Electronic Supplementary Information

Applications of the class II lanthipeptide protease LicP for sequence-specific, traceless peptide bond cleavage

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General methods

All polymerase chain reactions (PCRs) were carried out on a C1000 thermal cycler (Bio-Rad). DNA sequencing was performed by ACGT, Inc. Preparative HPLC was performed using a Waters Delta 600 instrument equipped with a Waters Delta-pak C4 column (15 µm 300 Å 25 x 100 mm). Solid phase extraction was performed with a Strata-X polymeric reversed phase column (Phenomenex) or Vydac BioSelect C4 reversed phase column. FPLC was carried out using an AKTA FPLC system (Amersham Pharmacia Biosystems). MALDI-TOF MS was

carried out on a Bruker Daltonics UltrafleXtreme MALDI TOF/TOF instrument (Bruker). The detection of peptides with low molecular weights (700-3,500 Da), peptides with medium molecular weights (3,500-20,000 Da) and proteins with high molecular weights (20,000-50,000 Da) was achieved by using different instrument settings optimized for these mass ranges.

Materials

All oligonucleotides were synthesized by Integrated DNA Technologies and used as received. Restriction endonucleases, DNA polymerases, and T4 DNA ligase were obtained from New England Biolabs. Media components were purchased from Difco Laboratories and Fisher Scientific. Chemicals were ordered from Sigma Aldrich or Fisher Scientific unless otherwise specified. Miniprep, gel extraction and PCR purification kits were purchased from Qiagen and 5 PRIME. Synthetic genes were obtained from IDT, Inc. For LanP from *Bacillus cereus* VD156, the DNA was ordered in two gBlocks, whereas for the substrate it was ordered as one oligonucleotide. An UltraClean microbial DNA isolation kit was obtained from Mo Bio Laboratories, Inc.

Strains and plasmids

Bacillus licheniformis ATCC 14580 and *Bacillus licheniformis* ATCC 9945A were obtained from American Type Culture Collection. *E. coli* DH5α and *E. coli* BL21 (DE3) cells were used as hosts for cloning and plasmid propagation, and hosts for protein expression, respectively. The expression vector pRSFDuet-1 was obtained from Novagen.

Extraction of genomic DNA from *B. licheniformis* ATCC 14580 and *B. licheniformis* ATCC 9945A

Bacteria were cultured in LB medium at 37 ^{o}C aerobically for 12 h and the genomic DNA was extracted using an UltraClean microbial DNA isolation kit following the manufacturer's protocol.

Construction of pRSFDuet-1 derivatives for expression of LicP-25-433 and LicA2

The genes *licP* and *licA2* were amplified from genomic DNA of *B. licheniformis* ATCC 14580 using appropriate primers and cloned into the multiple cloning site 1 (MCS1) of a pRSFDuet-1 vector to generate pRSFDuet-1/LicP-25-433 and pRSFDuet-1/LicA2 plasmids, respectively. Primer sequences are listed in **Table S2**.

Construction of pRSFDuet-1 derivatives for expression of NDVNPE-ProcA1.7 and NDVNPE-NisA

Engineered peptide genes were generated by multi-step overlap extension PCR. First, the amplification of the 5' leader part was carried out by 30 cycles of denaturing (95 °C for 10 s), annealing (55 °C for 30 s), and extending (72 °C for 15 s) using forward primers for *procA1*.7 and *nisA* and appropriate leader peptide reverse primers containing the mutations (**Table S2**) to generate a forward megaprimer (FMP). In parallel, PCR reactions using forward primers and reverse primers for *procA1*.7 and *nisA* core peptides (**Table S2**) were performed to produce the 3' core fragments (termed reverse megaprimer, RMP). The 5' FMP fragment and 3' RMP fragment were purified by 2% agarose gel, combined in equimolar amounts and amplified using the same PCR conditions as above with *procA1*.7 and *nisA* primers. The resulting PCR products were purified, digested and then cloned into the MCS1 of a pRSFDuet-1 vector to generate pRSFDuet-1/NDVNPE-ProcA1.7 and pRSFDuet-1/NDVNPE-NisA plasmids.

Construction of pRSFDuet-1 derivatives for expression of LicP-25-433-S376A, LicP-25-433-H186A, LicP-25-433-E100A, LicP-25-433-E100A-E102A, G-LicA2, NDVNPE-NisA-I1G, NDVNPE-NisA-I1T, NDVNPE-NisA-I1C, NDVNPE-NisA-I1L, NDVNPE-NisA-I1F, NDVNPE-NisA-I1W, NDVNPE-NisA-I1K, NDVNPE-NisA-I1E, LicA2-E–1A, LicA2-E–1D, LicA2-E–1Q, LicA2-P–2A, LicA2-N–3A, LicA2-V–4A, LicA2-V–4L, LicA2-V–4N, LicA2-V–4F, LicA2-D–5A, LicA2-D–5K, and LicA2-D–5V

