Supplementary Information

Substrate specificity of the lanthipeptide peptidase ElxP and the oxidoreductase ElxO

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Markov chain Monte Carlo phylogenetic tree analysis

The LanP sequences were aligned in ClustalX[1] using default parameters with iteration at each alignment step, and the alignments were manually fine-tuned afterwards. Bayesian inference was used to calculate posterior probability of clades utilizing the program MrBayes[2] (version 3.2). Final analyses consisted of two sets of eight chains each (one cold and seven heated), run to reach a convergence with standard deviation of split frequencies <0.005. Posterior probabilities were averaged over the final 75% of trees (25% burn in). The analysis utilized a mixed amino acid model with a proportion of sites designated invariant, and rate variation among sites modeled after a gamma distribution divided into eight categories, with all variable parameters estimated by the program based on BioNJ starting trees. Accession numbers are listed in the Supplementary Table 1.

Expression and purification of MBP-ElxP

The cloning of the gene encoding ElxP is described elsewhere.[3] An aliquot of 50 ng of *pelB-mbp-elxP-pET28b* was used to transform 50 µL of electrocompetent *Escherichia coli* BL21 (DE3) Rosetta 2 cells following standard procedures. After the incubation period, cells were plated on LB agar (LBA) plates supplemented with kanamycin (kan, 25 µg mL^{-1}) and chloramphenicol (cam, 12.5 µg mL^{-1}) and grown at 37 °C overnight. A single colony was used to inoculate 1 mL of fresh LB supplemented with kan and cam and 1 µL was plated for a second time on LBA plates supplemented with kan and cam. Plates were kept at 37 °C overnight. For overexpression, 1 mL of LB supplemented with kan and cam was used to scrape the cells out of the overnight LBA plates to be used as initial inoculum. Overexpression was performed in 6 L of LB supplemented with kan and cam with a starter OD_{600} of 0.025. Cultures were incubated at 37 °C, 250 rpm until the OD_{600} reached ~1.0. At this OD, cultures were chilled on ice for 15 min and protein expression was induced with 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Protein overexpression was carried out at 18 °C, 250 rpm for 16 h. The cells were harvested (6976 xg, 20 min, 4 °C), resuspended in 50 mL of lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% (v/v) glycerol, 1 mM EDTA) and lysed using an EmulsiFlex-C3 homogenizer with a pressure of less than 500 bar. To remove cell debris, the lysed fraction was centrifuged (22,789 xg, 4 °C, 30 min) and the supernatant was cleared through a 0.45 µm syringe-tip filter (Millipore). The protein was purified by affinity chromatography using an ÄKTA purifier (GE healthcare) equipped with an MBPTrap HP 5 mL column pre-packed with Dextrin Sepharose™ (GE healthcare) according to the manufacturer's protocol. After loading the supernatant into the column pre-equilibrated with lysis buffer, the column was washed with lysis buffer until a stable baseline based on absorbance at 280 nm for the absorbance was reached. Protein was eluted with a linear gradient from 0–100% (v/v) of elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 10 mM maltose) in lysis buffer over 30 min. Collected fractions were analyzed by SDS-PAGE using a 4–20% TGX mini-protean gel (BioRad) and visualized by Coomassie staining. Fractions containing the desired MBP-ElxP were collected and desalted using gel filtration chromatography on a HiLoad 16/60 column packed with Superdex 200 PG eluting with 1 mL min^{-1} of gel filtration buffer (300 mM NaCl, 20 mM Tris-HCl pH 8.0, 10% (v/v) glycerol). Protein was concentrated using an Amicon Ultra centrifuge tube of 30 kDa molecular weight cut off (MWCO) (3,488 xg, 30 min, 4 °C) (Millipore). The concentration of the purified protein was determined spectrophotometrically using a calculated molar extinction coefficient of 113,485 M^{-1} cm^{-1} and a molecular weight of 80.504 kDa. Purity was assessed by

SDS-PAGE analysis (Supplementary Figure 1). Aliquots were frozen in liquid nitrogen for future use and stored at –80 °C.

Cloning, expression, and purification of His_6-ElxA and mutant variants

The cloning of the gene encoding ElxA into *pET28b* is described elsewhere.[3] The ElxA leader peptide variants were constructed using QuikChange mutagenesis following a previously described method.[4] The *his_6-elxA-pET28b* was used as a template to introduce the different mutations by PCR using the corresponding primer pair listed in the Supplementary Table 5. A typical PCR reaction consisted of 1X HF Buffer, 0.2 mM DNTP, 1 μM forward and reverse primers, 10 ng template DNA, 3% (v/v) DMSO and 0.02 U μL^{-1} Phusion polymerase (NEB). After PCR, reactions were incubated at 37 °C for 2 h with DpnI (5 U). After treatment with DpnI, samples were purified using the QIAquick PCR purification kit (Qiagen). An aliquot of 10 μL was used to transform *E. coli* DH5α cells using the heat shock method and cells were plated on LBA plates supplemented with kan (50 μg mL^{-1}). Plates were incubated at 37 °C overnight. A single colony was inoculated in LB supplemented with kan, grown at 37 °C overnight, and plasmid was extracted using the QIAprep spin mini prep kit (Qiagen). The desired mutations were verified by DNA sequencing.

