Supporting Information

Production of Lantipeptides in *Escherichia coli*

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Materials

All oligonucleotides were purchased from Integrated DNA Technologies. Restriction endonucleases, DNA polymerases and T4 DNA ligase were purchased from New England Biolabs or Invitrogen. Media components for bacterial cultures were purchased from Difco laboratories. Chemicals were purchased from Fisher Scientific or from Aldrich unless noted otherwise. Endoproteinase GluC, LysC, and trypsin were purchased from Roche Biosciences or Worthington Biosciences. Factor Xa protease was purchased from New England Biolabs. Tobacco etch virus (TEV) protease was prepared as described previously.1 *E. coli* DH5α was used as host for cloning and plasmid propagation, and *E. coli* BL21 (DE3) was used as a host for co-expression.

General methods

Negative residue numbers are used for amino acids in the leader peptide counting backwards from the leader peptide cleavage site, i.e. the glycine residue immediately N-terminal to the cleavage site of ProcA1.7 is −1 and when substituted by arginine the mutant is denoted as ProcA1.7G−1R. Plasmids expressing ProcA1.7G−1R/pET15b, ProcA2.11/pET15b, ProcA3.2/pET15b, ProcA3.3/pET15b, and ProcM/pET28b were reported previously.2 All polymerase chain reactions (PCR) were carried out on a C1000™ thermal cycler (Bio-Rad). DNA sequencing was performed by the Biotechnology Center at the University of Illinois at Urbana-Champaign, using appropriate primers. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was carried out on a Voyager-DE-STR (Applied Biosystems). Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters).

Construction of pRSFDuet-1 derivatives for co-expression of ProcM and ProcA

The procM gene was cloned by PCR from ProcM/pET28b (see ref 2) and inserted into multiple cloning site-2 of the pRSFDuet-1 vector between the Ndel and KpnI restriction sites (for all primer sequences, see Table S1). The procA2.11G−1K, procA3.2TEV and procA3.3G−1K genes were generated using nested PCR with the originally reported expression plasmids2 as template. In the case of ProcA3.2, residues GVAGG at the C-terminus of the leader peptide were replaced with NLYFQ, the cleavage site recognized by TEV protease. The mutant procA genes (procA1.7G−1R, procA2.11G−1K, procA3.2TEV and procA3.3G−1K) were individually cloned into
multiple cloning site-1 of the pRSFDuet-1 vector, with the procM gene inserted in the multiple cloning site-2, between the EcoRI and NotI restriction sites. The sequences of the resulting plasmids were confirmed by DNA sequencing. The amino acid sequences of ProcA1.7G–1R, ProcA2.11G–1K, ProcA3.2TEV and ProcA3.3G–1K are shown in Figure S1.

**Molecular cloning of haloduracin expression constructs**

The pRSFDuet-1 vector was used for coexpression of hexa-histidine tagged HalA-Xa (HalA containing the Factor Xa cleavage site between the leader and core peptides) and untagged HalM protein. Multicloning site 1 (MCS1) of pRSFDuet-1 was used for hexa-histidine HalA-Xa fusions, and multicloning site 2 (MCS2) was used for production of untagged HalM. pET vectors containing HalA1-Xa, HalA2-Xa, HalM1, and HalM2 reported previously were used as templates for PCR amplification of HalA1-Xa, HalA2-Xa, HalM1, and HalM2, respectively. PCR primers (Table S2) were designed that added a SacI restriction site 5' and SbfI restriction site 3' to each HalA-Xa gene. An AsisI restriction site was added at the 5' end, while an XhoI site was added to the 3' end of each HalM gene. The PCR products were digested with the appropriate restriction enzymes and purified using a Qiagen PCR purification kit. The pRSFDUET-1 vector digested with the same restriction enzymes was added to a ligation reaction containing T4 ligase and the insert DNA. The amplified HalA1-Xa (or HalA2-Xa) gene was cloned into the MCS1 in pRSFDUET-1, and then HalM1 (or HalM2) gene was cloned into the MCS2 in the same vector. Chemically competent *E. coli* DH5α cells were transformed with each ligation mixture and plated on LB-agar containing kanamycin (50 mg/L) to screen for positive clones. Clones were confirmed by DNA sequencing at the UIUC Core Sequencing Facility.