The expression plasmids pRSFDuet-1/LicP-25-433-S376A, pRSFDuet-1/LicP-25-433-H186A, pRSFDuet-1/LicP-25-433-E100A, pRSFDuet-1/LicP-25-433-E100A-E102A, pRSFDuet-1/G-LicA2, pRSFDuet-1/NDVNPE-NisA-I1G, pRSFDuet-1/NDVNPE-NisA-I1T, pRSFDuet-1/NDVNPE-NisA-I1C, pRSFDuet-1/NDVNPE-NisA-I1L, pRSFDuet-1/NDVNPE-NisA-I1F, pRSFDuet-1/NDVNPE-NisA-I1W, pRSFDuet-1/NDVNPE-NisA-I1K, pRSFDuet-1/NDVNPE-NisA-I1E, pRSFDuet-1/LicA2-E–1A, pRSFDuet-1/LicA2-E–1D, pRSFDuet-1/LicA2-E–1Q, pRSFDuet-1/LicA2-P–2A, pRSFDuet-1/LicA2-N–3A, pRSFDuet-1/LicA2-V–4A, pRSFDuet-1/LicA2-V–4L, pRSFDuet-1/LicA2-V–4N, pRSFDuet-1/LicA2-V–4F, pRSFDuet-1/LicA2-D–5A, pRSFDuet-1/LicA2-D–5K, and pRSFDuet-1/LicA2-D–5V were generated using QuikChange methodology based on pRSFDuet-1/LicP-25-433, pRSFDuet-1/LicA2 and pRSFDuet-1/NDVNPE-NisA as templates. Primer sequences are listed in **Table S2**.

Construction of pRSFDuet-1 derivatives for co-expression of LicM2 with LicA2

The *licM2* gene was amplified from the genomic DNA of *B. licheniformis* ATCC 14580 using appropriate primers and cloned into the MCS2 of a pRSFDuet-1 vector to generate pRSFDuet-1/LicM2-2. The expression plasmid pRSFDuet-1/LicA2/LicM2-2 was constructed by inserting the *licA2* gene into the MCS1 of the pRSFDuet-1/LicM2-2 plasmid. Primer sequences are listed in **Table S2**.

Construction of a pET28b-MBP-BamL plasmid containing the LicP recognition sequence

Oligonucleotides corresponding to the LicP recognition sequence NDVNPE/SGS were inserted into the pET28b-MBP-BamL plasmid[1] in front of the DNA sequences corresponding to the TEV cleavage site using QuikChange methodology. Primer sequences are listed in **Table S2**.

Construction of pRSFDuet-1 derivatives for co-expression of LanM2-9945A with LanA2-9945A, and for expression of LanP-42-476-9945A

The genes for the LanM2 and LanA2 encoded in the genome of *B. licheniformis* ATCC 9945A (hereafter LanM2-9945A and LanA2-9945A, respectively) were amplified from the genomic DNA using appropriate primers and cloned into a pRSFDuet-1 vector to generate pRSFDuet-1/LanA2-9945A/LanM2-9945A-2 using Gibson assembly (LanA2 in MCS1 and LanM2 in MCS2). The gene encoding residues 42-476 of the class II LanP (designated LanP-42-476-9945A) was amplified from the genomic DNA of *B. licheniformis* ATCC 9945A using appropriate primers and cloned into the MCS1 of a pRSFDuet-1 vector to generate pRSFDuet-1/LanP-42-476-9945A using Gibson assembly. Primer sequences are listed in **Table S2**.

Construction of pRSFDuet-1 derivatives for expression of LanP and one LanA substrate encoded in the genome of *B. cereus* VD156

The *lanP* and *lanA3* gene were synthesized as codon-optimized dsDNA oligos and cloned into a pRSFDuet-1 vector between BamHI and NotI restriction sites via Gibson Assembly. For expression in *E. coli*, the N-terminal secretion signal of the protease (the first 27 amino acids shown in red) was removed. The synthetic gene sequences are listed in **Table S2**.

Expression and purification of LicP and LicP mutant proteins

E. coli BL21 (DE3) cells were transformed with one of the following plasmids: pRSFDuet-1/LicP-25-433, pRSFDuet-1/LicP-25-433-S376A, pRSFDuet-1/LicP-25-433-H186A,

pRSFDuet-1/LicP-25-433-E100A or pRSFDuet-1/LicP-25-433-E100A-E102A, and plated on an LB plate containing 50 mg/L kanamycin. A single colony was picked and grown in 20 mL of LB containing 50 mg/L kanamycin at 37 ^{o}C for 12 h and the resulting culture was inoculated into 2 L of LB containing 50 mg/L kanamycin. Cells were cultured at 37 ^{o}C until the OD at 600 nm reached 0.5, cooled and IPTG was added to a final concentration of 0.1 mM. The cells were cultured at 18 ^{o}C for another 10 h before harvesting. The cell pellet was resuspended on ice in LanP buffer (20 mM HEPES, 1 M NaCl, pH 7.5 at 25 ^{o}C) and lysed by homogenization. The lysed sample was centrifuged at 23,700×*g* for 30 min and the pellet was discarded. The supernatant was passed through 0.45-μm syringe filters and the protein was purified by immobilized metal affinity chromatography (IMAC) loaded with nickel as previously described.[2] The proteins were generally eluted from the column at an imidazole concentration between 150 mM and 300 mM and the buffer was exchanged using a GE PD-10 desalting column or a gel-filtration column pre-equilibrated with LanP buffer. Protein concentration was quantified by the absorbance at 280 nm. The extinction coefficient for His_6-LicP-25-433 was calculated as 46,300 M^{-1} cm^{-1}. His_6-LicP-25-433-S376A was predominantly expressed in inclusion bodies. Soluble protein was obtained by combining fractions eluted from the nickel column containing the desired protein and concentrating to a small volume. Gel filtration chromatography was not performed for the mutant protein. The yield was determined to be about 50 μg for 1 L of culture. Aliquoted protein solutions were flash-frozen and kept at −80 ^{o}C until further usage.

