SUPPLEMENTAL INFORMATION

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

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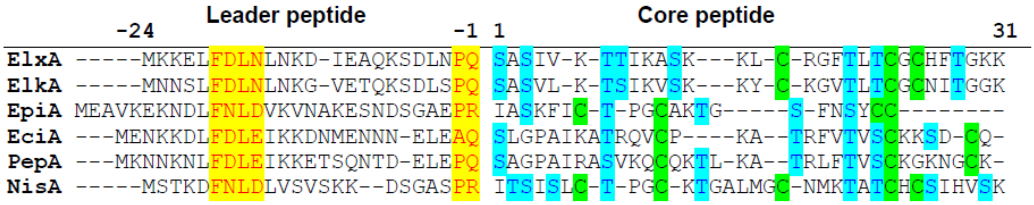
INVENTORY OF SUPPLEMENTAL INFORMATION

SUPPLEMENTAL DATA

A)



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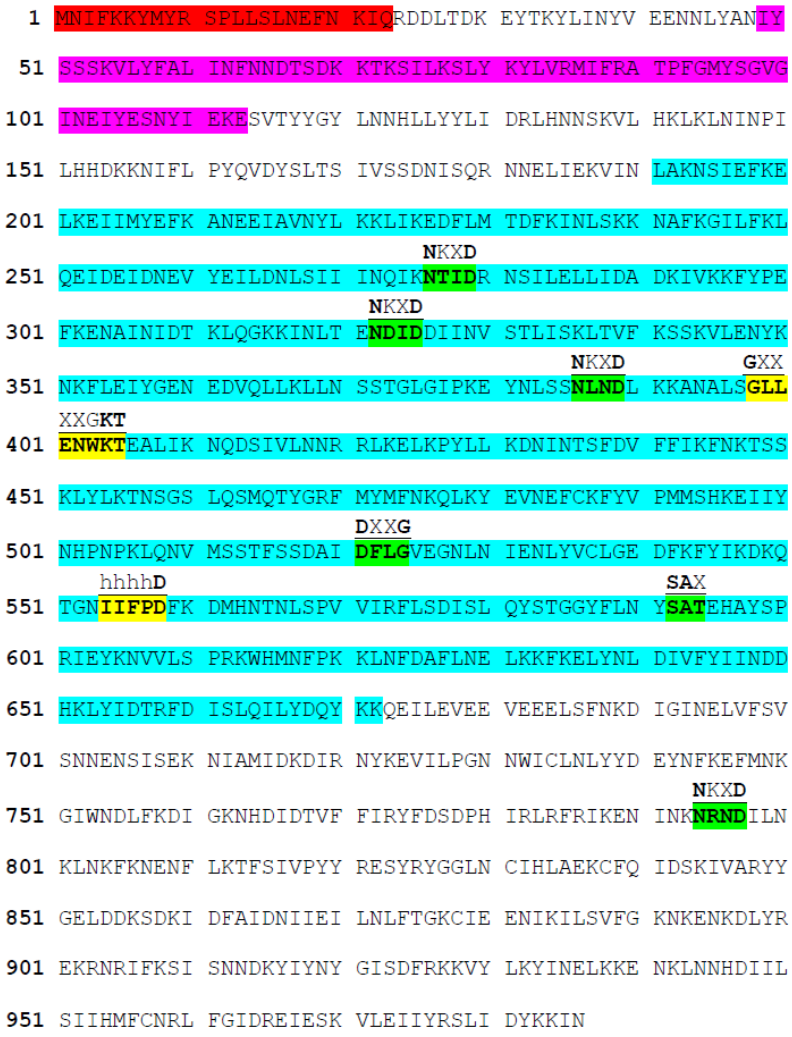

