positive fosmid, the fragment and the positive fosmid were mixed and treated with MuA transposase (Finnzymes) and the dialyzed DNA was used to transform *E. coli* WM4489. Sets of 192 clones were sequenced from the ends of the transposon insert using primers seqaetf and seqaetr at the Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign. Using *S. epidermidis* 15X154 as template, the specific primers elxBgapF and elxCgapR were used to amplify and sequence a DNA fragment of 1.2 kb, closing the sequence gap between the fosmids.

Construction of Plasmids pHis$_6$-ElxO, pHis$_6$-ElxA, and pHis$_6$-MBP-ElxP

The gene *elxO* was amplified by PCR from *S. epidermidis* 15X154 genomic DNA using a forward primer containing an NheI restriction site (elxO.NheI.F) and a reverse primer containing an XhoI site (elxO.XhoI.R). The PCR product and the vector pET28b (Novagen) were digested with restriction endonucleases NheI and XhoI (Invitrogen) and ligated using T4 DNA ligase (New England Biolabs) to produce the plasmid pHis$_6$-ElxO.

The gene *elxA* was amplified by PCR from *S. epidermidis* 15X154 genomic DNA using a forward primer containing an NdeI restriction site (elxA.NdeI.F) and a reverse primer containing an EcoRI site (elxA.EcoRI.R). The PCR product and the vector pET28b (Novagen) were digested with restriction endonucleases NdeI and EcoRI (Invitrogen) and ligated using T4 DNA ligase (New England Biolabs) to produce the plasmid pHis$_6$-ElxA.

The gene *elxP* was amplified by PCR from *S. epidermidis* 15X154 genomic DNA using a forward primer (elxP.28MBP.F) and a reverse primer (elxP.28MBP.R). The PCR product contained annealing regions to a modified pET28b vector (Novagen) that encodes for a fusion pelB, hexahistidine-tagged MBP. The purified PCR product was used as a primer to amplify the vector using Phusion Hot Start DNA polymerase (New England Biolabs), followed by treatment with DpnI (New England Biolabs) before transformation of *E. coli* DH5α cells to generate the plasmid pHis$_6$-MBP-ElxP that encodes for ElxP fused at its N terminus to a TEV protease cleavage site, an MBP tag, a hexahistidine tag, and a pelB signal peptide (Figure S3A). The correct sequences of the inserts were confirmed by sequencing at the Keck Center at the University of Illinois at Urbana-Champaign. *E. coli* BL21(DE3) Rosetta 2 cells were transformed with pHis$_6$-ElxO, pHis$_6$-ElxA, or pHis$_6$-MBP-ElxP, and the proteins were overexpressed and purified by IMAC (Supplemental Information).

His$_6$-ElxO and ElxP Activity Assays

His$_6$-ElxO (10 μM) and the peptide Pyr-AAIVK (1 mM) generated by Fmoc-based SPPS (Supplemental Information) were incubated with NADPH (1 mM) in assay buffer (100 mM HEPES [pH 7.5]) at room temperature for 6 hr. Reaction progress was monitored by UV-vis spectrophotometry, measuring the disappearance of the NADPH peak at 340 nm. Formation of reduced peptide was confirmed by LC-MS using an Agilent 1200 instrument equipped with a single-quadrupole multimode ESI/atmospheric pressure chemical ionization ion source mass spectrometry detector (Agilent) and a Synergi Fusion-RP column (4.6 mm i.d. × 150 mm length; Phenomenex). ElxP or His$_6$-MBP-ElxP (5 μM) and purified His$_6$-ElxA (Li et al., 2009) (50 μM) were incubated in the presence of an assay buffer (50 mM HEPES, [pH 7.5]) at room temperature for 2–3 hr. A control sample lacking enzyme was incubated under the same conditions. Cleavage of the peptide in the reaction sample was confirmed by MALDI-TOF MS as described above.