**Construction of pRSFDuet-1/NisA+NisB and pACYCDuet-1/NisC**

PCR amplification of *nisA*, *nisB* and *nisC* was performed using the following PCR cycle conditions: denaturation (98 °C for 20 s), annealing (60 °C for 20 s), and extension (72 °C for 30 s for *nisA*, 120 s for *nisB*, and 60 s for *nisC*) using the primers listed in Table S2 and genomic DNA of *Lactococcus lactis* as DNA template. The PCR reaction included 1× Phusion GC buffer (Finnzymes), DMSO (2%), Phusion hot start high fidelity DNA polymerase (0.5 U/µL) and primers (1 µM each). The amplifications were run on 1% agarose gel and the products were purified using a QIAquick Gel Extraction Kit (QIAGEN). The amplified *nisA* DNA fragment and the pRSFDuet-1 vector were digested with *Bam*HI and *Hind*III; PCR-amplified *nisC* and pACYCDuet-1 vector were digested with *Bgl*II and *Xho*I; and PCR amplified *nisB* was digested with *Nde*I and *Xho*I at 37 °C for 12 h. The digests were run on an agarose gel and the products extracted using a QIAquick Gel Extraction Kit (QIAGEN). The resulting DNA inserts were ligated with the respective digested vector at 4 °C for 15 h using T4 DNA ligase (Invitrogen). The ligation reaction mixtures were diluted 6 times with water prior to transformation. *E. coli* DH5α cells were transformed with the ligation product using heat shock and were plated on LB-kanamycin agar plates and grown at 37 °C for 15 h. Five colonies were picked and incubated in 5 mL of LB-kanamycin medium at 37 °C for 10 h, followed by isolation of the plasmids using a QIAprep Spin Miniprep Kit (QIAGEN). The sequences of the resulting plasmids were confirmed by DNA sequencing using the
appropriate primers by the Biotechnology Center of the University of Illinois at Urbana-Champaign. The plasmid vector pRSFDuet-1 containing the \textit{nisA} gene was digested with \textit{NdeI} and \textit{XhoI} and ligated with \textit{NdeI} and \textit{XhoI} digested PCR amplified \textit{nisB}. The ligation and transformation of \textit{E. coli} DH5\(\alpha\) cells was carried out as described above. The colonies were analyzed for insert and plasmids were isolated from 5 colonies and sequenced.

Overexpression and purification of His\(_6\)-tagged modified ProcA1.7G–1R, ProcA2.11G–1K, ProcA3.2TEV and ProcA3.3G–1K

\textit{E. coli} BL21 (DE3) cells transformed with a pRSFDuet-1 vector carrying the genes for one of the ProcA peptides as well as \textit{procM} were overexpressed in 2 L of LB medium containing 50 mg/L kanamycin at 37 °C until the O.D.\(_{600\text{nm}}\) reached 0.6–0.8. The culture was then induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and shaken continually at 18 °C for an additional 20 h. The induced cells were harvested by centrifugation (11,900 \( \times \) g for 20 min at 4 °C). The cell pellet was resuspended in 20 mL of LanA Start Buffer (20 mM NaH\(_2\)PO\(_4\), pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 pause, 15 min). The sample was centrifuged (23,700 \( \times \) g for 40 min at 4 °C) and the supernatant was kept for further purification. The pellet was resuspended in 20 mL of LanA Start Buffer to obtain any soluble proteins remaining in the pellet. Insoluble materials were removed from the combined soluble fractions by centrifugation (11,900 \( \times \) g for 20 min at 4 °C), and the resulting sample was clarified using a 0.45 μm syringe filter (Corning). The His\(_6\)-tagged peptides were then purified by immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare Life Sciences). The filtered sample was applied to the column and the column was washed with 2 column volumes of LanA Buffer 1 (6 M guanidine hydrochloride, 20 mM NaH\(_2\)PO\(_4\), pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole), followed by 2 ~ 3 column volumes of LanA Buffer 2 (4 M guanidine hydrochloride, 20 mM NaH\(_2\)PO\(_4\), pH 7.5 at 25 °C, 300 mM NaCl, 30 mM imidazole), and then eluted with 3 column volumes of LanA Elution Buffer (4 M guanidine hydrochloride, 20 mM Tris, pH 7.5 at 25 °C, 100 mM NaCl, 1 M imidazole).