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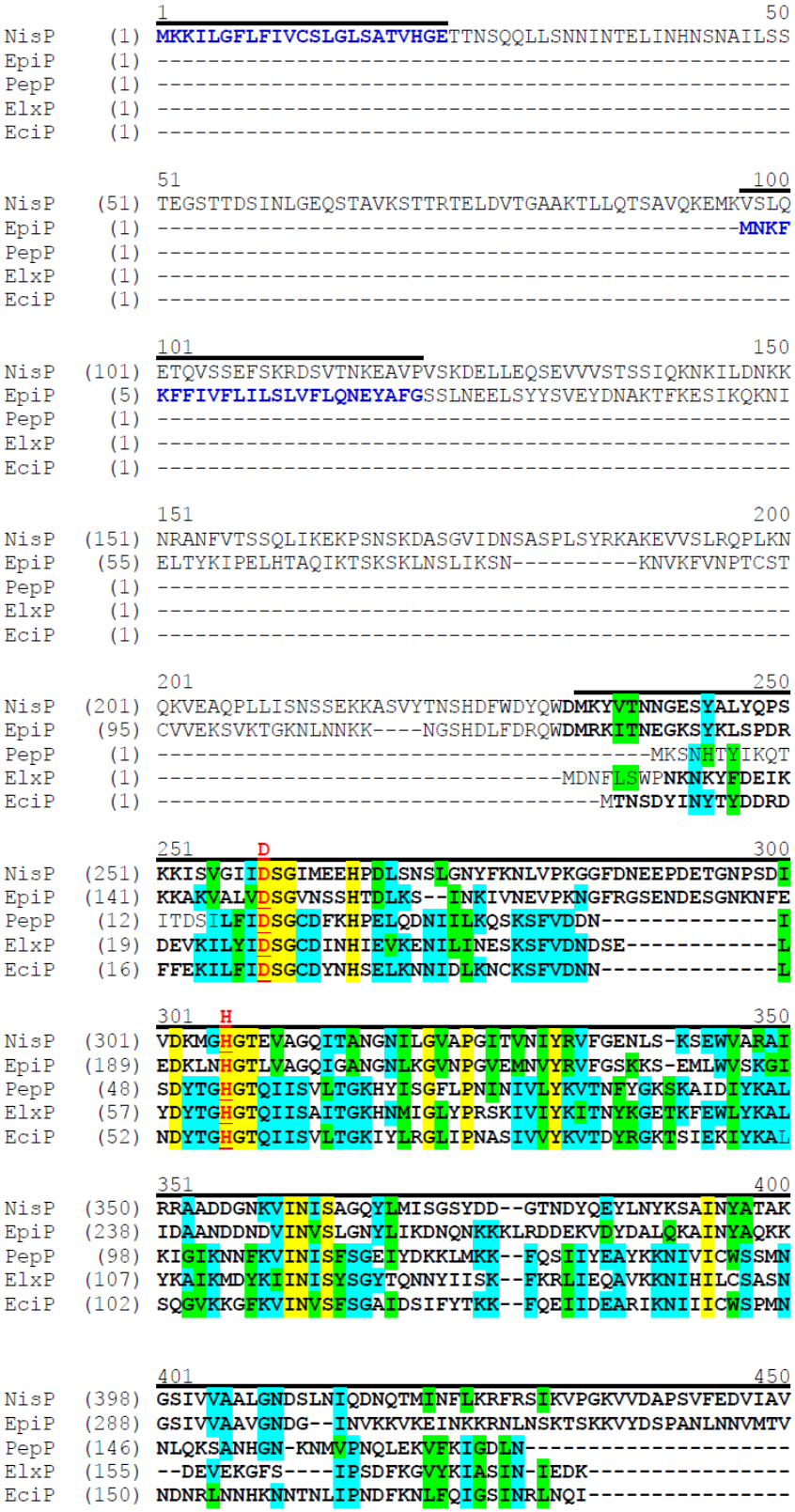
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PepP (1) ----------
ElxP (1) ----------
EciP (1) ----------
51 100
NisP (51) TEGSTTDSINLGEQSTAVKSTTRTELDVTGAAKTLLQTSAVQKEMKVSLQ
EpiP (1) ----------MNKF
PepP (1) ----------
ElxP (1) ----------
EciP (1) ----------
101 150
NisP (101) ETQVSSEFSKRDSVTNKEAVPVSKDELLEQSEVVVSTSSIQKNKILDNKK
EpiP (5) KFFIVFLILSLVFLQNEYAFGSSLNEELSYYSVEYDNAKTFKESIKQKNI
PepP (1) ----------
ElxP (1) ----------
EciP (1) ----------
151 200
NisP (151) NRANFVTSSQLIKEKPSNSKDASGVIDNSASPLSYRKAKEVVSLRQPLKN
EpiP (55) ELTYKIPELHTAQIKTSKSKLNSLIKSN----------KNVKFVNPTCST
PepP (1) ----------
ElxP (1) ----------
EciP (1) ----------
201 250
NisP (201) QKVEAQPLLISNSSEKKASVYTNSHDFWDYQWDMKYVTNNGESYALYQPS
EpiP (95) CVVEKSVKTGKNLNNKK----NGSHDLFDRQWDMRKITNEGKSYKLSPDR
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ElxP (1) ----------MDNFLSWPNKNKYFDEIK
EciP (1) ----------MTNSDYINYTYDDRD
251 D 300
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ElxP (19) DEVKILYIDSGCDINHIEVKENILINESKSFVDNDSE----------L
EciP (16) FFEKILFIDSGCDYNHSELKNNIDLKNCKSFVDNN----------L
301 H 350
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EpiP (189) EDKLNHGTLVAGQIGANGNLKGVNPGVEMNVYRVFGSKKS-EMLWVSKGI
PepP (48) SDYTGHGTQIISVLTGKHYISGFLPNINIVLYKVTNFYGKSKAIDIYKAL
ElxP (57) YDYTGHGTQIISAITGKHNMIGLYPRSKIVIYKITNYKGETKFEWLYKAL
EciP (52) NDYTGHGTQIISVLTGKIYLRGLIPNASIVVYKVTDYRGKTSIEKIYKAL