Production and Purification of Epilancin 15X

A medium composed of Lab-Lemco meat extract (10%; Oxoid), malt extract (3%; Becton Dickinson), ammonium chloride (20 mM), $Ca(OH)_2$ (0.4%), and NaCl (2%) was inoculated with an overnight preculture of *S. epidermidis* 15X154 in LB broth (1/100 dilution). Cells were incubated at 37°C with shaking for 12 hr and harvested by centrifugation. The supernatant was filtered through a 0.22 μm pore size filter and heated at 80°C for 1 hr to deactivate proteases. Solid $(NH_4)_2SO_4$ was added to the culture supernatant to reach 80% saturation at 4°C and stirred for 4 hr followed by centrifugation. The pellet was resuspended in water and loaded onto a Vydac C_4 reverse-phase solid-phase extraction column (214SPE1000; Discovery Sciences). The column was washed with 25% acetonitrile in ammonium acetate buffer (20 mM, pH 5.0) to remove impurities and the peptide was eluted with 80% methanol in 0.1% trifluoroacetic acid (TFA)/water. The lantibiotic was further purified by HPLC using an Agilent 1200 instrument (Agilent) equipped with a Vydac 214TP54 C_4 reverse-phase column (4.6 mm i.d. × 250 mm L; Discovery Sciences). A gradient of 50%–60% B (0.1% TFA in methanol) in A (0.1% TFA in water) over 50 min was used. The fractions corresponding to the major peak were collected. Analysis of the final product by ESI-MS on a Waters Synapt instrument showed a mass of 3172.6817 Da (calculated monoisotopic mass of epilancin 15X: 3172.6977 Da).

Incubation of Peptides with *A. proteolytica* Aminopeptidase

Epilancin 15X was incubated with *A. proteolytica* aminopeptidase (10 U/ml; Sigma-Aldrich) in assay buffer (40 mM Tris-HCl [pH 8.0]) at room temperature for 24 hr. Samples containing purified lacticin 481 with or without protease were incubated under the same conditions for 12 hr. The reaction products were analyzed by MALDI-TOF or ESI-MS/MS and tested by agar-diffusion bioactivity assays using M17 agar medium supplemented with 0.5% glucose and *L. lactis* subsp. *cremoris* HP as indicator strain.

The peptide Lac-AAIVKBBIKA, generated from Pyr-AAIVKBBIKA enzymatically with His$_6$-ElxO as described above, and the control peptide AAIVKBBIKA were also incubated with the peptidase under similar conditions for 6 hr. The peptides were then purified by solid-phase extraction using Discovery DSC-18 columns (1 ml, 50 mg; Supelco) and 0.1% formic acid in water or 0.1% formic acid in 60% acetonitrile as washing and eluting solvents, respectively, and analyzed by ESI-MS using a ZMD quadrupole instrument (Waters) at the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.05.007.

ACKNOWLEDGMENTS

We thank M. Ekkelenkamp and Prof. J. Verhoef (Utrecht University Hospital) for a sample of *S. epidermidis* 15X154, and William Metcalf and Mr. Jun Kai Zhang for helpful advice on molecular genetics and for the strains and plasmids used for generating the genomic library. We also thank Ms. Laura Guest at the Core DNA Sequencing Facility, Ms. Neha Garg for help in the screening of the genomic library, and Ms. Isabel Neacato for help in cloning. This work was supported by a grant from the National Institutes of Health (GM58822 to W.A.v.d.D.). J.E.V. was supported by the National Institutes of Health under Ruth L. Kirschstein National Research Service Award T32 GM070421.

Received: September 26, 2010
Revised: April 15, 2011
Accepted: May 3, 2011
Published: July 28, 2011

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Breukink, E., and de Kruijff, B. (2006). Lipid II as a target for antibiotics. Nat. Rev. Drug Discov. *5*, 321–332.

Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O.P., Sahl, H., and de Kruijff, B. (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science *286*, 2361–2364.

Brötz, H., Josten, M., Wiedemann, I., Schneider, U., Götz, F., Bierbaum, G., and Sahl, H.G. (1998). Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol. *30*, 317–327.

Castiglione, F., Lazzarini, A., Carrano, L., Corti, E., Ciciliato, I., Gastaldo, L., Candiani, P., Losi, D., Marinelli, F., Selva, E., et al. (2008). Determining the structure and mode of action of microbisporicin, a potent lantibiotic active against multiresistant pathogens. Chem. Biol. *15*, 22–31.