Overexpression and purification of wild type ElxA and mutant variants was carried out using a previously described method with minor modifications.[3] An aliquot of 50 ng of recombinant DNA from wild type ElxA and each of the mutants described above was used to transform 50 μL of electrocompetent *E. coli* BL21 (DE3) cells and plated on LBA supplemented with kan (50 μg mL^{-1}) at 37 °C overnight. A single colony was used to inoculate a starter inoculum of LB supplemented with kan and incubated at 37 °C for 12 h. After the incubation period, 6 L of Terrific Broth (TB) supplemented with kan, and glycerol (4 mL L^{-1}), were inoculated with the starter culture to obtain an initial OD_{600} of 0.025. Flasks were incubated at 37 °C, 250 rpm, and peptide expression was induced at 18 °C, 250 rpm for 18 h with 1.0 mM IPTG when the OD_{600} reached 1.0. Cells were harvested by centrifugation (6,976 xg, 20 min, 4 °C), resuspended in 30 mL of LanA Buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4 pH 7.5, 500 mM NaCl and 0.5 mM imidazole) and lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 s pause, 15 min). Cell debris was removed by centrifugation (22789 xg, 30 min, 4 °C) and supernatant was filtered through a 0.45 μm syringe filter unit. Purification was carried out using a 5 mL HisTrap column pre-packed with Ni Sepharose™. After loading the supernatant into the column, the column was washed with 10 column volumes (CV) of LanA Buffer 1 followed by 10 CV of LanA Buffer 2 (4 M guanidine hydrochloride, 20 mM NaH_2PO_4 pH 7.5, 300 mM NaCl and 30 mM imidazole) to remove any non-specifically bound proteins, followed by peptide elution in 3 CV of LanA Elution Buffer (4 M guanidine hydrochloride, 20 mM Tris HCl pH 7.5, 100 mM NaCl, 1 M imidazole). To remove excess salts, the peptide was purified by reversed phase high performance liquid chromatography (RP-HPLC) using a C_4 Waters Delta Pak cartridge column with a linear gradient of 2% (v/v) of solvent A [80% (v/v) MeCN, 20% (v/v) H_2O, 0.086% (v/v) trifluoroacetic acid (TFA)] in solvent B [0.1% (v/v) TFA in H_2O] to 75% (v/v) solvent A over 30 min. Fractions were analyzed for the desired peptide by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Fractions containing peptide were freeze dried using a lyophilizer (Labconco) and stored at −20 °C. The purity of each peptide was assessed by analytical HPLC.

Cloning, expression, and purification of His_6-NisA and mutant variants

The cloning of the gene encoding NisA into *pRSF Duet-1* is described elsewhere.[5] The NisA leader peptide variants were constructed using QuikChange mutagenesis. The *his_6-nisA-pRSF Duet-1* plasmid was used as a template to introduce the different mutations by PCR using the corresponding primer pair listed in the Supplementary Table 5. PCR conditions, overexpression, and purification of peptides were performed as described above.

Determination of ElxP kinetic parameters

The kinetic parameters of MBP-ElxP were determined using an HPLC based assay following a previously described method.[6] The peptidase activity of tagged ElxP was assayed in a 100 μL reaction mixture containing 50 mM Tris pH 8.0, and various concentrations of wild type His_6-ElxA or mutant variants. The reaction was started by adding MBP-ElxP to a final concentration such as to consume less than 10% of the initial substrate concentration in the time frame of the assay. The enzyme concentration ranged from 0.25 μM to 1 μM. The reaction was incubated at room temperature and quenched in 0.1% (v/v) TFA and 5 mM

tris(2-carboxyethyl)phosphine (TCEP) at different time points. The reactions were loaded on a Hypersil Gold C_4 (250 x 4.6 mm, 5 μ) analytical column (Thermo Fisher Scientific) connected to an Agilent 1260 Liquid Chromatography (HPLC) system (Agilent Technologies). Product formation was detected by monitoring the increase in the peak area of the leader peptide at 220 nm (Supplementary Figure 2). The leader peptide was separated from the unmodified core and precursor peptide using a linear gradient from 2% to 75% (v/v) of solvent A [80% (v/v) MeCN, 20% (v/v) H_2O, 0.086 % (v/v) TFA] in solvent B [0.1% (v/v) TFA in H_2O] over 30 min at a flow rate of 1 mL min^{-1} at room temperature. The concentration of the leader peptide was calculated by converting the area under the leader peptide peak to leader peptide concentration using a calibration curve made from purified leader peptide (Supplementary Figure 3). Rates of leader peptide formation were then plotted against substrate concentration and the resulting graph was fit to the Michaelis-Menten equation. Values were plotted as the average and standard error of two independent experiments. Reactions were performed with tagged enzymes and substrates unless otherwise noted.