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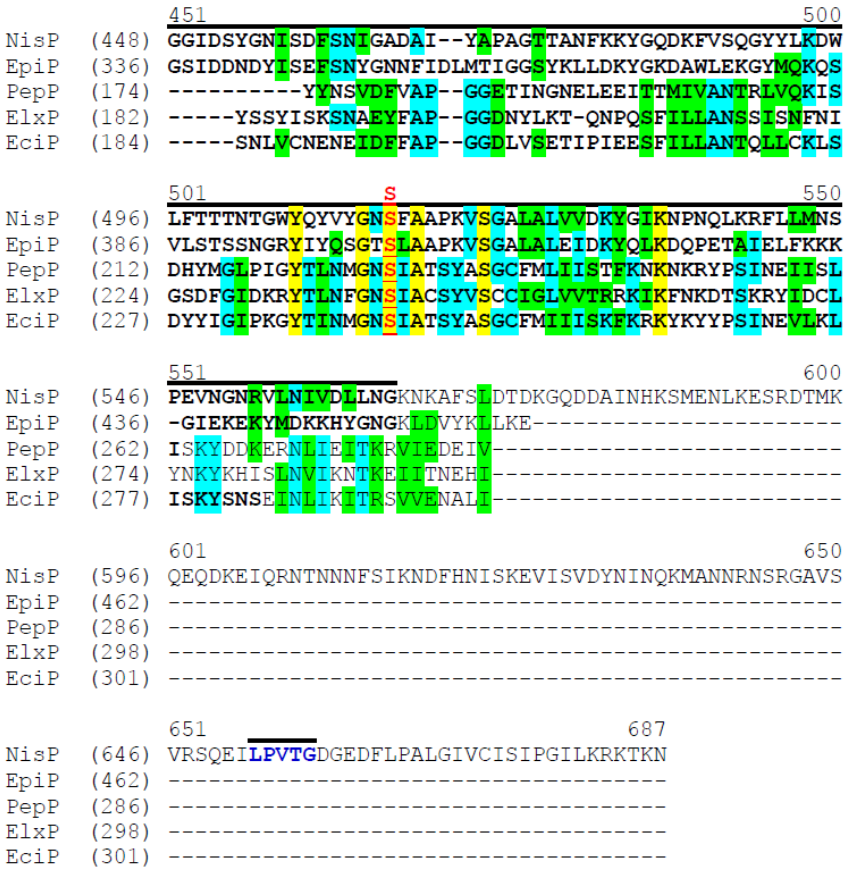
ElxP (107) YKAIKMDYKIINISYSGYTQNNYIISK--FKRLIEQAVKKNIHILCSASN
EciP (102) SQGVKKGFKVINVSFSGAIDSIFYTKK--FQEIIDEARIKNIIICWSPMN
401 450
NisP (398) GSIVVAALGNDSLNIQDNQTMINFLKRFRSIKVPGKVVDAPSVFEDVIAV
EpiP (288) GSIVVAAVGNDG--INVKKVKEINKKRNLNSKTSKKVYDSPANLNNVMTV
PepP (146) NLQKSANHGN-KNMVPNQLEKVFKIGDLN----------
ElxP (155) --DEVEKGFS----IPSDFKGVYKIASIN-IEDK----------
EciP (150) NDNRLNNHKNNTNLIPNDFKNLFQIGSINRLNQI----------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ElxP (182) -----YSSYISKSNAEYFAP--GGDNYLKT-QNPQSFILLANSSISNFNI
EciP (184) -----SNLVCNENEIDFFAP--GGDLVSETIPIEESFILLANTQLLCKLS
501 S 550
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ElxP (224) GSDFGIDKRYTLNFGNSIACSYVSCCIGLVVTRRKIKFNKDTSKRYIDCL
EciP (227) DYYIGIPKGYTINMGNSIATSYASGCFMIIISKFKRKYKYYPSINEVLKL
551 600
NisP (546) PEVNGNRVLNIVDLLNGKNKAFSLDTDKGQDDAINHKSMENLKESRDTMK
EpiP (436) -GIEKEKYMDKKHYGNGKLLVYKLLKE----------
PepP (262) ISKYDDKERNLIEITKRVIEDEIV----------
ElxP (274) YNKYKHISLNVIKNTKEIITNEHI----------
EciP (277) ISKYSNSEINLIKITRSVVENALI----------
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NisP (596) QEQDKEIQRNTNNNFSIKNDFHNISKEVISVDYNINQKMANNRNSRGAVS
EpiP (462) ----------
PepP (286) ----------
ElxP (298) ----------
EciP (301) ----------
651 687
NisP (646) VRSQEILPVTGDGEDFLPALGIVCISIPGILKRKTKN
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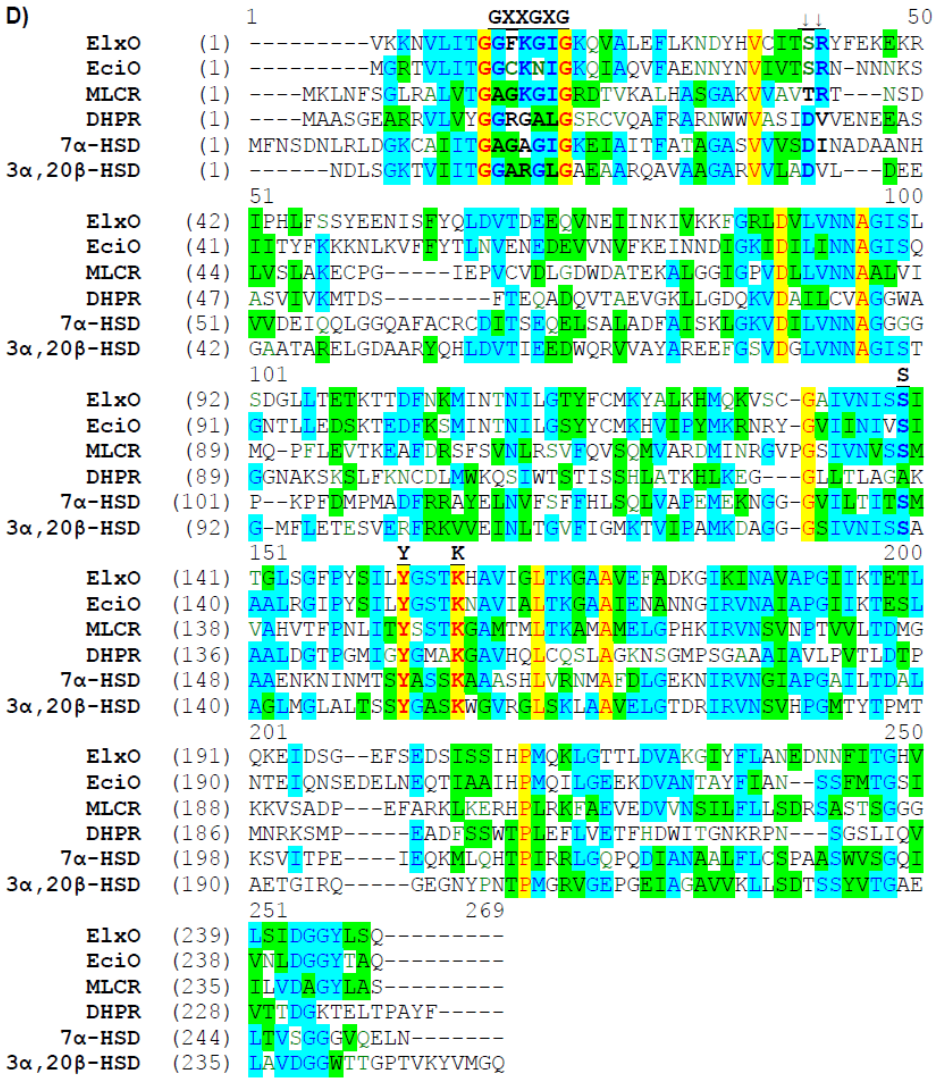
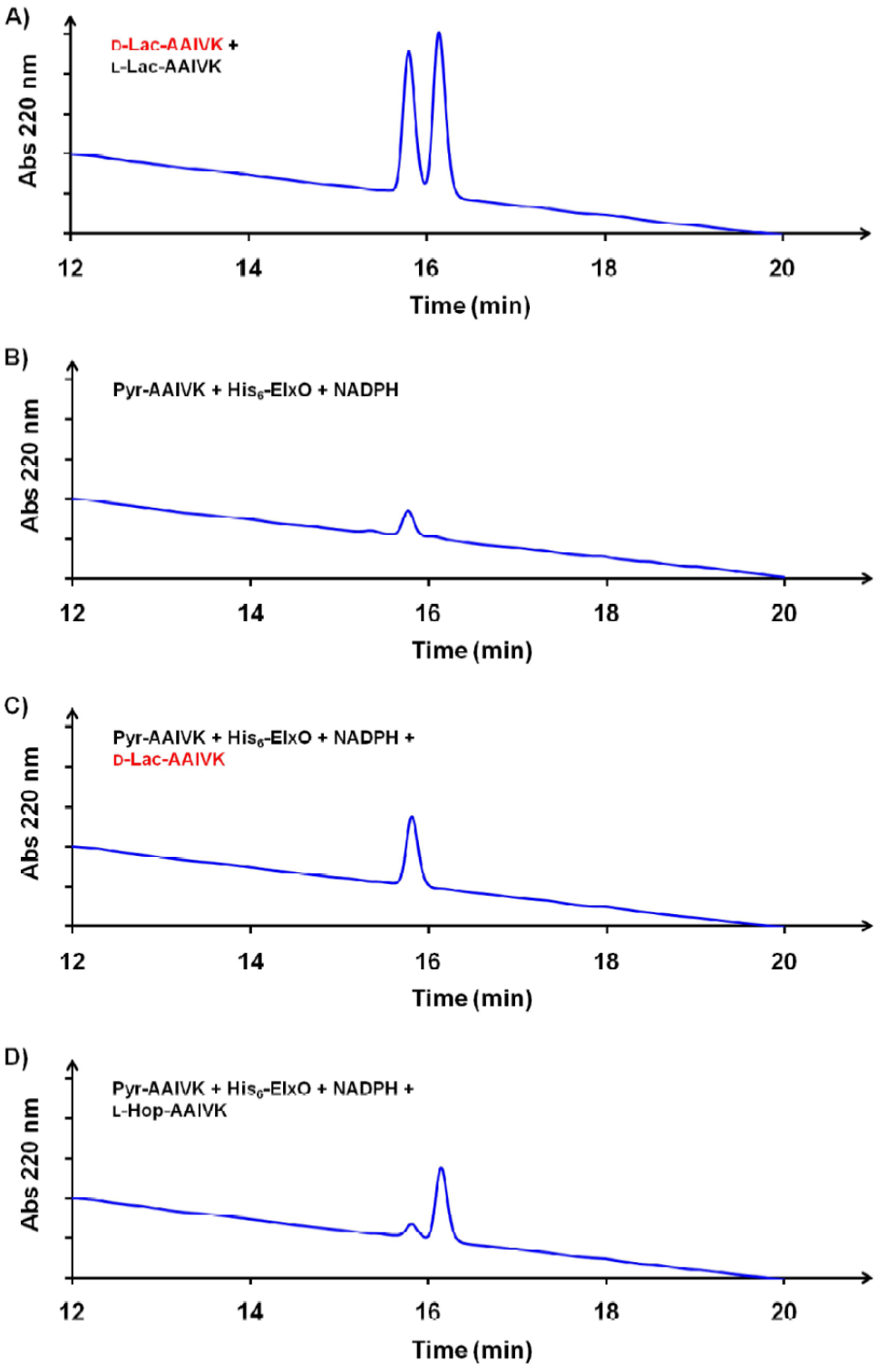