Expression and purification of modified His_6-LicA2

Modified LicA2 was obtained using a procedure similar to that reported previously using the corresponding co-expression plasmid pRSFDuet-1/LicA2/LicM2-2.[3,4]

Expression and purification of unmodified His_6-LicA2, His_6-G-LicA2, His_6-NDVNPE-ProcA1.7, His_6-NDVNPE-NisA, His_6-NDVNPE-NisA-I1G, His_6-NDVNPE-NisA-I1T, His_6-NDVNPE-NisA-I1C, His_6-NDVNPE-NisA-I1L, His_6-NDVNPE-NisA-I1F, His_6-NDVNPE-NisA-I1W, His_6-NDVNPE-NisA-I1K, and His_6-NDVNPE-NisA-I1E

E. coli BL21 (DE3) cells were transformed with one of the following plasmids: pRSFDuet-1/LicA2, pRSFDuet-1/G-LicA2, pRSFDuet-1/NDVNPE-ProcA1.7, pRSFDuet-1/NDVNPE-NisA, pRSFDuet-1/NDVNPE-NisA-I1G, pRSFDuet-1/NDVNPE-NisA-I1T, pRSFDuet-1/NDVNPE-NisA-I1C,

pRSFDuet-1/NDVNPE-NisA-I1L, pRSFDuet-1/NDVNPE-NisA-I1F, pRSFDuet-1/NDVNPE-NisA-I1W, pRSFDuet-1/NDVNPE-NisA-I1K or pRSFDuet-1/NDVNPE-NisA-I1E. Then the cells were plated on an LB plate containing 50 mg/L kanamycin. A single colony was picked and grown in 10 mL of LB containing 50 mg/L kanamycin at 37 °C for 12 h and the resulting culture was inoculated into 1 L of LB containing 50 mg/L kanamycin. Cells were cultured at 37 °C until the OD at 600 nm reached 0.5 and IPTG was added to a final concentration of 0.2 mM. The cells continued to be cultured at 37 °C for another 3 h before harvesting. The cell pellet was resuspended at room temperature in LanA start buffer (20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and lysed by sonication. The sample was centrifuged at 23,700×*g* for 30 min and the supernatant was discarded. The pellet was then resuspended in LanA buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole) and sonicated again. The insoluble portion was removed by centrifugation at 23,700×*g* for 30 min and the soluble portion was passed through 0.45-μm syringe filters. His-tagged peptides were purified by IMAC as previously described.[2] The eluted fractions were desalted using reversed phase HPLC or a Strata X polymeric reversed phase SPE column. The desalted peptides were lyophilized and stored at −20 °C for future use.

Expression and purification of unmodified His_6-LicA2-E–1A, His_6-LicA2-E–1D, His_6-LicA2-E–1Q, His_6-LicA2-P–2A, His_6-LicA2-N–3A, His_6-LicA2-V–4A, His_6-LicA2-V–4L, His_6-LicA2-V–4N, His_6-LicA2-V–4F, His_6-LicA2-D–5A, His_6-LicA2-D–5K, and His_6-LicA2-D–5V

E. coli BL21 (DE3) cells were transformed with one of the following plasmids: pRSFDuet-1/LicA2-E–1A, pRSFDuet-1/LicA2-E–1D, pRSFDuet-1/LicA2-E–1Q, pRSFDuet-1/LicA2-P–2A, pRSFDuet-1/LicA2-N–3A, pRSFDuet-1/LicA2-V–4A, pRSFDuet-1/LicA2-V–4L, pRSFDuet-1/LicA2-V–4N, pRSFDuet-1/LicA2-V–4F, pRSFDuet-1/LicA2-D–5A, pRSFDuet-1/LicA2-D–5K or pRSFDuet-1/LicA2-D–5V. The cells were then plated on an LB plate containing 50 mg/L kanamycin. A single colony was picked and grown in 7 or 20 mL of LB containing 50 mg/L kanamycin at 37 °C for 14.5–16.5 h and the resulting culture was used to inoculate 750 mL of LB containing 50 mg/L kanamycin. Cells were cultured at 37 °C until the OD at 600 nm reached 0.5–0.6 and IPTG was added to a final concentration of 0.2 mM. The cells continued to be cultured at 37 °C for another 3 h before harvesting. The cell pellet was resuspended in LanA start buffer and lysed by sonication. The sample was centrifuged at 15,377×*g* for 30 min and the supernatant was discarded. The pellet

was then resuspended in LanA buffer 1 and sonicated again. The insoluble portion was removed by centrifugation at 15,377×*g* for 30 min and the soluble portion was passed through 0.45-μm syringe filters. His-tagged peptides were purified by IMAC as previously described.[2] Eluted fractions were desalted using a Vydac Bioselect C4 reversed phase SPE column. The desalted peptides were lyophilized, dissolved in water to a final concentration of 3 mg/mL and stored at −20 °C for future use.