Synthesis of ElxO substrate analogs

Peptides were synthesized on a 0.1 mmol or 0.15 mmol scale by Fmoc-based SPPS using a PS3 peptide synthesizer (Protein Technologies) or an Apex SC-396 Peptide Synthesizer (Advanced Chemtech). Fmoc groups were removed during the deprotection steps with 20% (v/v) piperidine in *N*,*N*-dimethylformamide (DMF) for 3 × 3 min. Coupling of the amino acids was performed for 1–1.5 h with 0.5 or 0.75 mmol (five equiv.) of each Fmoc-AA and using DMF as solvent and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) with 0.4 M *N*-methylmorpholine (NMM) or hydroxybenzotriazole (HOBt) with diisopropylcarbodiimide (DIC) as activating reagents. Coupling reactions of pyruvic acid or α-ketobutyric acid were performed twice for 1–1.5 h using 5 equiv. of acid in the presence of HOBt and DIC. After completion of synthesis, peptidyl-resins were washed with DMF and ethanol, and dried under reduced pressure. Peptides were cleaved from the resins using a mixture of TFA/water/phenol (90:5:5) for 2 h. For Pyr-MAIVK a cleavage cocktail containing TFA/water/phenol/thioanisole /mercaptoethanol (82.5:5:5:5:2.5) was used, and for Pyr-YAIVK, Pyr-TAIVK, Pyr-RAIVK, and Obu-RAIVK a cleavage mixture of TFA/water/phenol/thioanisole (85:5:5:5) was used. The cleavage solutions were evaporated in a rotary evaporator to remove TFA and the peptides were precipitated from the solution with cold diethyl ether. The precipitated materials were resuspended in 0.1% (v/v) TFA in acetonitrile/water (50:50) and lyophilized to dryness. The synthesis of Glx-AAIVK was performed by oxidation of SAAIVK (5 mM) with sodium periodate (10 mM) in sodium phosphate buffer (40 mM, pH 7.5) for 5 min, followed by quenching with sodium sulfite (40 mM).[7] The peptides were purified by semi preparative reversed phase HPLC using an Agilent 1200 instrument equipped with an Eclipse XDB-C18 column (9.4 mm x 250 mm, Agilent) or a Synergi Fusion-RP column (9.4 mm × 150 mm, Phenomenex) and a variable wavelength detector set at 220 nm. The mobile phase was 0.1% (v/v) formic acid in water (solvent A) and acetonitrile (solvent B). A gradient of 2-30% (v/v) solvent B in solvent A and a flow rate of 4 mL min^{-1} were used. The masses of the purified peptides were determined by ESI-MS using a Waters ZMD quadrupole Instrument at the Mass Spectrometry Laboratory of the University of Illinois at Urbana-Champaign.

Production of dihydrolactocin S

Synthetic lactocin S (50 μM), obtained from Prof. J. Vederas (University of Alberta),[8] was incubated with His_6-ElxO (50 μM) and NADPH (10 mM) in assay buffer (100 mM HEPES, 500 mM NaCl, pH 7.5) at room temperature for 12 h. The formation of reduced peptide was confirmed by LC-MS using a Waters SYNAPT™ mass spectrometry system equipped with a ACQUITY UPLC®, an ESI ion source, a quadrupole time-of-flight detector, and a ACQUITY Bridged Ethyl Hybrid (BEH) C8 column (2.1 mm × 50 mm, 1.7 μm, Waters). A gradient of 3-97% (v/v) B (0.1% (v/v) formic acid in methanol) in A (0.1% (v/v) formic acid in water) over 12 min was used.

Determination of the ElxO x-ray crystal structure

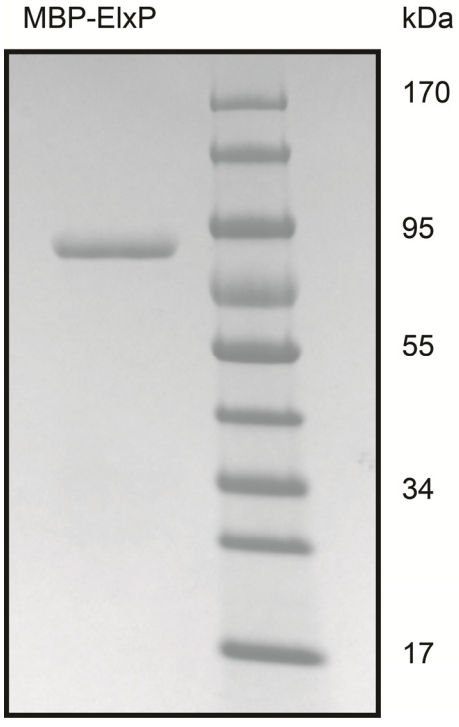
Apo-ElxO was concentrated to a final concentration of 0.4 mM by centrifugal filters, and incubated with five-fold excess of freshly prepared NADPH. The solution was incubated on ice for 2 h prior to crystallization. Crystals of the ElxO wild type enzyme in complex with NADPH were grown using the hanging drop vapor diffusion technique. Commercially available sparse matrix screens were used to identify initial crystallization conditions using the Hampton Natrix crystallization screen. The initial