D)
GXXGXG
ElxO
EciO
MLCR
DHPR
7α-HSD
3α,20β-HSD

Figure S1, related to Figure 2. A) Sequence alignment of ElxA with related lantibiotic precursor peptides. ElxA: epilancin 15X, ElkA: epilancin K7, EpiA: epidermin, EciA: epicidin 280, PepA: Pep5, NisA: nisin A. B) Potential binding motifs and protein domain analysis of ElxB. Lantibiotic dehydratase - N terminus (pfam04737, pink) and C terminus (pfam04738, blue) domains and an N-terminal signal peptide (red) are present in ElxB. Potential Walker A and B motifs (yellow) and GTP binding sites (green) are almost perfectly conserved. h, stands for hydrophobic residue. C) Sequence alignment of different lantibiotic proteases. Shown are the proteases involved in the biosynthesis of nisin A (NisP), epidermin (EpiP), Pep5 (PepP), epilancin 15X (ElxP), and epicidin 280 (EciP). The N-terminal signal peptide of NisP and EpiP as well as the C-terminal anchor signal in NisP are shown in blue bold letters. The central subtilisin-like serine protease domains are shown in black bold letters. D) Sequence alignment of ElxO and several other short-chain dehydrogenase reductase proteins. The highly conserved residues Ser139, Tyr152, and Lys156, and the GXXGXG motif, are all shown above the sequences in bold underlined font. EciO: epicidin 280 oxidoreductase, MLCR: Mouse lung carbonyl reductase, DHPR: Dihydropteridine reductase, 7α-HSD: 7α-hydroxysteroid dehydrogenase, 3α,20β-HSD: 3α,20β-hydroxysteroid dehydrogenase. E) Domain analysis of ElxT. The N-terminal transmembrane domains are highlighted in blue, the putative Walker A and B motifs in yellow and the C-motif characteristic of ABC transporters in green. F) Domain analysis of ElxI2. The transmembrane domains are highlighted in blue and the conserved Abi motifs in green.


A)
D-Lac-AAIVK +
L-Lac-AAIVK
Abs 220 nm
12
14
16
18
20
Time (min)
B)
Pyr-AAIVK + His6-ElxO + NADPH
Abs 220 nm
12
14
16
18
20
Time (min)
C)
Pyr-AAIVK + His6-ElxO + NADPH +
D-Lac-AAIVK
Abs 220 nm
12
14
16
18
20
Time (min)
D)
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L-Hop-AAIVK
Abs 220 nm
12
14
16
18
20
Time (min)

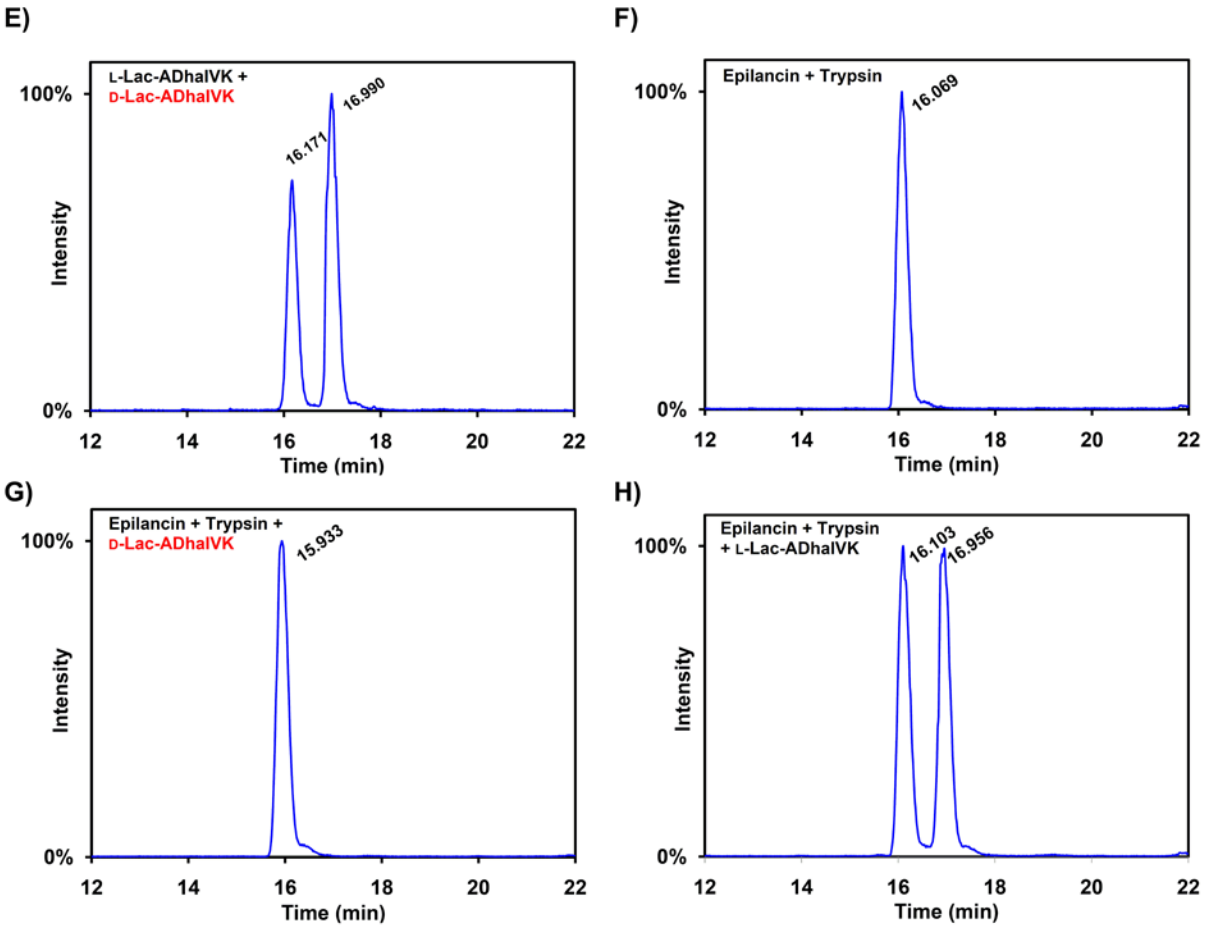

Figure S2, related to Figure 3. Determination of the stereochemical configuration of the N-terminal lactate (Lac) in epilancin 15X.

The two possible diastereoisomers of Lac-AAIKV or Lac-ADhaIKV have different retention times when analyzed by reverse-phase HPLC or UPLC, allowing the determination of the stereochemical configuration of Lac in epilancin 15X. A) A mixture of chemically synthesized D-Lac-AAIVK and L-Lac-AAIVK analyzed by HPLC. B) The reaction product of Pyr-AAIVK after incubation with His_6-ElxO and NADPH analyzed under the same conditions as A). C) A mixture of the enzymatic reaction product of Pyr-AAIVK and D-Lac-AAIVK produced a single peak. D) The enzymatic product of Pyr-AAIVK combined with L-Lac-AAIVK produced two well separated peaks. E) A mixture of chemically synthesized D-Lac-ADhaIVK and L-Lac-ADhaIVK analyzed by UPLC. F) The reaction product of epilancin 15X after digestion with trypsin analyzed under the same conditions as E). G) A mixture of the enzymatic digestion product of epilancin 15X and D-Lac-AAIVK produced a single peak. H) The enzymatic digestion product of epilancin 15X combined with with L-Lac-AAIVK produced two well separated peaks. The peptides were detected using a quadrupole time-of-flight MS detector scanning the mass range 570 - 573 Da.