Intermolecular cleavage of His_6-LicP-25-433-S376A by His_6-LicP-25-433

His_6-LicP-25-433-S376A and His_6-LicP-25-433 proteins were both diluted with LanP buffer to a final concentration of 0.2 mg/mL. Parallel reactions were set up for His_6-LicP-25-433 with a final protein concentration of 0.1 mg/mL in LanP buffer, His_6-LicP-25-433-S376A with a final protein concentration of 0.1 mg/mL in LanP buffer, and His_6-LicP-25-433-S376A and His_6-LicP-25-433 combined with a final protein concentration of 0.1 mg/mL each. The three reactions were allowed to proceed at room temperature for 0, 2, 4, 7 and 19 h before being stopped by addition of SDS loading buffer and boiling at 95 °C for 10 min and analyzed by SDS-PAGE.

Removal of leader peptides of modified or linear LicA2

Modified or linear LicA2 peptides were dissolved in H_2O to make a 3 mg/mL solution (340 μM). To a 17 μL solution of peptide (final peptide concentration 290 μM), 2 μL of 500 mM HEPES buffer (pH 7.5) was added followed by 1 μL of 1 mg/mL LicP (final protein concentration 1.1 μM). The reaction was incubated at room temperature for 6 h followed by MS analysis.

Proteolytic cleavage of the leader peptides of engineered peptides

NDVNPE-ProcA1.7 was dissolved in H_2O to a final concentration of 3 mg/mL (250 μM), whereas for NDVNPE-NisA and its mutant peptides, a 10 mg/mL peptide solution was made (1.3 mM). For NDVNPE-ProcA1.7, 15 μL of peptide solution (final peptide concentration 190 μM) was pre-mixed with 1 μL of 50 mM DTT and 2 μL of 500 mM HEPES buffer (pH 7.5), to which 2 μL of 0.1 mg/mL LicP (final protein concentration 210 nM) was added. The reaction was incubated at room temperature for 4 h before analysis. For NDVNPE-NisA-I1T and NDVNPE-NisA-I1C, 1 μL of peptide (final peptide concentration 65 μM) was pre-mixed with 1 μL of 50 mM DTT and 2 μL of 500 mM HEPES buffer (pH 7.5) in 14 μL H_2O, then 2 μL of 0.1 mg/mL LicP (final protein concentration 210 nM) was added. The reaction was incubated at room temperature for 20 h before analysis. For NDVNPE-NisA and other NisA mutant peptides,

1 mg/mL LicP (final protein concentration 2.1 μM) was employed instead of 0.1 mg/mL LicP and the reaction was kept at room temperature for 30 h before analysis.

Sequential proteolytic cleavage of modified LicA2

HPLC-purified LicM2-modified LicA2 was dissolved in H_2O to a final concentration of 3 mg/mL (340 μM). To a 17 μL solution of peptide (final peptide concentration 290 μM), 2 μL of 500 mM HEPES buffer (pH 7.5) was added followed by 1 μL of 0.5 mg/mL AspN. The reaction mixture was kept at room temperature for 12 h, and then 0.5 μL of 0.1 mg/mL LicP (final protein concentration 50 nM) was added. The reaction was then incubated at room temperature for one more hour. MALDI-TOF MS analysis was performed after each step.

Assay of modified and linear LicA2 peptides treated independently with LicP using HPLC and SDS-PAGE

Three *in vitro* assays were conducted to compare the time dependence of LicP activity toward modified and linear LicA2 peptides at one concentration using HPLC and SDS-PAGE analysis. The relative activity was further analyzed in a competition assay using MS analysis (see below). As discussed in the main text, Michaelis-Menten kinetic experiments were not possible due to poor solubility of the peptides. Although 3 mg/mL stock solutions of modified and linear LicA2 could be prepared, these solutions were made from HPLC-purified peptides and therefore contain residual TFA. LicA2 is soluble at lower pH but upon incubation under physiological conditions (pH 7.5), both modified and linear LicA2 precipitate. Therefore, the following in vitro assays were heterogeneous due to poor solubility of the peptides.

1) Linear wild type LicA2 (100 μM) was incubated with 0.4 μM His_6-LicP-25-433 and 2 mM DTT in 50 mM HEPES (pH 7.5) buffer at room temperature with a total reaction volume of 75 μL. After 5 min, 15 min, 30 min and 2 h, the reaction was centrifuged for 30 s to 1 min at 2000x*g* and a 13.6 μL aliquot was removed and quenched by addition of 3.5 μL 5% aqueous formic acid to give a final concentration of 1%.