The elution fractions were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) using a Waters Delta-pak™ C4 15 μm 300 Å 25 \( \times \) 100 mm PrepPac® Cartridge. Solvents for the RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile / 20% water). A gradient of 2-100% of solvent B was executed for over 45 min at a flow rate of 8 mL/min, and peptides were detected by absorbance at 220 nm. The fractions were analyzed by MALDI-ToF-MS. All the fractions containing the desired product were combined and the organic solvents were removed by rotary evaporation, followed by lyophilization overnight. The product was kept at −20 °C for short-term storage and −80 °C for long-term storage. The yields of the peptides were 22.6 mg, 62.0 mg, 57.9 mg, and 61.7 mg, respectively, for modified His\(_6\)-ProcA1.7G–1R, His\(_6\)-ProcA2.11G–1K, His\(_6\)-ProcA3.2TEV, and His\(_6\)-ProcA3.3G–1K from 2 L of cell culture.
Protease cleavage and tandem mass spectrometry analysis of prochlorosins

Modified ProcA mutants were cleaved by a commercial protease (trypsin for ProcA1.7G−1R, LysC for ProcA2.11G−1K and ProcA3.3G−1K, and TEV protease for ProcA3.2TEV) to remove the leader peptide. MALDI-TOF mass spectra of the modified peptides treated by proteases are shown in Figure 3 of the main text.

The ring topology of lantipeptides can be deduced from their MS fragmentation pattern.1 A sample of 5 μL of each protease cleavage reaction mixture was injected to a BEH C8 column (1.7 μm, 1.0× 100 mm), and the fully modified product was purified by UPLC using a gradient of 3% mobile phase B (0.1% formic acid in methanol) to 97% mobile phase B in mobile phase A (0.1% formic acid in water) over 12 min. Mass spectra were acquired in ESI positive mode in the range of 50-2000 m/z. The capillary voltage was 3500 V, and the cone voltage was 40 V. The other parameters used were as follows: 120 °C source temperature; 300 °C desolvation temperature, 150 L/h cone gas flow, and 600 L/h desolvation gas flow. A transfer collision energy of 4 V was used for both MS and tandem MS, while the trap collision energy was set to 6 V for MS and a 20–55 V ramp for MSMS depending on the peptide. Glu-fibrinopeptide B (Sigma) was directly infused as the lock mass. The tandem mass spectra were processed with MaxEnt3 and analyzed by Protein/Peptide Editor in BioLynx 4.1. The software for analyzing both precursor-ion and fragment-ion mass was set to report any mass within 0.3 amu of the calculated expected values. Tandem mass spectra of the fully modified peptides treated by proteases (ProcA1.7G−1R core peptide with five dehydrations, ProcA2.11G−1K core peptide with five dehydrations, ProcA3.2TEV core peptide with three dehydrations, and ProcA3.3G−1K core peptide with three dehydrations) are shown in Figure S4. Fragmentation patterns of these core peptides were consistent with the ring topology of prochlorosins previously obtained in vivo and in vitro.2 Therefore, they are denoted as “Pcn in E. coli” (Pcn is short for prochlorosin, i.e. prochlorosin 1.7 is denoted as Pcn1.7).