condition (Natrix 39) was optimized to 0.1 M ammonium acetate, 0.02 M magnesium chloride, 0.05 M HEPES-Na (pH 7.5), 8% (v/v) PEG 800, and 5% (v/v) glycerol. Crystals were obtained by adding 1 μL of the protein to an equal volume of the crystallization mother liquor equilibrated against the same solution at 9 °C. Crystals were vitrified by soaking in crystallization condition supplemented with 30% (v/v) glycerol prior to direct immersion in liquid nitrogen. All X-ray diffraction data were collected at the Life Sciences Collaborative Access Team (Sector-21) Argonne National Laboratory (Lemont, IL).

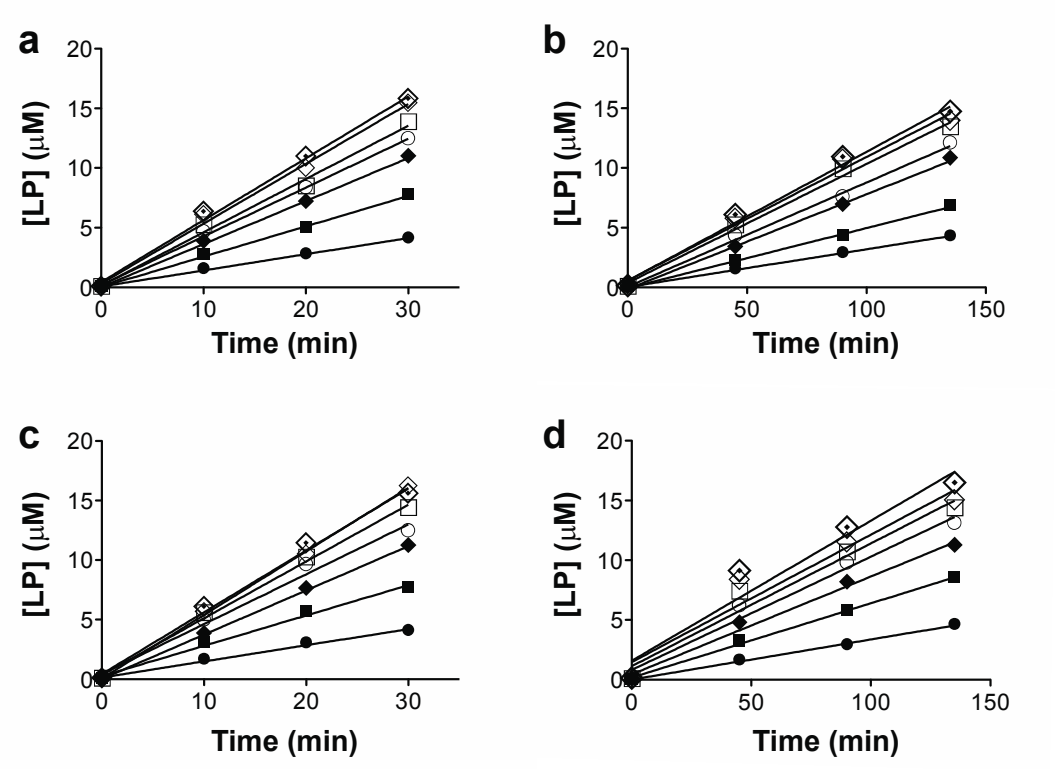
The diffraction data was indexed and scaled using HKL2000[9] to a limiting resolution of 1.85 Å. The coordinates from three models (PDB ids: 2HQI, 2UVD, and 2CFC) were superimposed to generate a search model for molecular replacement. All water and ligand atoms from the search model were deleted, and non-conserved amino acid side chains reduced to alanine. A five-fold redundant native dataset for the ElxO-NADPH complex crystal was used.