2) Modified wild type LicA2 (100 μM) was incubated with 0.4 μM His_6-LicP-25-433 and 2 mM DTT in 50 mM HEPES (pH 7.5) buffer at room temperature with a total reaction volume of 50 μL. After 5 min, 15 min and 30 min, quenched aliquots were prepared as described above.

3) Modified wild type LicA2 (100 μM) was incubated with 0.1 μM His_6-LicP-25-433 and 2 mM DTT in 50 mM HEPES (pH 7.5) buffer at room temperature with a total reaction volume of 64 μL. After 5 min, 15 min, 30 min and 2h, quenched aliquots were prepared as described above.

Samples for HPLC analysis were prepared as follows: 7.5 μL of each formic-acid quenched sample from the linear LicA2 assay was combined with 12.5 μL of 32 mM TCEP followed by incubation at room temperature for at least 3 h in order to fully reduce disulfides. To 7.5 μL of each formic-acid quenched sample from assays involving modified LicA2 was added 12.5 μL H_2O. Aliquots of 30 μM modified LicA2 and 30 μM linear LicA2, which was incubated in 20 mM TCEP at room temperature for 3 h, were used as precursor peptide standards. HPLC was conducted using an Agilent 1260 Liquid Chromatography (HPLC) system (Agilent Technologies) and absorbance was monitored at 220 nm. The samples (5 μL) were injected on a C18 Mass Spec Vydac column (#218MS5120). A linear gradient of 2:98 to 100:0 B:A over 45 min (B = 80% MeCN/20% H_2O/0.086% TFA; A = 0.1% aqueous TFA) was used with a flow rate of 0.1 mL/min, giving a retention time of 34.1 min for leader peptide and retention times of 37.5 min and 41.4 min for linear and modified LicA2 precursor peptides, respectively. The identity of peaks in the HPLC traces was confirmed by MALDI-TOF MS analysis.

Samples for SDS-PAGE analysis were prepared as follows. To 4.5 μL of each formic-acid quenched sample was added 1.5 μL of 95:5 4X NuPAGE LDS sample buffer:β-mercaptoethanol. Standard samples of 60 μM linear and modified LicA2 were prepared from 1.5 μL of 3 mg/mL peptide (336 and 344 μM), 4.8 μL H_2O and 2.1 μL 95:5 4X NuPAGE LDS sample buffer:β-mercaptoethanol. The Polypeptide Standards (#161-0326) sample was prepared from 20 μL of 20-fold diluted 161-0326, 10 μL H_2O and 10 μL 95:5 4X NuPAGE LDS sample buffer:β-mercaptoethanol. All SDS-PAGE samples were heated for 10 min at 70 ºC and then loaded on a 10-20% Mini-Protean Tris-Tricine gel (#456-3116) adding 5 μL per lane. The gel was run at 100 V for 125 min in 100 mM Tris, 100 mM Tricine, and 0.1% SDS buffer while cooling the entire apparatus in ice. The gel was then subjected to consecutive coomassie and silver staining as described below. The gels were rocked for 1 h in 50% MeOH/7% AcOH followed by rocking for 45 min in coomassie stain (0.25% coomassie/50% MeOH/10% AcOH), rinsing with H_2O, rocking overnight in 20% MeOH/10% AcOH, and then rinsing with H_2O again. All of the following steps were conducted with agitation on a Barnstead LabLine multipurpose rotator. The gels were washed with 50% MeOH/7% AcOH for 1.5 h, followed by washing with H_2O (3 x 10 min). The gels were then sensitized 1 min in 0.02% $Na_2S_2O_3$, then washed with H_2O (2 x 1 min), followed by staining for 30 min in a solution containing 44 mL of H_2O, 5.9 mL of 0.1 M $AgNO_3$, and 37.5 μL of 37% formaldehyde. The gels were then washed with H_2O (< 1 min) and developed for < 5 min in a solution containing 100 mL of 3% Na_2CO_3, 2 mL of 0.02% $Na_2S_2O_3$, and 50 μL of 37% formaldehyde. Developing was quenched by washing with 12%

AcOH for 30 min, followed by washing with H_2O (2 x 30 min). Images of gels were acquired using an HP scanjet 8250.

Competition assay of LicP activity with modified and linear LicA2

To a reaction vessel containing 70 μL deionized H_2O, 5 μL each of 3 mg/mL modified LicA2 and linear G-LicA2 peptides were added (final peptide concentration 17 μM each) followed by 10 μL of 500 mM HEPES buffer (pH 7.5). Then, 10 μL of 0.01 mg/mL LicP was supplied (final protein concentration 21 nM) and the reaction was incubated at room temperature before being quenched by addition of formic acid to a final concentration of 1% at different time points. To observe the complete consumption of both peptides, substrates were incubated as above except that 10 μL of 1 mg/mL LicP was added (final protein concentration 2.1 μM). The reaction mixture was kept at room temperature for 12 h before being quenched with 1% formic acid for MS analysis.