Chatterjee, C., Miller, L.M., Leung, Y.L., Xie, L., Yi, M., Kelleher, N.L., and van der Donk, W.A. (2005a). Lacticin 481 synthetase phosphorylates its substrate during lantibiotic production. J. Am. Chem. Soc. *127*, 15332–15333.

Chatterjee, C., Paul, M., Xie, L., and van der Donk, W.A. (2005b). Biosynthesis and mode of action of lantibiotics. Chem. Rev. *105*, 633–684.

Cooper, L.E., McClerren, A.L., Chary, A., and van der Donk, W.A. (2008). Structure-activity relationship studies of the two-component lantibiotic haloduracin. Chem. Biol. *15*, 1035–1045.

Cotter, P.D., Deegan, L.H., Lawton, E.M., Draper, L.A., O'Connor, P.M., Hill, C., and Ross, R.P. (2006). Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. Mol. Microbiol. *62*, 735–747.

Datta, V., Myskowski, S.M., Kwinn, L.A., Chiem, D.N., Varki, N., Kansal, R.G., Kotb, M., and Nizet, V. (2005). Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. Mol. Microbiol. *56*, 681–695.

Ekkelenkamp, M.B., Hanssen, M., Hsu, S.-T.D., de Jong, A., Milatovic, D., Verhoef, J., and van Nuland, N.A. (2005). Isolation and structural characterization of epilancin 15X, a novel lantibiotic from a clinical strain of *Staphylococcus epidermidis*. FEBS Lett. *579*, 1917–1922.

Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protoc. *2*, 953–971.

Geissler, S., Gotz, F., and Kupke, T. (1996). Serine protease EpiP from *Staphylococcus epidermidis* catalyzes the processing of the epidermin precursor peptide. J. Bacteriol. *178*, 284–288.

Goto, Y., Li, B., Claesen, J., Shi, Y., Bibb, M.J., and van der Donk, W.A. (2010). Discovery of unique lanthionine synthetases reveals new mechanistic and evolutionary insights. PLoS Biol. *8*, e1000339.

Heidrich, C., Pag, U., Josten, M., Metzger, J., Jack, R.W., Bierbaum, G., Jung, G., and Sahl, H.G. (1998). Isolation, characterization, and heterologous expression of the novel lantibiotic epicidin 280 and analysis of its biosynthetic gene cluster. Appl. Environ. Microbiol. *64*, 3140–3146.

Hoffmann, A., Schneider, T., Pag, U., and Sahl, H.G. (2004). Localization and functional analysis of PepI, the immunity peptide of Pep5-producing *Staphylococcus epidermidis* strain 5. Appl. Environ. Microbiol. *70*, 3263–3271.

Horner, T., Zahner, H., Kellner, R., and Jung, G. (1989). Fermentation and isolation of epidermin, a lanthionine containing polypeptide antibiotic from *Staphylococcus epidermidis*. Appl. Microbiol. Biotechnol. *30*, 219–225.

Horner, T., Ungermann, V., Zahner, H., Fiedler, H.P., Utz, R., Kellner, R., and Jung, G. (1990). Comparative studies on the fermentative production of lantibiotics by staphylococci. Appl. Microbiol. Biotechnol. *32*, 511–517.

Hosoda, K., Ohya, M., Kohno, T., Maeda, T., Endo, S., and Wakamatsu, K. (1996). Structure determination of an immunopotentiator peptide, cinnamycin, complexed with lysophosphatidylethanolamine by 1H-NMR1. J. Biochem. *119*, 226–230.

Hsu, S.T., Breukink, E., Tischenko, E., Lutters, M.A., de Kruijff, B., Kaptein, R., Bonvin, A.M., and van Nuland, N.A. (2004). The nisin-lipid II complex reveals a pyrophosphate cage that provides a blueprint for novel antibiotics. Nat. Struct. Mol. Biol. *11*, 963–967.

Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzàlez-Duarte, R., Jeffery, J., and Ghosh, D. (1995). Short-chain dehydrogenases/reductases (SDR). Biochemistry *34*, 6003–6013.

Kallberg, Y., Oppermann, U., Jornvall, H., and Persson, B. (2002). Short-chain dehydrogenases/reductases (SDRs): coenzyme-based functional assignments in completed genomes. Eur. J. Biochem. *269*, 4409–4417.