HPLC purification of prochlorosins modified in E. coli

The protease cleavage reactions were quenched with 0.5% TFA, and the desired products purified by analytical RP-HPLC using a VydaC™ C18 5 μm 300 Å 4.6 i.d. × 250 mm column. Solvents for RP-HPLC were solvent A (0.1% TFA in water) and solvent B (0.086% TFA in 80% acetonitrile / 20% water). A gradient of 0.8-100% of solvent B was executed for over 50 min at a flow rate of 1 mL/min, and peptides were detected by absorbance at 220 nm. The fractions were tested by MALDI-ToF-MS for the desired peptides. All the fractions containing the desired fully modified product were combined and the solvents were removed by lyophilization overnight. The product was kept at −20 °C for short-term storage and −80 °C for long-term storage. The yields of the peptides with the purity shown in Figure S2 were 14% (0.16 mg), 19% (0.20 mg), 12% (0.17 mg), and 86% (1.09 mg) for Pcn1.7, Pcn2.11, Pcn3.2, and Pcn3.3, respectively, starting from 6.0 mg of modified precursor peptides before proteolysis. MALDI MS spectra of purified prochlorosins are shown in Figure S3.

Incorporation of para-benzoil phenylalanine (pBpa or B) in ProcA3.2

The nucleotides encoding phenylalanine at position 26 of ProcA3.2TEV were substituted by the amber codon TAG. The mutant was generated by nested PCR using primers 5’- GCA GAC AAT ACA ATT GTC CCT TGC TGC TAG CAC CAG TG
-3' (3.2_F26B_FP) and 5'- ATA ATT TAG CGG CCG CTC ACT GGT GCT AGC AG -3' (3.2_F26B_RP). The vector procA3.2TEV/procM/pRSFDuet-1 was used as the template.

*E. coli* BL21 (DE3) cells transformed with a pDule-Tyr vector (obtained from Professor Peter Schultz, the Scripps Research Institute) and a pRSFDuet-1 vector carrying both procA3.2TEV_F26B and procM were grown in 400 mL of glycerol minimal medium, supplemented with metals (for components, see Ref. 4 and 5), containing 50 mg/L kanamycin, 12.5 mg/L tetracycline and an 18-amino-acid solution (for components, see Ref. 4 and 5). The pDule-Tyr vector carrying an orthogonal aminoacyl tRNA synthetase-tRNA <sub>CUA</sub> pair can incorporate p-benzoyl-L-phenylalanine (pBpa) at the position encoded by the amber codon TAG. The culture was shaken at 37 °C until the O.D. 600 nm reached 0.5, then pBpa dissolved in 1 M NaOH solution was added to the culture to obtain a final concentration of 1 mM pBpa. After shaken for 0.5 h, the culture was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and shaken continually at 18 °C for an additional 20 h. The induced cells were harvested by centrifugation (11,900×g for 20 min at 4 °C). The cell pellet was resuspended in 10 mL of LanA Start Buffer and lysed by sonication (35% amplitude, 4.0 s pulse, 9.9 pause, 15 min). The sample was centrifuged (23,700×g for 40 min at 4 °C) and the supernatant was kept for further purification. The pellet was resuspended in 10 mL of LanA Start Buffer again. The soluble portions after centrifugation (11,900×g for 20 min at 4 °C) were combined and clarified using 0.45 μm syringe filters (Corning), then purified by immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare Life Sciences). The filtered sample was applied to the column and the column was washed with 2 column volumes of LanA Buffer 1, followed by 2 ~ 3 column volumes of LanA Buffer 2, and then eluted by 3 column volumes of LanA Elution Buffer. The elution fractions were desalted and purified by solid phase extraction (SPE) using a 3 mL Vydac® BioSelect™ reversed-phase C4 column. A step gradient of 2% solvent B (0.086% TFA in 80% acetonitrile / 20% water) in solvent A (0.1% TFA in water), 50% solvent B in solvent A, and 100% solvent B was applied for the purification. The fractions were tested by MALDI-ToF-MS for the desired peptides. The majority of ProcA3.2TEV with Phe26 substituted with pBpa eluted in fractions of 50% solvent B in solvent A.