Comparison of the proteolytic activity of LicP and TEV on MBP-BamL

A sample of 1 mL of MBP-BamL (50 μM) was incubated with the same molar amount of LicP or TEV (final concentration 0.54 μM) at 4 °C. At different time points, the reaction was quenched by adding an equal volume of loading dye and heating for 10 min at 90 °C. The results were analyzed by Coomassie-stained SDS-PAGE. The size difference between the product bands is a consequence of slightly different recognition site locations in the construct.

LicP assay and SDS-PAGE analysis for wild type linear His_6-LicA2 and linear His_6-LicA2 mutants

Linear LicA2 peptides (100 μM) were incubated with 0.4 μM His_6-LicP-25-433 and 2 mM DTT in 50 mM HEPES buffer (pH 7.5) at room temperature with a total reaction volume of 300 μL. The reactions were heterogeneous as a consequence of the poor solubility of the peptides. After 15 min, 30 min, 1 h, 2 h, 4 h and 7.5 h, the reactions were centrifuged for 30 s at 2000x*g* and 40 μL aliquots were removed and quenched by addition of 10.4 μL 5% aqueous formic acid to give a final concentration of 1%.

Samples for SDS-PAGE analysis were prepared as follows. To 5.1 μL of each formic-acid quenched sample was added 1.7 μL of 95:5 4X NuPAGE LDS sample buffer:β-mercaptoethanol. Standard samples of 60 μM LicA2 substrates were prepared from 1.5 μL of 3 mg/mL peptide (334 – 338 μM), 4.8 μL H_2O and 2.1 μL 95:5 4X NuPAGE LDS sample

buffer:β-mercaptoethanol. Standard samples of 0.24 μM LicP were prepared from 5.1 μL 0.32 μM LicP and 1.7 μL of 95:5 4X NuPAGE LDS sample buffer:β-mercaptoethanol. Polypeptide Standards (#161-0326) samples were prepared from 20 μL of 20-fold dilute 161-0326, 10 μL H_2O, and 10 μL 95:5 4X NuPAGE LDS sample buffer:β-mercaptoethanol. All SDS-PAGE samples were heated for 10 min at 70 ºC and then loaded on a 10-20% Mini-Protean Tris-Tricine gel (#456-3116) adding 5 μL per lane. The gels were run at 100 V for 125 min in 100 mM Tris, 100 mM Tricine, 0.1% SDS buffer while cooling the entire apparatus in ice. The gels were subjected to consecutive coomassie and silver staining as described on page S9.

Identification of the cleavage sites of the LanP proteases encoded in the genomes of *B. licheniformis* 9945A and *B. cereus* VD156

The LanP proteases from *B. licheniformis* 9945A and *B. cereus* VD156 were purified using a procedure similar to that described for LicP. A His_6-tagged version of dehydrated and cyclized LanA2 encoded in the genome of *B. licheniformis* 9945A was obtained by co-expression of the precursor peptide with its corresponding LanM synthetase in *E. coli* using the same procedure as described for LicA2/LicM2. Linear LanA3 encoded in the genome of *B. cereus* VD156 was also obtained by expression in *E. coli* as an N-terminal His_6-tagged peptide. After IMAC and HPLC purification, the peptides were incubated with their corresponding purified proteases and the results were analyzed using MALDI-TOF MS.

Purification and crystallization of LicP

Purification of LicP for crystallization employed a slightly modified procedure from that described above. Briefly, *E. coli* cells containing overexpressed LicP were lysed by sonication and the lysates were clarified by centrifugation at 4 °C. The clear supernatant containing the soluble fraction was loaded onto a 5 mL immobilized metal ion affinity resin column (Hi-Trap Ni-NTA, G.E. Healthcare) pre-equilibrated with binding buffer (1 M NaCl, 5% glycerol, 20 mM Tris, pH 8.0, 4 °C). The column was washed with 50 mL of 88:12 (v:v) binding buffer: elution buffer (1 M NaCl, 250 mM imidazole, 20 mM Tris, pH 8.0), and the protein was eluted by a linear gradient to 100% elution buffer. Coomassie-stained SDS-PAGE was used to analyze fractions for purity and the cleanest fractions were combined. Samples were further fractionated using anion exchange chromatography (Hi-Trap Q HP, GE Healthcare), and appropriate fractions were combined and adjusted to pH 8.0 using HCl (LicP eluted at ~400 mM NaCl, 20 mM Tris, pH 8.9, 4 °C). The protein was concentrated using Amicon Ultra-4 centrifugal filters (10 kDa molecular weight cut-off, Millipore) and stored in liquid nitrogen until needed. Some aliquots of protein solution were further purified by size exclusion chromatography (Superdex HiLoad 75

16/60, GE Healthcare) in 300 mM KCl, 20 mM HEPES, pH 7.5 buffer. The final concentration of purified protein was quantified by Bradford analysis (Thermo Scientific).