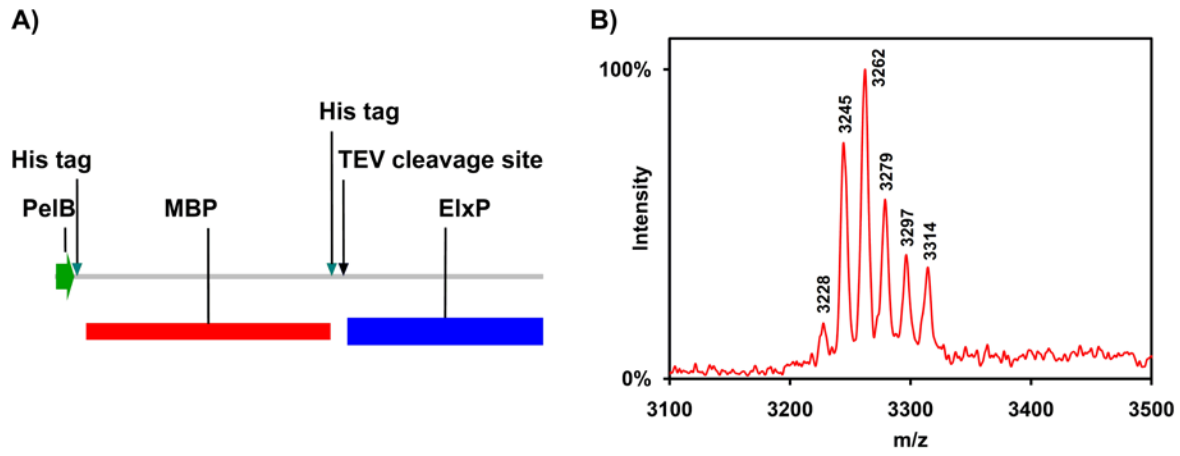

Figure S3, related to Figure 4. A) Scheme of ElxP fused to a TEV protease cleavage site, a MBP tag, a hexahistidine tag, and a pelB signal peptide encoded by pHis$_6$-MBP-ElxP. B) MALDI-TOF MS spectrum of a mixture of peptides obtained after co-expression of *elxA*, *elxB*, and *elxC* in *E. coli* BL21(DE3), purification by IMAC, and cleavage of leader peptide after incubation with His$_6$-MBP-ElxP. Peaks corresponding to one to fivefold dehydrated ElxA (calculated m/z = 3299, 3281, 3263, 3245, and 3227) were observed.

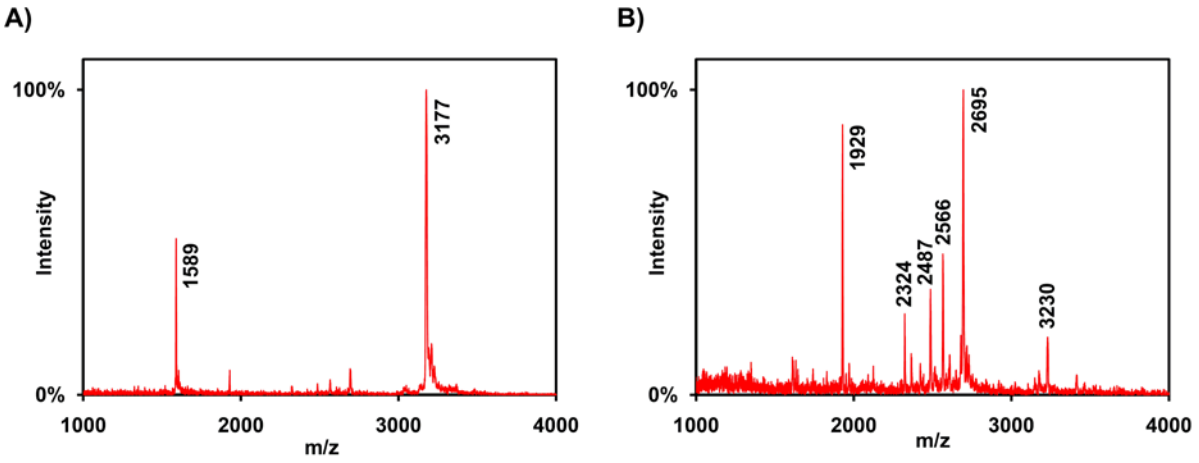

Figure S4, related to Figure 5. MALDI-TOF MS analyses of epilancin 15X after treatment with *A. proteolytica* aminopeptidase. (A)Epilancin 15X is resistant to the protease. The peaks at m/z = 3177 and 1589 corresponds to the $[M+H]^+$ and $[M+2H]^{2+}$ ions of epilancin 15X (expected mass 3175 Da), respectively. The small peaks on the baseline are also present in a control sample containing aminopeptidase but lacking bacteriocin (B) and are not epilancin degradation products.

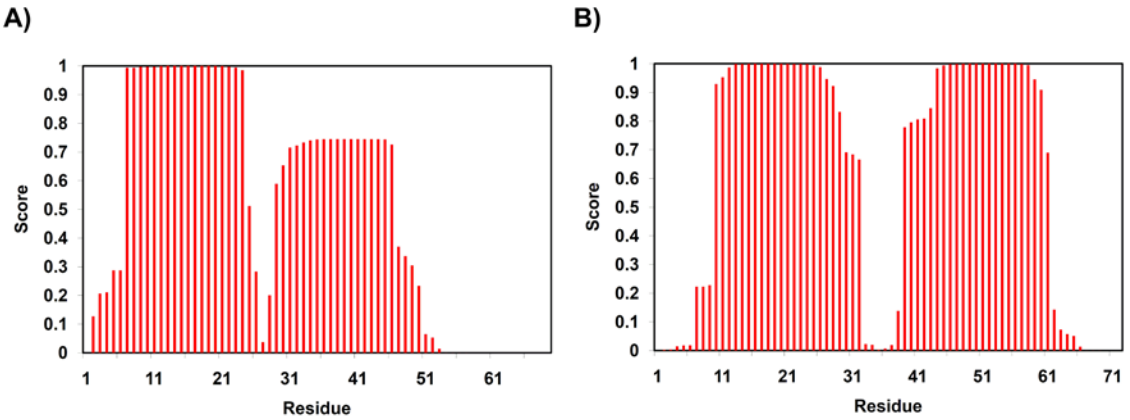

Figure S5, related to Table 1. Domain analysis of PepI (A) and ElxI1 (B). Both proteins are predicted to contain two highly hydrophobic α-helical domains followed by strongly hydrophilic C-terminal segments. The helical regions were predicted using TMHMM 2.0 (Emanuelsson, et al., 2007).