An 18 μL sample of 50% solvent B eluted fraction was incubated with 0.03 U endoproteinase GluC in 50 mM tris buffer at room temperature for 6 h. The mixture was quenched with 0.5% (f.c.) TFA, zip-tipped and subjected to MALDI-ToF-MS (for the spectrum, see Figure 3d and Figure S5).

**Overexpression and purification of HalA peptides**

Electrocompetent *E. coli* BL21 (DE3) cells were transformed with the pDUET construct containing both the N-terminal hexa-histidine HalA-Xa fusion gene and the corresponding HalM gene. Cultures were inoculated from single colony transformants and grown overnight at 37 °C in LB broth supplemented with 50 mg/L kanamycin. The overnight culture was used to inoculate 2 L of LB broth, and cells were grown at 37 °C to O.D. 600 ≈0.6-0.8. Expression was induced by the addition of 0.5 mM IPTG, and the culture was incubated at 18 °C for 18 h. Cells were harvested by centrifugation at 6500×g for 20 min at 4 °C. The pellet (≈6 g) was resuspended in 30 mL of start buffer containing
20 mM sodium phosphate, pH 7.5, 20% glycerol, 500 mM NaCl, and 0.5 mM imidazole. The cell paste was subjected to sonication to lyse the cells. Cell debris was removed by centrifugation at 16,500×g for 20 min at 4 °C. The pellet was discarded and the supernatant containing the soluble peptide was passed through a 0.45 μm filter. The peptides were purified by immobilized metal affinity chromatography (IMAC) and reverse-phase high-performance liquid chromatography as described in previous work. The peptide sample was lyophilized and stored at −20 °C. Typical peptide yields from each construct were 1-2 mg of final, dried peptide per liter of overexpressed cells. The peptide product showed complete conversion as determined by MALDI-TOF and Tandem Mass Spectrometry (Figure 3 of the main text).

**Overexpression and purification of modified nisin**

Chemically competent BL21(DE3) cells were co-transformed with pRSFDuet-1 containing the nisA and nisB genes and pACYCDuet-1 containing nisC. Overnight culture grown from a single colony transformant was used as inoculum to grow 1.5 L of terrific broth medium containing 50 mg/L kanamycin and 25 mg/L chloramphenicol at 37 °C until the OD₆₀₀nm reached between 0.6 and 0.8. The incubation temperature was then changed to 18 °C and the culture was induced with 0.5 mM IPTG. The induced cells were shaken continually at 18 °C for an additional 15 h. The cells were harvested by centrifugation (11,900×g for 10 min, Beckman JLA-10.500 rotor). The cell pellet was resuspended in 45 mL of start buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, containing a protease inhibitor cocktail from Roche Applied Science) and lysed by MultiFlex C3 homogenizer (Avestin). The sample was centrifuged at 23,700×g for 30 min at 4 °C. The supernatant was loaded onto a HiTrap HP nickel affinity column (GE Healthcare) pre-equilibrated with start buffer. Following loading, the column was washed with wash buffer (start buffer + 30 mM imidazole). The peptide was eluted from the column using elution buffer (start buffer + 1 M imidazole). The pellet was homogenized using a sonicator (35 % amplitude, 4.4 s pulse, 9.9 s pause for total 20 min) in start buffer to remove any soluble proteins. The pellet from this step was resuspended in 30 mL of denaturing buffer (6 M guanidine hydrochloride, 20 mM NaH₂PO₄, 500 mM NaCl, pH 7.5). The insoluble portion was removed by centrifugation and supernatant was loaded onto a HiTrap HP nickel affinity column. The column was washed with denaturing buffer containing 30 mM imidazole and eluted with 15 mL denaturing buffer containing 1 M imidazole. Desalting of the eluent from both supernatant and pellet fractions was performed using preparative scale RP-HPLC using a Waters Delta-pak C4 15μm 300 Å 25X100 mm PrepPak Cartridge. A gradient of 2-100% of solvent B (0.086 % TFA in 80% acetonitrile/20% water) was used. Modified prenisin began to elute at 52% B. The fractions containing modified prenisin were lyophilized and analyzed by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS).