Protein from both anion exchange and size exclusion purifications was used for crystallization by the hanging drop vapor diffusion. Samples purified by anion exchange afforded crystals using a precipitant solution containing 0.05 M cadmium sulfate, 0.1 M HEPES, pH 7.5, 1 M sodium acetate and 8% formamide added to a LicP solution (8.9 mg/mL) in a 1:1 ratio (v/v). Protein purified using size exclusion chromatography afforded crystals when mixed with a solution containing 0.2 M di-ammonium hydrogen citrate, 20% w/v PEG 3350, 2% tacsimate pH 7.0, and 4 mM HEPES pH 6.8. Crystals were stepwise equilibrated with incremental concentrations of glycerol or PEG 3350 up to a final concentration of 30% prior to vitrification by direct immersion in liquid nitrogen.

All diffraction data were collected at insertion device synchrotron beam lines (LS-CAT Sector 21 ID, Advanced Photon Source, Argonne, IL). All data were indexed and scaled using either the HKL2000[5] or XDS package[6]. Crystallographic phases were determined by the molecular replacement method as implemented in Phaser[7] using the coordinates of the S8 peptidase from *S. aureus* determined by the Center for Structural Genomics of Infectious Diseases (PDB Code = 3QFH; 40% sequence identity). There are 8 copies of LicP in the crystallographic assymmetric unit and non-crystallographic symmetry averaging yielded maps of exceptional quality, allowing nearly the entire polypeptide chain to be manually fitted and adjusted using COOT.[8] Cross-validation, using 5% of the data for the calculation of the free R factor,[9] was utilized throughout model building process in order to monitor building bias. The stereochemistry of all of the models was routinely monitored throughout the course of refinement using PROCHECK.[10] Relevant data collection and refinement parameters are provided in **Table S1**. The coordinates for the LicP structure can be accessed under code 4ZOQ in the Protein Data Bank.

Table S1. Data collection, phasing and refinement statistics.

	Native 1
Data collection	
a, b, c (Å)	71.0, 112.8, 114.8
α, β, γ (°)	82.2, 89.7, 82.9
Resolution (Å)[1]	50.00 -2.35 (2.45 - 2.35)
Total reflections	568.612
Unique reflections	143,709
R_{sym} (%)	8.6 (88.5)
I/σ(I)	13.14 (1.73)
Completeness (%)	98.5 (97.8)
Redundancy	4.0 (4.0)
Refinement	
Resolution (Å)	25.0 – 2.35
No. reflections used	143,685
R_{work} /R_{free}[3]	19.9/24.9
Number of atoms	
Protein	24,462
Solvent	1,100
B-factors	
Protein	51.8
Peptide	49.4
R.m.s deviations	
Bond lengths (Å)	0.011
Bond angles (°)	1.54

1. Highest resolution shell is shown in parenthesis.
2. Figure of merit- Probability weighted average of the cosine of the phase error, before and after density modification.
3. 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.

Table S2. Primer sequences for cloning of *licP-25-433*, *licM2*, *licA2*, *licP-25-433-S376A*, *licP-25-433-H186A*, *licP-25-433-E100A*, *licP-25-433-E100A-E102A, G-licA2*, *NDVNPE-ProcA1.7*, *NDVNPE-NisA*, *NDVNPE-NisA-I1G*, *NDVNPE-NisA-I1T*, *NDVNPE-NisA-I1C*, *NDVNPE-NisA-I1L*, *NDVNPE-NisA-I1F*, *NDVNPE-NisA-I1W*, *NDVNPE-NisA-I1K, NDVNPE-NisA-I1E*, *MBP-BamL-NDVNPE*, *licA2-E–1A*, *licA2-E–1D*, *licA2-E–1Q*, *licA2-P–2A*, *licA2-N–3A*, *licA2-V–4A*, *licA2-V–4L*, *licA2-V–4N, licA2-V–4F*, *licA2-D–5A*, *licA2-D–5K*, *licA2-D–5V, LanA2-9945A, LanM2-9945A, LanP-42-476-9945A*.