Table S1. Microorganisms and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
WM4489	*E. coli* DH10B derivative: mcrA Δ(mrr hsdRMS mcrBC) ϕ80(ΔlacM15) ΔlacX74 endA1 recA1 deoR Δ(ara-leu)7697 araD139 galU galK nupG rpsL λattB::pAE12(PrhaB::trfA33 ΔoriR6K-cat::frt5)	(Eliot, et al., 2008)
DH5*α*	λpir/ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1	(Grant, et al., 1990)
Rosetta2	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pRARE2 pLysS	Novagen
Staphylococcus epidermidis 15X154	Epilancin 15X producer strain	(Ekkelenkamp, et al., 2005)
carnosus TM300	Epilancin 15X sensitive strain	G. Bierbaum, University of Bonn
Lactococcus		
lactis subsp. *lactis* CNRZ 481	Lacticin 481 producer strain	(Piard, et al., 1990)
lactis subsp. *cremoris* HP ATCC 12602	Lacticin 481 sensitive strain	ATCC[a]
Plasmids		
pACYCDuet-1	Cm^R *E. coli* T7 based vector for coexpression vector of two target genes	Novagen
pAE5	Source of mini-Mu transposon	(Eliot, et al., 2008)
pElxC	*elxC* cloned in pACYCDuet-1 vector	This study
pET28b	Kan^R *E. coli* T7 based histidine-tag fusion expression vector	Novagen
$pHis_6$-ElxA	*elxA* cloned in pET28b vector	This study
$pHis_6$-ElxO	*elxO* cloned in pET28b vector	This study
$pHis_6$-MBP-ElxP	*elxP* cloned in pET28b vector	This study
pRSFDuet-1	Kan^R *E. coli* T7 based histidine-tag fusion vector for coexpression of two target genes	Novagen
$pHis_6$-ElxA.ElxB	*elxA* and *elxB* cloned in pRSFDuet-1 vector	This study
pJK050	*oriV*, *oriS*, copy-control cosvector, Cm^R	(Eliot, et al., 2008)

[a]ATCC, American Type Culture Collection, Manassas, VA.

Table S2. Primers used in this study

Name	Sequence
elxA.BamHI.F	5'- GCG AGC CAG GAT CCG ATG AAA AAA GAA TTA TTT GAT TTA AAT CTT AAT AAA G -3'
elxA.EcoRI.R	5'- GGC GCG CCG AAT TCT TAT TTT TTA CCA GTA AAG TG -3'
elxA.NdeI.F	5'- GGC GCG CCC ATA TGA AAA AAG AAT TAT TTG ATT TAA ATC TTA AT -3'
elxA.NotI.R	5'- GCA TTA TGC GGC CGC TTA TTT TTT ACC AGT AAA GTG ACA TCC ACA AGT TAG -3'
elxA-F1	5'-ATG AAT AAC GAA TTA TT(C/T) (A/G)AT TTG GAT C-3'
elxA-R2	5'-CTT TGT AGA GGA TTT ACA CTA ACT TG-3'
elxB.Bglll.F	5'- GAA GGA GAT ATA CAT ATG GCA GAT CTC ATG AAC ATC TTC AAA AAA T -3'
elxB.XhoI.R	5'- CGG TTT CTT TAC CAG ACT CGA GTT AGT TGA TTT TTT TGT AG -3'
elxBgapF	5'- GTT ATT TCT TGA ATT ATT CTG CTA CAG AGC -3'
elxC.NdeI.F	5'- GGA TAT ACA TAT GGA AAA TAG TAT CCA AAA ATC CTT ATC ATA CCT TTC AG -3'
elxC.XhoI.R	5'- ACC AGA CTC GAG TTA CGC AAA ACA AAA CAA TTT ATA CC -3'
elxC-596F	5'-AT(C/T) TAG G(A/T)T A(T/C)G C(A/T/G)C ATG G(A/T)A T-3'
elxC-781R	5'- CC(A/G) TA(A/G) CAC CAA (C/G)C(A/G) T(C/T)T CT-3'
elxCgapR	5'- AAC CCA TAA CAC CAG TAA ATA ATG AAG TAG -3'
elxO.NheI.F	5'- GGT TGG TTG CTA GCA TGA AAA AAA ATG TTC TTA TTA CAG -3'
elxO.XhoI.R	5'- AAG TCG ACC TCG AGT TAT TGG GAT AAA TAT CCT C -3'
elxP.28MBP.F	5'- GAA CCT GTA CTT CCA ATC CGG ATC CAT GGA TAA TTT TCT TAG TTG GCC TAA TAA A -3'
elxP.28MBP.R	5'- GGT GGT GGT GGT GGT GCT CGA GTT AAA TAT GTT CAT TAG TAA TAA TCT CCT TTG TG -3'
seqaetf	5'- TCG CCT TCT TGA CGA GTT CT -3'
seqaetr	5'- TAG GAA CTT CGG GAT CCG TT- 3'

SUPPLEMENTAL METHODS

Organisms, media, and growth conditions

The strains and plasmids used in this study are listed in Table S1. *S. epidermidis* 15X154 and *S. carnosus* TM300 were grown on Luria – Bertani (LB) or Mueller Hinton (Oxoid) solid agar or liquid broth at 37 °C. *E. coli* strains were routinely grown in LB solid agar or broth supplemented appropriately at 37 °C. Strain *E. coli* WM4489 (Blodgett, et al., 2005; Eliot, et al., 2008), used as the host for genomic library construction, was supplemented with maltose (10 mM) before phage transfection, while strains containing fosmid pJK050 (Blodgett, et al., 2005; Eliot, et al., 2008) or derivatives were supplemented with rhamnose (10 mM) preceding fosmid isolation. For agar diffusion bioactivity assays, 25 mL of agar medium inoculated with culture (1/100 dilution) were poured into a sterile plate. Aliquots of antibacterial compounds were placed into wells made on the solidified agar and the plates were incubated at 37 or 30 °C

overnight. The bioactivity of the sample was confirmed if a clear inhibition zone surrounding the well was formed.

The strains *L. lactis* subsp. *lactis* CNRZ481 and *L. lactis* subsp. *cremoris* HP were grown in Elliker broth medium without gelatin and supplemented with sodium β-glycerophosphate (15 g/L) as buffering agent (EG'P medium) or in M17 medium (BD) containing 0.5% glucose, respectively.

Genomic DNA Isolation

An aliquot of *S. epidermidis 15X154* culture was pelleted by centrifugation and cells were washed with TE25S buffer (25 mM Tris-HCl, 25 mM EDTA, 0.3 M sucrose, pH 8). Cells were then lysed with 2 mg/mL of lysozyme (Sigma-Aldrich) and 50 μg/mL of lysostaphin (Sigma-Aldrich) in TE25S buffer at 37 °C for 60 min. The washed protoplasted cells were incubated with proteinase K (0.15 mg/mL, Sigma-Aldrich) in TE25S buffer at 50 °C for 30 min. Sodium dodecyl sulfate (SDS) was added to 0.5% (w/v) final concentration and gently mixed for 10 min at room temperature. The cell lysate was mixed with an equal volume of saturated buffer of phenol, chloroform, and isoamyl alcohol (25:24:1). After centrifugation, the aqueous layer was removed and mixed gently with an equal volume of a mixture of chloroform and isoamyl alcohol (24:1). The top layer was removed again and 0.1 volumes of 5 M NaCl and 0.7 volumes of isopropanol were added to precipitate the DNA. The DNA was washed three times with 70% ethanol and once with 100% ethanol, air-dried, and resuspended in TE buffer (pH 8.0).