Kjeldgaard, M., Nyborg, J., and Clark, B.F.C. (1996). The GTP binding motif: variations on a theme. FASEB J. *10*, 1347–1368.

Kjos, M., Snipen, L., Salehian, Z., Nes, I.F., and Diep, D.B. (2010). The Abi proteins and their involvement in bacteriocin self-immunity. J. Bacteriol. *192*, 2068–2076.

Kuipers, A., De Boef, E., Rink, R., Fekken, S., Kluskens, L.D., Driessen, A.J., Leenhouts, K., Kuipers, O.P., and Moll, G.N. (2004). NisT, the transporter of the lantibiotic nisin, can transport fully modified, dehydrated and unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. J. Biol. Chem. *279*, 22176–22182.

Kupke, T., Kempter, C., Gnau, V., Jung, G., and Götz, F. (1994). Mass spectroscopic analysis of a novel enzymatic reaction. Oxidative decarboxylation of the lantibiotic precursor peptide EpiA catalyzed by the flavoprotein EpiD. J. Biol. Chem. *269*, 5653–5659.

Levengood, M.R., Knerr, P.J., Oman, T.J., and van der Donk, W.A. (2009). In vitro mutasynthesis of lantibiotic analogues containing nonproteinogenic amino acids. J. Am. Chem. Soc. *131*, 12024–12025.

Li, B., and van der Donk, W.A. (2007). Identification of essential catalytic residues of the cyclase NisC involved in the biosynthesis of nisin. J. Biol. Chem. *282*, 21169–21175.

Li, B., Yu, J.P., Brunzelle, J.S., Moll, G.N., van der Donk, W.A., and Nair, S.K. (2006). Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. Science *311*, 1464–1467.

Li, B., Cooper, L.E., and van der Donk, W.A. (2009). In vitro studies of lantibiotic biosynthesis. Methods Enzymol. *458*, 533–558.

Majer, F., Schmid, D.G., Altena, K., Bierbaum, G., and Kupke, T. (2002). The flavoprotein MrsD catalyzes the oxidative decarboxylation reaction involved in formation of the peptidoglycan biosynthesis inhibitor mersacidin. J. Bacteriol. *184*, 1234–1243.

Maqueda, M., Sanchez-Hidalgo, M., Fernandez, M., Montalban-Lopez, M., Valdivia, E., and Martinez-Bueno, M. (2008). Genetic features of circular bacteriocins produced by Gram-positive bacteria. FEMS Microbiol. Rev. *32*, 2–22.

McClerren, A.L., Cooper, L.E., Quan, C., Thomas, P.M., Kelleher, N.L., and van der Donk, W.A. (2006). Discovery and in vitro biosynthesis of haloduracin, a two-component lantibiotic. Proc. Natl. Acad. Sci. USA *103*, 17243–17248.

Meyer, C., Bierbaum, G., Heidrich, C., Reis, M., Suling, J., Iglesias-Wind, M.I., Kempter, C., Molitor, E., and Sahl, H.G. (1995). 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. Eur. J. Biochem. *232*, 478–489.

Müller, W.M., Schmiederer, T., Ensle, P., and Süssmuth, R.D. (2010). In vitro biosynthesis of the prepeptide of type-III lantibiotic labyrinthopeptin A2 including formation of a C-C bond as a post-translational modification. Angew. Chem. Int. Ed. Engl. *49*, 2436–2440.

Oman, T.J., and van der Donk, W.A. (2010). Follow the leader: the use of leader peptides to guide natural product biosynthesis. Nat. Chem. Biol. *6*, 9–18.

Pei, J., and Grishin, N.V. (2001). Type II CAAX prenyl endopeptidases belong to a novel superfamily of putative membrane-bound metalloproteases. Trends Biochem. Sci. *26*, 275–277.

Reis, M., Eschbach-Bludau, M., Iglesias-Wind, M.I., Kupke, T., and Sahl, H.G. (1994). Producer immunity towards the lantibiotic Pep5: identification of the immunity gene pepI and localization and functional analysis of its gene product. Appl. Environ. Microbiol. *60*, 2876–2883.