Following rigid body refinement of the initial MR solution, phases from the resultant model were further improved by two fold density averaging allowing for a significant portion of the model to be manually rebuilt using XtalView.[10] Further manual fitting was interspersed with automated rebuilding using ARP/wARP[11] and rounds of refinement using REFMAC5.[12, 13] Cross-validation, using 5% of the data for the calculation of the free R factor,[14] was utilized throughout the model building process in order to monitor building bias. Although clear density for NADPH could be observed prior to crystallographic refinement, the ligand was manually built into the model only after the free R factor dropped below 30%. The stereochemistry of the models was routinely monitored throughout the course of refinement using PROCHECK.[15] Crystallographic data summary for ElxO is presented in Supplementary Table 4.

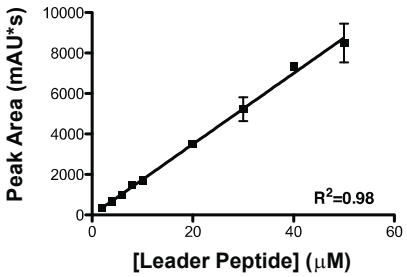
Supplementary Figures and Tables:



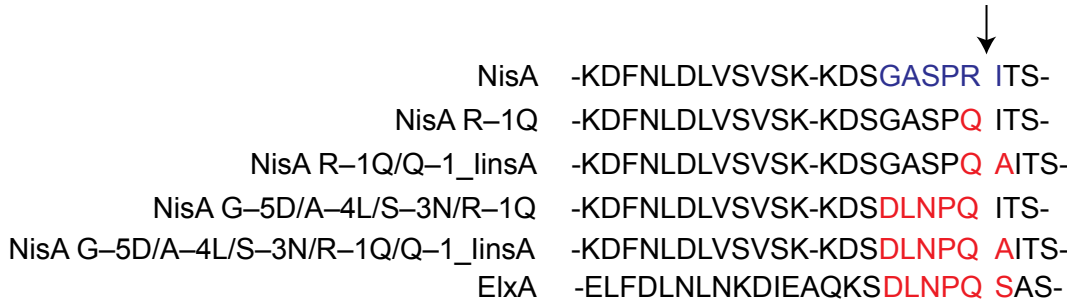
Supplementary Figure 1. SDS-PAGE analysis of purified recombinant MBP tagged ElxP.



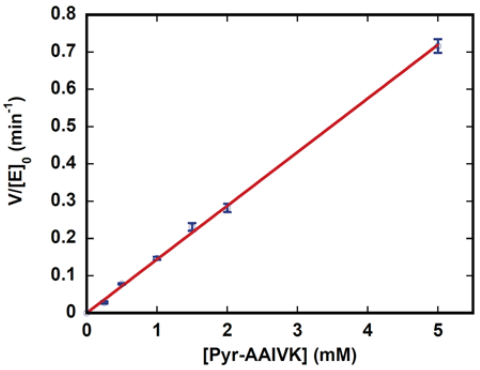
Supplementary Figure 2. Time dependent formation of the *N*-terminal leader peptide upon treatment of His_6-ElxA and its variants with MBP-ElxP. a) His_6-ElxA, b) His_6-ElxA Q–1A, c), His_6–ElxA P–2A, and d) His_6-ElxA L–4A were incubated at different concentrations (● 50 μM, ■ 100 μM, ◆ 150 μM, ○ 200 μM, □ 250 μM, ◇ 300 μM, ⟐ 350 μM) with MBP-ElxP and leader peptide formation (LP) was monitored at different time points by RP-HPLC. Data was fit to a linear regression to obtain enzymatic rates.



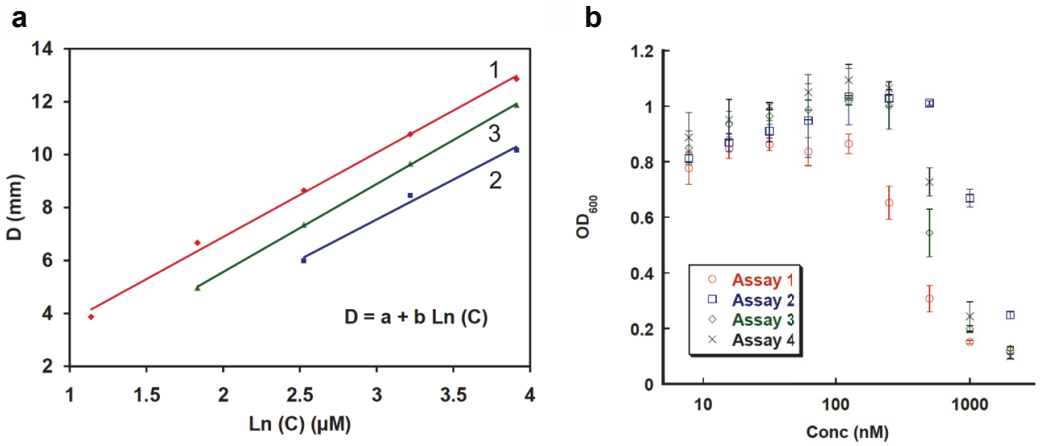
Supplementary Figure 3. ElxA leader peptide standard curve. The absorbance of different concentrations of purified His tagged ElxA leader peptide was determined by analytical HPLC using absorbance at 220 nm for detection. Linearity was observed up to 50 μM leader peptide. Data plotted represents the average of two independent runs and error bars represent the standard error associated with the measurements.