Overproduction and purification of His_6-ElxO

Electrocompetent *E. coli* BL21 (DE3) Rosetta 2 cells were transformed with pHis_6-ElxO and a single colony was grown in LB medium supplemented with 50 μg/mL kanamycin and 12.5 μg/mL chloramphenicol. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM when OD_{600} = 0.7 and the culture was incubated for additional 16 h at 18 °C. The cells were harvested by centrifugation and the pellet was resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM imidazole, pH 7.5) and stored at -80 °C until used.

The cell pellet was thawed, lysozyme was added to a final concentration of 1 mg/mL, and the suspension was passed through a French-press. The protein was purified by immobilized metal ion affinity chromatography (IMAC) using an ÄKTApurifier (Amersham Biosciences, GE Healthcare) equipped with a HisTrap HP 5 mL column prepacked with Ni Sepharose™ (GE Healthcare). A gradient of 0-100% buffer B (20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4) in buffer A (20 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4) over 20 CV was used to elute the proteins. The fractions containing His_6-ElxO were concentrated using an Amicon Ultracel 10k filter (Millipore). Imidazole was removed by using a PD-10 desalting column (GE Healthcare) and the protein was eluted and stored in buffer (50 mM Tris-HCl, 300 mM NaCl, 20% glycerol, pH 7.5). The native molecular weight of His_6-ElxO was determined by size exclusion chromatography using an ÄKTApurifier equipped with a Superdex 200 HR 10/30 GL column (GE Healthcare) and utilizing cytrochrome c (12.4 kDa), carbonic anydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), and blue dextran (2000 kDa) as standards (MWGF200, Sigma-Aldrich).

Overexpression and purification of His_6-ElxA

Electrocompetent *E. coli* BL21 (DE3) Rosetta 2 cells were transformed with pHis_6-ElxA and a single colony was inoculated in 5 mL of LB medium containing 50 µg/mL kanamycin and 12.5 µg/mL chloramphenicol and grown for 12 h with shaking. An aliquot of 1 mL was used to inoculate 100 mL LB cultures containing the same antibiotics followed by overnight incubation at 37 °C. Finally, an aliquot of 20 mL of the overnight culture was used to inoculate 2 L cultures. The cells were grown at 37 °C until OD_{600} = 1.0. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM and the cultures were incubated for 5 h at 37 °C. The cells were harvested by centrifugation and resuspended in lysis buffer (20 mM NaH_2PO4, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol, pH 7.5). After sonication and centrifugation, the pellets were resuspended in denaturing buffer (6 M guanidine hydrochloride, 20 mM NaH_2PO4, 0.5 mM imidazole, 500 mM NaCl, pH 7.5) and sonicated and centrifuged again. The supernatants were loaded onto a HisTrap HP 5 mL column prepacked with Ni Sepharose™ (GE Healthcare) and washed with buffer (4 M guanidine hydrochloride, 20 mM NaH_2PO4, 30 mM imidazole, 300 mM NaCl, pH 7.5). The peptides were then eluted with elution buffer (20 mM Tris-HCl, 100 mM NaCl, 1 M imidazole, 4 M guanidine hydrochloride, pH 7.5). Finally, the peptides were purified by C_4 semi preparative reverse phase HPLC using an Agilent 1200 instrument (Agilent) equipped with a Delta-Pak C4 column (25 mm i.d. x 100 mm L, Waters) and a variable wavelength detector set at 220 nm. The mobile phase was 0.1% trifluoroacetic acid (TFA) in water (A) and 0.086% TFA in 80% acetonitrile / 20% water (B). A gradient of 2-100% B in A over 60 min and a flow rate of 8 mL/min were used. The masses of the purified peptides were determined by MALDI-TOF MS on a Voyager DE-STR Biospectrometry Workstation using α-cyano-4-hydroxycinnamic acid as matrix at the Mass Spectrometry Laboratory of the University of Illinois at Urbana Champaign.

Overexpression and purification of His_6-MBP-ElxP

Electrocompetent *E. coli* BL21 (DE3) Rosetta 2 cells were transformed with pHis_6-MBP-ElxP and a single colony was resuspended in 100 µL of LB and plated on an LB agar plate containing 50 µg/mL kanamycin and 12.5 µg/mL chloramphenicol and grown overnight at 37 °C. The colonies were scraped-off and resuspended in 2 mL of LB medium and used to inoculate a 2 L culture. The culture was incubated at 37 °C until OD_{600} = 1.0. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and the culture was incubated for an additional 16 h at 18 °C. The cells were harvested by centrifugation and the cell pellet was resuspended in 20 mL of lysis buffer (50 mM Na_2HPO4, 500 mM NaCl, 20 mM imidazole, 5 mg/mL lysozyme, pH 7.5) After incubation at 4 °C for 45 min, the cells were lysed using French-press. The suspension was centrifuged for 45 min at 16,000 × g at 4 °C and the supernatant was filtered through a 0.45 µm syringe-tip filter (Millipore). The protein was purified by IMAC using an ÄKTApurifier (Amersham Biosciences, GE Healthcare) equipped with a HisTrap HP 5 mL column prepacked with Ni Sepharose™ (GE Healthcare). After loading and washing the column with 15 column volumes (CV) of buffer A (20 mM Na_2HPO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4), a gradient of 0-100% buffer buffer B (20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole, pH 7.4) in buffer A over 20 CV was used to elute the proteins. The fractions containing His_6-MBP-ElxP, as confirmed by SDS-PAGE analysis, were concentrated using an Amicon Ultracel 50k filter (Millipore). Imidazole was removed by using a PD-10 desalting column (GE Healthcare) and the protein was eluted from the column using an elution buffer containing glycerol (50 mM HEPES, 300 mM NaCl, 20%

glycerol, pH 7.5). Aliquots were frozen in liquid N_2 and stored at -80 °C. An aliquot containing His_6-MBP-ElxP was incubated with TEV protease (0.1 mg TEV/mg protein) in the presence of TCEP (1 mM) at 4 °C overnight. The suspension was incubated with Ni-NTA agarose beads (Qiagen) for 30 min with shaking at 4 °C. The supernatant was recovered and aliquots were frozen in liquid N_2 and stored at -80 °C.

Cloning and coexpression of *elxA*, *elxB*, and *elxC*

The *elxA* gene was amplified by PCR from *S. epidermidis* 15X154 genomic DNA using a forward primer containing a *BamH*I restriction site (elxA.BamHI.F) and a reverse primer containing a *Not*I site (elxA.NotI.R). The PCR product and the vector pRSFDuet-1 (Novagen,) were digested with restriction endonucleases *BamH*I and *Not*I (New England Biolabs) and ligated using T4 DNA ligase (New England Biolabs) to produce the plasmid pDuetHis_6-ElxA encoding ElxA fused at its N-terminus to a His_6-tag.

A sequence optimized synthetic *elxB* gene (Geneart) was amplified by PCR using a forward primer containing a *Bgl*II restriction site (elxB.Bglll.F) and a reverse primer containing a *Xho*I restriction site (elxB.XhoI.R). The PCR product and the vector pDuetHis_6-ElxA were digested with restriction endonucleases *Bgl*II and *Xho*I (Invitrogen) and ligated using T4 DNA ligase (New England Biolabs) to produce the plasmid pHis_6-ElxA.ElxB encoding for ElxA fused at its N-terminus to a hexahistidine tag and for untagged ElxB.