Shi, Y., Yang, X., Garg, N., and van der Donk, W.A. (2011). Production of lantipeptides in *Escherichia coli*. J. Am. Chem. Soc. *133*, 2338–2341.

Skaugen, M., Abildgaard, C.I., and Nes, I.F. (1997). Organization and expression of a gene cluster involved in the biosynthesis of the lantibiotic lactocin S. Mol. Gen. Genet. *253*, 674–686.

Sollenberger, P.Y., and Martin, R.B. (1970). Mechanism of enamine hydrolysis. J. Am. Chem. Soc. *92*, 4261–4270.

Suter-Crazzolara, C., and Unsicker, K. (1995). Improved expression of toxic proteins in *E. coli*. Biotechniques *19*, 202–204.

Tanaka, N., Nonaka, T., Nakamura, K.T., and Hara, A. (2001). SDR structure, mechanism of action, and substrate recognition. Curr. Org. Chem. *5*, 89–111.

Taubes, G. (2008). The bacteria fight back. Science *321*, 356–361.

Uguen, P., Hindre, T., Didelot, S., Marty, C., Haras, D., Le Pennec, J.P., Vallee-Rehel, K., and Dufour, A. (2005). Maturation by LctT is required for biosynthesis of full-length lantibiotic lacticin 481. Appl. Environ. Microbiol. *71*, 562–565.

van de Kamp, M., Horstink, L.M., van den Hooven, H.W., Konings, R.N., Hilbers, C.W., Frey, A., Sahl, H.G., Metzger, J.W., and van de Ven, F.J. (1995a). Sequence analysis by NMR spectroscopy of the peptide lantibiotic epilancin K7 from *Staphylococcus epidermidis* K7. Eur. J. Biochem. *227*, 757–771.

van de Kamp, M., van den Hooven, H.W., Konings, R.N., Bierbaum, G., Sahl, H.G., Kuipers, O.P., Siezen, R.J., de Vos, W.M., Hilbers, C.W., and van de Ven, F.J. (1995b). Elucidation of the primary structure of the lantibiotic epilancin K7 from *Staphylococcus epidermidis* K7. Cloning and characterisation of the epilancin-K7-encoding gene and NMR analysis of mature epilancin K7. Eur. J. Biochem. *230*, 587–600.

van der Meer, J.R., Polman, J., Beerthuyzen, M.M., Siezen, R.J., Kuipers, O.P., and de Vos, W.M. (1993). Characterization of the *Lactococcus lactis* nisin A operon genes nisP, encoding a subtilisin-like serine protease involved in precursor processing, and nisR, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. *175*, 2578–2588.

van der Meer, J.R., Rollema, H.S., Siezen, R.J., Beerthuyzen, M.M., Kuipers, O.P., and de Vos, W.M. (1994). Influence of amino acid substitutions in the nisin leader peptide on biosynthesis and secretion of nisin by *Lactococcus lactis*. J. Biol. Chem. *269*, 3555–3562.

Verhoef, J., Milatovic, D., and Ekkelenkamp, M.B. March 17, 2005. Antimicrobial compounds. Patent WO/2005/023852 (http://www.wipo.int/patentscope/search/en/WO2005023852).

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O.P., Bierbaum, G., de Kruijff, B., and Sahl, H.G. (2001). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. *276*, 1772–1779.

Willey, J.M., and van der Donk, W.A. (2007). Lantibiotics: peptides of diverse structure and function. Annu. Rev. Microbiol. *61*, 477–501.

Worobo, R.W., Van Belkum, M.J., Sailer, M., Roy, K.L., Vederas, J.C., and Stiles, M.E. (1995). A signal peptide secretion-dependent bacteriocin from *Carnobacterium divergens*. J. Bacteriol. *177*, 3143–3149.

Xie, L., Chatterjee, C., Balsara, R., Okeley, N.M., and van der Donk, W.A. (2002). Heterologous expression and purification of SpaB involved in subtilin biosynthesis. Biochem. Biophys. Res. Commun. *295*, 952–957.

Xie, L., Miller, L.M., Chatterjee, C., Averin, O., Kelleher, N.L., and van der Donk, W.A. (2004). Lacticin 481: in vitro reconstitution of lantibiotic synthetase activity. Science *303*, 679–681.