Supplementary Figure 4. Leader peptide sequences of NisA variants. The cleavage recognition sequence of NisA is shown in blue. Amino acid mutations in the NisA variants are shown in red. ElxA leader peptide sequence is shown for reference. LanP cleavage site is shown with an arrow.

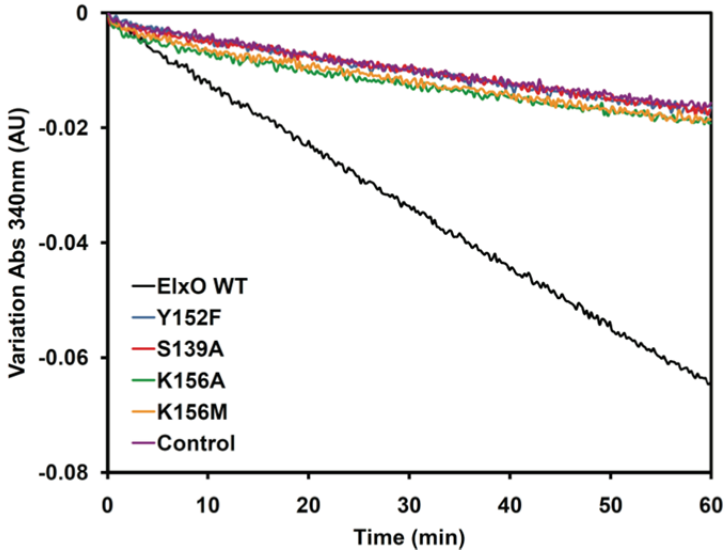


Supplementary Figure 5. Dependence of the reaction rate of reduction by His_6-ElxO on the Pyr-AAIVK concentration using 2.5 mM NADPH as co-substrate. Saturation of the enzyme with the peptide substrates was not achieved even at high peptide concentration (5 mM).



	Critical conc. (μM)	MIC (μM)
Assay 1	0.8	0.5
Assay 2	1.6	2.0
Assay 3	1.4	1.0
Assay 4	N.D.	1.0

Supplementary Figure 6. a) The sizes of the inhibition zones of the serial dilution agar diffusion bioactivity assays (Figure 6) were determined and fit to a linear model to establish the critical concentration (D = 0).[16] Assay 1: sample containing enzymatically synthesized dihydrolactocin S, assay 2: control sample containing lactocin S but lacking ElxO, assay 3: control sample containing lactocin S but lacking cofactor, assay 4: control sample containing lactocin S but lacking enzyme and cofactor. N.D., not determined. b) Apparent minimal inhibitory concentrations (MIC) determined by a serial dilution bioactivity assay in liquid media of a solution containing dihydrolactocin S produced enzymatically compared with the control samples containing lactocin S. The MIC values were determined based on the smallest concentration of antibiotic that caused OD_{600} to be less than 0.3.



Supplementary Figure 7. Consumption of NADPH (2.5 mM) over time upon reduction of Pyr-AAIVK (5.0 mM) by wild-type His_6-ElxO (black) or the mutants Y152F (blue), S139A (red), K156A (green), or K156M (orange) compared with a control sample lacking peptide (purple).

Supplementary Table 1. Accession numbers of proteases used to construct phylogenetic tree (Figure 2b)

Protease	Accession Number	Organism
CylA	AFJ74725.1	*Enterococcus faecalis*
LicP	AAU42937.1	*Bacillus licheniformis* DSM 13
NisP	ADJ56357.1	*Lactococcus lactis* subsp. lactis
NiqP	BAG71484.1	*Lactococcus lactis*
SlvP	AEX55163.1	*Streptococcus salivarius*
EpiP	CAA44257.1	*Staphylococcus epidermidis*
GdmP	ABC94907.1	*Staphylococcus gallinarum*
BsAa associated	BAB95626.1	*Staphylococcus aureus* subsp. aureus MW2
BsaA1 associated	YP_005737288.1	*Staphylococcus aureus* subsp. ED133
NsuP	ABA00872.1	*Streptococcus uberis*
ElxP	AFN69433.1	*Staphylococcus epidermidis*
EciP	CAA74349.1	*Staphylococcus epidermidis*
PepP	CAA90024.1	*Staphylococcus epidermidis*
Bsn5 associated	YP_004206152.1	*Bacillus subtilis* BSn5

Supplementary Table 2. MBP-ElxP cleavage of wild type and His_6-ElxA mutant peptides*