The *elxC* gene was amplified by PCR from *S. epidermidis* 15X154 genomic DNA using a forward primer containing an *Nde*I restriction site (elxC.NdeI.F) and a reverse primer containing a *Xho*I site (elxC.XhoI.R). The PCR product and the vector pACYCDuet-1 (Novagen) were digested with restriction endonucleases *Nde*I and *Xho*I (Invitrogen) and ligated using T4 DNA ligase (New England Biolabs) to produce the plasmid pElxC.

Electrocompetent *E. coli* BL21 (DE3) cells were co-transformed with pHis_6-ElxA.ElxB and pElxC and a single colony was inoculated in 5 mL of LB medium containing 50 µg/mL kanamycin and 12.5 µg/mL chloramphenicol and grown for 12 h with shaking. An aliquot of 2 mL was used to inoculate 200 mL LB cultures containing the same antibiotics followed by incubation at 37 °C until OD_{600} = 0.7. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cultures were incubated for 16 h at 18 °C. The cells were harvested by centrifugation and resuspended in lysis buffer (20 mM NaH_2PO4, 500 mM NaCl, 20 mM imidazole, 2 mg/mL lysozyme, pH 7.4). After sonication and centrifugation, the supernatant was loaded onto a HisTrap HP 5 mL column prepacked with Ni Sepharose™ (GE Healthcare) and washed with buffer (20 mM NaH_2PO4, 500 mM NaCl, 20 mM imidazole, pH 7.4). The peptide was then eluted with elution buffer (20 mM NaH_2PO4, 500 mM NaCl, 500 mM imidazole, pH 7.4) and loaded onto a Vydac® C_4 reverse phase solid phase extraction column (214SPE1000, Discovery Sciences). The column was washed with 5% methanol in 0.1% TFA/water to remove impurities and the peptide was eluted with 80% methanol in 0.1% TFA/water and 80% acetonitrile in 0.1% TFA/water. After lyophilization, the peptide was resuspended in buffer (50 mM HEPES, 500 mM NaCl, pH 7.5) and incubated with His_6-MBP-ElxP at room temperature overnight to cleave the leader peptide. Ni-NTA agarose (Qiagen) was added and the supernatant was recovered and analyzed by MALDI-TOF MS.

Synthesis of substrate analogue and products

The peptides Pyr-AAIVK, D-Lac-AAIKV, L-Lac-AAIVK, ASIVK, Pyr-AAIVKBBIKA, and AAIVKBBIKA were synthesized by Fmoc-based SPPS using a PS3 peptide synthesizer

(Protein Technologies). Fmoc groups were removed during the deprotection steps with 20% piperidine in DMF. Coupling of the amino acids was performed using DMF as solvent and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) and 0.4 M N-methylmorpholine (NMM) as activating reagents. Coupling reactions of pyruvic acid or L/D-lactic acid were performed using hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC) as activating reagents. Coupling of unprotected Ser in ASIVK was performed using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT) and N,N'-diisopropylethylamine (DIPEA) as activating reagents. ADhaIVK was produced after solid phase dehydration of Ser with large excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and CuCl as described elsewhere (Jiménez, et al., 2002). D-Lac-ADhaIKV and L-Lac-ADhaIVK were produced after coupling of L/D-lactic acid to ADhaIVK as described above. Peptides were cleaved from the resins using a mixture of TFA/water/phenol (90:5:5) or TFA/ triisopropylsilane (95:5) in the case of D/L-Lac-ADhaIKV. The cleavage solutions were concentrated by rotary evaporation and the peptides were precipitated from the solution with cold diethyl ether. The peptide was purified by C_{18} semi preparative reverse phase HPLC using an Agilent 1200 instrument (Agilent) equipped with an Eclipse XDB-C18 column (9.4 mm i.d. x 250 mm L, Agilent). A gradient of 2-30% B (acetonitrile) in A (0.1% formic acid) in 30 min was used. The masses of the purified peptides were determined by ESI-MS using a Waters ZMD quadrupole Instrument (Waters) at the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign.

Determination of stereochemical configuration of N-terminal Lac

After incubation of Pyr-AAIVK (1 mM) with ElxO (10 µM) and NADPH (1 mM) in reaction buffer (100 mM HEPES, 500 mM NaCl, pH 7.5), the reaction mixture and the synthetic peptides D-Lac-AAIKV and L-Lac-AAIVK were analyzed individually or combined by HPLC using an Agilent 1200 instrument (Agilent) equipped with a Synergi Fusion-RP column (4.6 mm i.d. × 150 mm L, Phenomenex). A gradient of 0-70% B (methanol) in A (0.1% formic acid in water) over 30 min was used and absorbance at 210 nm was monitored.

Epilancin 15X (30 µM) was incubated with trypsin (5 µM, Sigma-Aldrich) in buffer (50 mM HEPES, pH 7.5) at room temperature for 3 h. The resulting proteolyzed peptide and the synthetic peptides D-Lac-ADhaIKV and L-Lac-ADhaIVK were analyzed individually or combined by LC-MS using a Waters SYNAPT^{TM} mass spectrometry system equipped with a ACQUITY UPLC®, an ESI ion source, a quadrupole time-of-flight detector (Waters) scanning a range 570 - 573 Da, and a ACQUITY Bridged Ethyl Hybrid (BEH) C18 column (2.1 mm i.d. × 50 mm L, 1.7 µm, Waters). A gradient of 9-12% B (0.1% formic acid in acetonitrile) in A (0.1% formic acid in water) over 15 min was used.

Production and purification of lacticin 481

Production of lacticin 481 was performed as suggested before (Piard, et al., 1990; Piard, et al., 1992). In brief, *L. lactis* subsp. *lactis* CNRZ481 was grown for 9 hours in EG'P media (Elliker broth medium without gelatin and supplemented with sodium β-glycerophosphate), maintaining the culture pH at 5.5 by adding aliquots of 3 M ammonium hydroxide (Sigma-Aldrich). In previous studies, the purification of lacticin 481 and other lantibiotics have been performed using a complex combination of chromatographic techniques and/or organic solvent extractions with low recovery yields and high amounts of oxidized products that are difficult to separate (Allgaier, et al., 1986; Bailey and Hurst, 1971; Cheeseman and Berridge, 1957; Piard,

et al., 1992). To purify lacticin 481, the culture supernatant was heat-treated to deactivate proteases and the bacteriocin was concentrated with an ammonium sulfate precipitation step saturating the solution at 60% at 4 °C. After resuspension of the pellet in a Sorensen buffer (pH 6.0), lacticin 481 was further purified by C4 reverse phase solid phase extraction (214SPE1000, Discovery Sciences) using 20 mM ammonium acetate and acetonitrile as eluting solvents. The bacteriocin was eluted with 32% acetonitrile, while most of the impurities were eluted at lower concentrations of organic solvent. The fraction containing lacticin 481 was concentrated under vacuum and 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 30-40% B (acetonitrile) in A (20 mM, ammonium acetate, pH 5.5) over 30 minutes was used. A yield of 1.1 mg of lacticin 481 per liter of culture was obtained, compared with a yield of 0.24 mg per liter reported previously (Piard, et al., 1992). The identity and purity of lacticin 481 were confirmed by MALDI-TOF MS.

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