**Factor Xa cleavage of modified HaLA-Xa**

Purified HaLA-Xa peptides were dissolved (final concentration 1 mg mL⁻¹) in Factor Xa assay buffer containing 50 mM Tris, 100 mM NaCl, 2 mM CaCl₂, and 5 mM TCEP, pH 8.0. Factor Xa was added to a final concentration of 0.03 mg mL⁻¹. Cleavage reactions were incubated at 25 °C for 5 h and were quenched with 1% TFA. Reactions were lyophilized to dryness, resuspended in sterile water, and analyzed by MALDI-MS.
Agar diffusion growth inhibition assay with haloduracin

The growth inhibition activity of Halα/β was determined against Lactococcus lactis HP in combination with wild type Halβ/α peptide (1:1 ratio) obtained from the producing strain. Both Halα and Halβ wild type peptides (in combination) were used as controls. Liquid molten GM17 agar (25 mL; 4% M17, 0.5% glucose, 1.5% agar) was cooled to 42 °C and seeded with 200 μL of dense overnight culture (approx $10^8$-$10^9$ CFU mL$^{-1}$) of the indicator strain Lactococcus lactis HP. After agar solidification in a Petri dish, samples were applied to a small well created in the medium. Assay samples were typically diluted in 10 μL of sterile water to give a final concentration of 5 μM. Plates were incubated for 15 h at room temperature and antibacterial activity was qualitatively determined by the presence or absence of a zone of growth inhibition.

Cleavage of modified prenisin with trypsin and antimicrobial activity assay

An aliquot of 100 μL of 500 μM modified prenisin was incubated with 3 μL of 30 μM trypsin (Worthington Biochemicals) at room temperature for 3 h. The resulting mixture was checked by MALDI-TOF MS and the proteolytic fragment corresponding to mature nisin was observed calcd: 3354 (nisin); found 3356 (M+H). Inhibitory activity assays were carried out using solid agar diffusion. GM17 agar was melted in a microwave, cooled to 45 °C and mixed with 150 μL of overnight grown Lactococcus lactis HP (10$^8$-10$^9$ cfu). After the agar was solidified, 10 μL of 5 μM of heterologously expressed and trypsin cleaved nisin was spotted on the surface. As a control, nisin purified from commercial Nisaplin (Danisco) using RP-HPLC was also spotted. Nisin purified by HPLC after production in E. coli is shown in Figure S6.

Tandem mass spectrometry analysis to confirm the correct ring topology of nisin

Commercial, HPLC-purified nisin and in vivo produced (E. coli), and then trypsin treated nisin were subjected to ESI-MSMS analysis using collision induced dissociation. Similar fragmentation patterns were observed for both samples (Figure S7). To carry out ESI-MSMS, 10 μL of each nisin was injected to a BEH C8 column (1.7 μm, 1.0 100 mm), and separated by UPLC using a gradient of 3% mobile phase A (0.1% formic acid in water) to 97 % mobile phase B (0.1% formic acid in methanol) over 12 min. The instrument settings used included capillary voltage and cane voltage of 3500 V and 40 V respectively, 120 °C as source temperature; 300 °C as desolvation temperature, cone gas flow of 150 L/h, and desolvation gas flow of 600 L/h. A transfer collision energy of 4 V was used for both MS and tandem MS, while the trap collision energy was set to 6 V for MS and a fixed voltage 28.4 V was used for MSMS.