Peptide	Full length (calcd)	Leader (calcd)	Unmodified core (calcd)	Result
His_6-ElxA wt	8161 (8162)	4865 (4864)	3315 (3316)	+++
Q–1A	8107 (8105)	4809 (4807)	3315 (3316)	+
P–2A	8134 (8136)	4838 (4838)	3314 (3316)	++
N–3A	8118 (8119)	4822 (4821)	3313 (3316)	++
L–4A	8119 (8120)	4822 (4822)	3314 (3316)	+
D–5A	8115 (8118)	4819 (4820)	3313 (3316)	+
F–19A	8086 (8086)	4789 (4788)	3315 (3316)	++
D–18A	8118 (8118)	4818 (4820)	3314 (3316)	+++
L–17A	8118 (8120)	4823 (4822)	3315 (3316)	+++
N–16A	8119 (8119)	4819 (4821)	3314 (3316)	+++

* *Observed masses are shown with the calculated mass in parenthesis. Reactions were analyzed by MALDI-TOF MS. Results are based on the amount of substrate left after the assay based on normalized ion intensities. Complete cleavage (+++), partial cleavage (++), marginal cleavage (+).*

Supplementary Table 3. Peptides tested for reduction by ElxO; all peptides were substrates as confirmed by LC-MS analysis.

Entry		Substrate	
		Pyr-GAIVK	Pyr-IAIVK
19	Non-polar	Pyr-VAIVK	Pyr-FAIVK
20	Polar	Pyr-TAIVK	Pyr-YAIVK
21	Basic	Pyr-HAIVK	
22	Pyr analogs	Pyr-ASIVK	
23	Obu analogs	Obu-AAAVK	Obu-AAIAK
		Pyr-AAVLK	Pyr-LGPAIK
24	Lantibiotic analogs	Pyr-APVLA	Obu-AGPAIR
		Pyr-BPVLAAVAVAKKK	

B stands for L-2-aminobutyric acid, Pyr stands for pyruvyl, and Obu stands for 2-oxobutyryl

Supplementary Table 4. Data collection, phasing and refinement statistics

	ElxO-NADPH	Y152F ElxO-NADPH
PDB Codes		
Data collection		
Space Group	C2	C2
a, b, c (Å), β (°)	131.5, 57.2, 82.3, 121.0	131.4, 52.3, 82.4, 121.3
Resolution (Å)[1]	50-1.9 (1.97-1.9)	50-1.85 (1.92-1.85)
R_{sym} (%)[2]	6.7 (22.3)	5.4 (26.8)
I/σ(I)	23.4 (6.0)	29.8 (4.2)
Completeness (%)	97.8 (86.0)	95.4 (78.4)
Redundancy	6.1 (5.2)	5.0 (3.9)
Refinement		
Resolution (Å)	25.0-1.9	25.0-1.85
No. reflections	35,510	40,626
R_{work} / R_{free}[4]	16.1/19.6	17.9/20.5
Number of atoms		
Protein	3842	3840
NADP(H)	96	96
Water	409	328
B-factors		
Protein	24.5	28.8
NADP(H)	18.2	22.4
Water	33.3	35.2
R.m.s deviations		
Bond lengths (Å)	0.007	0.007
Bond angles (°)	1.23	1.19

1. Highest resolution shell is shown in parenthesis.
2. $R_{sym} = \Sigma |(I_i - \langle I_i \rangle | \Sigma I_i$ where I_i = intensity of the *i*th reflection and $\langle I_i \rangle$ = mean intensity.
3. Mean figure of merit (acentric and centric)
4. R-factor = $\Sigma(|F_{obs}|-k|F_{calc}|)/\Sigma |F_{obs}|$ and R-free is the R value for a test set of reflections consisting of a random 5% of the diffraction data not used in refinement.