References

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**Table S1.** Primer sequences for cloning and mutagenesis of ProcAs.

| Primer Name | Primer Sequence (5’-3’) |
|-------------|-------------------------|
| ProcA1.7_EcoRI_FP_Duet | GGT GCG AGG AAT TCG ATG AAG CAT AGA CAA CTA AAT CTG |
| ProcA1.7_NotI_RP_Duet | ATA ATA TCG CGG CCG CTC AGC ACA TTT TCC C |
| ProcA2.11_EcoRI_FP_Duet | GGT GAG TGG AAT TCG ATG TCA GAA GAA CAA CTC AAA GC |
| ProcA2.11_NotI_RP_Duet | GTA ATT TAG CGG CCG CCT AGC AAC AGG TAC C |
| ProcA2.11_G-1K_FP | GAA AGT GTG GCT GGC AAA GGG AGG ATT GAT ACC |
| ProcA2.11_G-1K_RP | GTA ATC AAT CCT CCC TTT GCC AGC AAC ACT TTC |
| ProcA3.2_EcoRI_FP_Duet | GGT GAG TGG AAT TCG ATG TCA GAA GAA CAA CTC AAG GC |
| ProcA3.2_NotI_RP_Duet | ATA ATT TAG CGG CCG CCT ACG TCT GCC AGC |
| ProcA3.2_TEV_FP | GAT GAT GAG CTG GAA AAT TTT TAT TTT CAA GGG GGA GGC TGT GAC |
| ProcA3.2_TEV_RP | GTC ACA GCC TCC CCC TTT AAA ATA CAA ATT TTT CAG CTC ATC ATC |
| ProcA3.3_EcoRI_FP_Duet | GGT GAG TGG AAT TCG ATG TCA GAA GAA CAA CTC AAG GC |
| ProcA3.3_NotI_RP_Duet | ATA ATT TAG CGG CCG CCT ATG CGC GGC |
| ProcA3.3_G-1K_FP | CCG CTA GCG GCA AAG GCG ATA CCG |
| ProcA3.3_G-1K_RP | CGG TAT CGC CTT TGC CGC TAG CGG |