Supplementary Table 5. Oligonucleotides used in this study

Name	5' Sequence 3'
ElxA Q-1A F	GCA CAA AAA AGT GAC CTA AAT CCG GCA TCA GCT AGT ATT GTT AAA ACA AC
ElxA Q-1A R	GTT GTT TTA ACA ATA CTA GCT GAT GCC GGA TTT AGG TCA CTT TTT TGT GC
ElxA P-2A F	GCA CAA AAA AGT GAC CTA AAT GCG CAA TCA GCT AGT ATT GTT AAA ACA AC
ElxA P-2A R	GTT GTT TTA ACA ATA CTA GCT GAT TGC GCA TTT AGG TCA CTT TTT TGT GC
ElxA N-3A F	ATC GAG GCA CAA AAA AGT GAC CTA GCA CCG CAA TCA GCT
ElxA N-3A R	ACT TTT TTG TGC CTC GAT ATC TTT ATT AAG ATT TAA ATC AAA TAA TTC TTT TTT C
ElxA L-4A F	ATC GAG GCA CAA AAA AGT GAC GCA AAT CCG CAA TCA GCT
ElxA L-4A R	ACT TTT TTG TGC CTC GAT ATC TTT ATT AAG ATT TAA ATC AAA TAA TTC TTT TTT C
ElxA D-5A F	ATC GAG GCA CAA AAA AGT GCA CTA AAT CCG CAA TCA GCT
ElxA D-5A R	ACT TTT TTG TGC CTC GAT ATC TTT ATT AAG ATT TAA ATC AAA TAA TTC TTT TTT C
ElxA F-19A F	GGC AGC CAT ATG AAA AAA GAA TTA GCT GAT TTA AAT CTT AAT AAA GAT ATC G
ElxA F-19A R	CGA TAT CTT TAT TAA GAT TTA AAT CAG CTA ATT CTT TTT TCA TAT GGC TGC C
ElxA D-18A F	CAG CCA TAT GAA AAA AGA ATT ATT TGC TTT AAA TCT TAA TAA AGA TAT CGA GGC
ElxA D-18A R	GCC TCG ATA TCT TTA TTA AGA TTT AAA GCA AAT AAT TCT TTT TTC ATA TGG CTG
ElxA L-17A F	GCC ATA TGA AAA AAG AAT TAT TTG ATG CAA ATC TTA ATA AAG ATA TCG AGG C
ElxA L-17A R	GCC TCG ATA TCT TTA TTA AGA TTT GCA TCA AAT AAT TCT TTT TTC ATA TGG C
ElxA N-16A F	GCC ATA TGA AAA AAG AAT TAT TTG ATT TAG CTC TTA ATA AAG ATA TCG AGG CAC
ElxA N-16A R	GTG CCT CGA TAT CTT TAT TAA GAG CTA AAT CAA ATA ATT CTT TTT TCA TAT GGC
NisA R-1Q F	CAT CAC CAC AGA TTA CAA GTA TTT CGC TAT GTA CAC CCG GTT G
NisA R-1Q R	CTT GTA ATC TGT GGT GAT GCA CCT GAA TCT TTC TTC GAA ACA G
NisA R-1Q Q-1_I1insA F	GAT TCA GGT GCA TCA CCA CAG GCA ATT ACA AGT ATT TC
NisA R-1Q Q-1_I1insA R	TGA TGC ACC TGA ATC TTT CTT CGA AAC AGA TAC CAA ATC
NisA G-5D A-4L S-3N R-1Q F	GTT TCG AAG AAA GAT AGC GAT CTG AAT CCA CAG ATT ACA AGT ATT TCG
NisA G-5D A-4L S-3N R-1Q R	ATC TTT CTT CGA AAC AGA TAC CAA ATC CAA GTT AAA ATC
NisA G-5D A-4L S-3N R-1Q Q-1_I1insA F	GTT TCG AAG AAA GAT TCA GAT CTG AAT CCG CAG GCA ATT ACA AGT ATT TC
NisA G-5D A-4L S-3N R-1Q Q-1_I1insA R	TGA ATC TTT CTT CGA AAC AGA TAC CAA ATC CAA GTT AAA ATC TTT TGT ACT C
ElxO. S139A.FP	GGA AAT CCG CTT AAT CCT GTA ATA GCA GAA ATA TTT ACT ATT GCT CC
ElxO. S139A.RP	GGA GCA ATA GTA AAT ATT TCT GCT ATT ACA GGA TTA AGC GGA TTT CC
ElxO.Y152F.FP	GCG GAT TTC CTT ACT CTA TAT TAT TCG GTA GCA CAA AAC ATG CTG
ElxO.Y152F.RP	CAG CAT GTT TTG TGC TAC CGA ATA ATA TAG AGT AAG GAA ATC CGC
ElxO.K156A.FP	CCT TTA GTT AAA CCA ATA ACA GCA TGT GCT GTG CTA CCG TAT AAT ATA GAG TAA GG
ElxO.K156A.RP	CCT TAC TCT ATA TTA TAC GGT AGC ACA GCA CAT GCT GTT ATT GGT TTA ACT AAA GG
ElxO.K156M.FP	CCT TTA GTT AAA CCA ATA ACA GCA TGC ATT GTG CTA CCG TAT AAT ATA GAG TAA GG
ElxO.K156M.RP	CCT TAC TCT ATA TTA TAC GGT AGC ACA ATG CAT GCT GTT ATT GGT TTA ACT AAA GG

Supplementary Table 6. Microorganisms used in this study

Strain	Relevant characteristics	Source or reference
Escherichia coli DH5α (plasmid maintenance)	λpir/ϕ80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1	*17*
Escherichia coli BL21 DE3 (protein expression)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
Escherichia coli Rosetta2 (protein expression)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pRARE2 pLysS	Novagen
Lactobacillus sake L45	Lactocin S producer strain	*18*
Pediococcus acidilactici Pac1.0	Lactocin S sensitive strain	*18*

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