**Table S2.** Primer sequences for nisin and haloduracin (5’-3’)

| Prime r Name | Primer Sequence (5’-3’) |
|--------------|-------------------------|
| pHalA1-Xa-5’ | CGCCACTCGGAGCTCGATGACAATAATTTTTT AAAAA |
| pHalA1-Xa-3’ | ATAGTGATCCTGCAAGGTTATTTGCAAGAAGGCATG |
| pHalA2-Xa-5’ | GCCACTCGGAGCTCGATGTAATATTCAAAG |
| pHalA2-Xa-3’ | ATATAGATCTCTGCAAGTGGTTAACGATGCT |
| pHalM1-5’ | TCACTATGCGATCGATGAGAAATTTCAAG |
| pHalM1-3’ | ATATATGCTCGAGTTATGATTAGTCAGCCTATGC |
| pHalM2-5’ | TCACTATGCGATCGATGAAAAACTTCCTC TAA |
| pHalM2-3’ | ATGTCGTCGTCGATGTTATCCTGTCAATG |
| NisA_BamHI_F | CTA GAT GGA TCC GAT GAC TAA AAG TTT TAA CTG TTG |
| NisA_HindIII_R | CTA GAG TTT ATT TGG TTA CGT GAA TAC TAC AAT G |
| NisB_Ndel_F | AAG CAG CGG CAT ATG ATA AAA AGT TCA TTT AAA GCT CAA CCG |
| NisB_Xhol_R | CTA GCT CGA GTC ATT TCA TGT ATT CTT CCG AAA CAA ACA ACC |
| NisC_BglII_F | CTA GGG AAG ATC TGA ATA AAA AAA ATA TAA AAA GAA ATG TTG |
| NisC_Xhol_R | CTA GCT CGA GTC ATT TCC TCT CTC CTC TCA AAA AAA CAAT CGT C |
Figure S1. Precursor peptide sequences of the ProcA and HalA lantipeptides used in this study. The leader peptides are highlighted in yellow. Black arrows indicate the cleavage sites. Mutated amino acids introduced for commercial protease cleavage are in bold font and underlined. Cysteine residues in the core peptides are highlighted in red. Serine and threonine residues in the core peptides are highlighted in green. Serine residues that were not dehydrated in HalA1 and HalA2 core peptides after HalM modification were highlighted in light blue. Red lines indicate the rings formed. Purple lines indicate putative ring topology after ProcM modifications.2 Tandem mass spectrometry has confirmed the existence of overlapping rings at the C-terminal core peptide regions of Pcn2.11 and Pcn3.2 obtained both in vivo and in vitro.7 However, how the two rings overlap is currently not known and one possible ring topology is shown.
Figure S2. Analytical RP-HPLC of purified prochlorosins. A) Pcn 2.11. B) Pcn3.2. C) Pcn3.3.
Figure S3. MALDI-MS spectra of four prochlorosins modified by ProcM in *E. coli* after commercial proteases cleavage and HPLC purification. A) Pcn1.7 from *E. coli*. B) Pcn2.11 from *E. coli*. C) Pcn3.2 from *E. coli*. D) Pcn3.3 from *E. coli*. 
Figure S4. ESI-MSMS of four ProcA peptides processed by ProcM in *E. coli* and cleaved by commercial proteases. The observed fragmentation patterns of the core peptides are consistent with the ring topology of prochlororosins previously obtained *in vivo* and those modified *in vitro*. Therefore, they are denoted as “Pcn in *E. coli*”. A) ProcA1.7G-1R modified by ProcM in *E. coli* and treated with trypsin *in vitro*. B) ProcA2.11G-1K modified by ProcM in *E. coli* and treated with LysC *in vitro*. C) ProcA3.2TEV modified by ProcM in *E. coli* and cleaved by TEV protease.
in vitro. D) ProcA3.3G-1K modified by ProcM in E. coli and treated with LysC in vitro. E) Fragmentation patterns of the four prochlorosins in ESI-MSMS. The N-terminal threonine of ProcA1.7G-1R core peptide is converted to dehydrobutyrine by ProcM. This dehydrobutyrine hydrolyzes to 2-oxobutyryl (Obr) group after trypsin cleavage at the cleavage site. Dehydrated serine residues (dehydroalanine, Dha) and threonine residues (dehydrobutyrine, Dhb) are highlighted in green. Other colors have the same meaning as in Figure S1.

Figure S5. MALDI-MS spectrum of ProcA3.2TEV with Phe26 substituted by pBpa, modified by ProcM in E. coli and treated with GluC to remove the major part of the leader peptide. The remaining residues of the leader peptides after GluC cleavage are highlighted in yellow in the amino acid sequence shown in the figure. “B” representing the incorporation of pBpa is highlighted in grey. Cysteine residues are highlighted in red. Serine and threonine residues are highlighted in green. After modification by ProcM, these serine and threonine residues were dehydrated, which caused a mass shift in the MALDI-MS compared with the calculated mass of unmodified peptides with the same sequence (denoted as M in the figure). A small amount of peptide resulting from translation termination at the amber stop codon is seen around 3100 Da. The ion with three dehydrations in the core peptide was the major product along with a minor amount of product with two dehydrations, as shown in the inset.
Figure S6. HPLC trace showing the purity of nisin produced in *E. coli*. The peak at 33 min is fully modified nisin.
Figure S7. ESI-MSMS analysis of commercial nisin (top) and in vivo produced and trypsin cleaved nisin (bottom). The b3 ion seen in both samples and the y30 ion seen in the bottom sample are uncommon for lanthionine rings but are consistently observed for intact nisin. Dha5 may be involved in this unusual cleavage of the A-ring in nisin.