Primer Name	Primer Sequence (5'-3')
LicP_25_BamHI_FP	AAAAA GGATCCG AAAGAACAAGCAGGAGAACAG
LicP_NotI_RP	AAAAA GCGGCCGC TCACTCCTTG TTCATCATTT T
LicA2_BamHI_FP	AAAAA GGATCCG ATGAAAACAA TGAAAAATTC A
LicA2_NotI_RP	AAAAA GCGGCCGC CTAGCATCGG CTTGTACACT T
LicM2_NdeI_FP	AAAAA CATATG GTTTTCT TCGCCAAAGG GATG
LicM2_KpnI_RP	AAAAA GGTACC TCACCTGCCC GTCGGAATAT C
G-LicA2_ _QC_FP	CCAGGAT GGT ATGAAAAC AATGAAA AATTCAGCTGCCCGT
G-LicA2_QC_RP	GTTTTCAT ACC ATCCTGG CT GTGGTGATGA TGGTGATGG
ProcA1.7_EcoRI_FP	GGT GCG AGG AAT TCG ATG AAG CAT AGA CAA CTA AAT CTG
ProcA1.7_NotI_RP	ATA ATA TCG CGG CCG CTC AGC ACA TTT TCC C
NisA_BamHI_FP	CTA GAT GGA TCC GAT GAG TAC AAA AGA TTT TAA CTT GG
NisA_HindIII_RP	CTA GAA GCT TTT ATT TGC TTA CGT GAA TAC TAC AAT G
LicP-S376A_QC_FP	GGAACA GCA TTGGCC GCCCCG CAGGTAGCT
LicP-S376A_QC_RP	GG CCAA TGC TGT TCC GTATGAG AGGGAATATC CCTTTGGGAT
LicP-H186A_QC_FP	ACA GGA GCC GGAAC ACAA ACAGCCGGGATGATCAATATC
LicP-H186A_QC_RP	G TTCC GGC TCC TGT CGGATCT CCGGATACAG GC
LicP-E100A_QC_FP	CAGTAAAC GCA ACGGAATC A GTCATCAGCGGTTCGCC
LicP-E100A_QC_RP	GATTCCGTTGCGTTTACTG CTGTATT TGCAATCGGC TTTTCAATGAC
LicP-E100A-E102A_QC_FP	GCAACG GCA TCAGTC ATCAGCGGTTCGCCTG
LicP-E100A-E102A _QC_RP	GACTGA TGC CGTTGC GTTTA CTGCTGTATT TGCAATCGGC
ProcA1.7_core_FP	ACCATTGGGGGA ACCATTGTG
ProcA1.7- NDVNPE _RP	GGTTCC CCCAATGGT TTCAGGATTGACGTCATT CAG CTCAGCATCA GACAGGT
NisA_core _FP	ATTACAAGTATTTCGCTATGT
NisA- NDVNPE _RP	CGAAATACTT GTAAT TTCAGGATTGACGTCATT ATCTTTC

	TTCGAAACAG ATA
NDVNPE-NisA-I1G_QC_FP	CAATCCTGAA GGT ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1G_QC_RP	GAAATACTTGT ACC TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
NDVNPE-NisA-I1C_QC_FP	CAATCCTGAA TGT ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1C_QC_RP	GAAAT ACTTGT ACA TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
NDVNPE-NisA-I1T_QC_FP	CAATCCTGAA ACC ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC AG
NDVNPE-NisA-I1T_QC_RP	GAAATACTTGT GGT TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACCA
NDVNPE-NisA-I1L_QC_FP	CAATCCTGAA CTT ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1L_QC_RP	GAAATACTTGT AAG TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
NDVNPE-NisA-I1F_QC_FP	CAATCCTGAA TTT ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1F_QC_RP	GAAATACTTGT AAA TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
NDVNPE-NisA-I1W_QC_FP	CAATCCTGAA TGG ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1W_QC_RP	GAAATACTTGT CCA TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
NDVNPE-NisA-I1K_QC_FP	CAATCCTGAA AAA ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1K_QC_RP	GAAATACTTGT TTT TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
NDVNPE-NisA-I1E_QC_FP	CAATCCTGAA GAA ACAAGTATTTC GCTATGTACACC CGGTTGTAAAAC
NDVNPE-NisA-I1E_QC_RP	GAAATACTTGT TTC TTCAGGATTG ACGTCATTATCTTTCTTCGAAACAGATACC
MBP-BamL-NDVNPE_QC_FP	AATGACGTCAATCCTGAA TCTGGTTCT

	GAGAACCTGTACTTCCAATCC
MBP-BamL-NDVNPE_QC_RP	TTCAGGATTGACGTCATTAGATCCACGCG GAACCAG
LicA2-E–1A_QC_FP	TCAATCCT GCA ACAACTC CTGCTACAACCTCTTCTTGG AC
LicA2-E–1A_QC_RP	GAGTTGT TGC AGGATTGA CGTCATTTCC TCCTACCAAA GC
LicA2-E–1D_QC_FP	TCAATCCT GAT ACAACTC CTGCTACAACCTCTTCTTGG AC
LicA2-E–1D_QC_RP	GAGTTGT ATC AGGATTGA CGTCATTTCC TCCTACCAAA GC
LicA2-E–1Q_QC_FP	TCAATCCT CAA ACAACTC CTGCTACAACCTCTTCTTGG AC
LicA2-E–1Q_QC_RP	GAGTTGT TTG AGGATTGA CGTCATTTCC TCCTACCAAA GC
LicA2-P–2A_QC_FP	ACGTCAAT GCT GAA ACAA CTCCTGCTACAACCTCTTCTTG
LicA2-P–2A_QC_RP	TTGTT TC AGC ATTGA CGT CATTTCC TCCTACCAAA GCTTTCAATT
LicA2-N–3A_QC_FP	GACGTC GCT CCTGAA ACAACTCCTGCTACAACCTCT
LicA2-N–3A_QC_RP	TTCAGG AGC GACGTC ATTTCC TCCTACCAAA GCTTTCAATT
LicA2-V–4A_QC_FP	AAATGAC GCC AATCCTG AA ACAACTCCTGCTACAACC TCTT
LicA2-V–4A_QC_RP	CAGGATT GGC GTCATTT CC TCCTACCAAA GCTTTCAATT CC
LicA2-V–4L_QC_FP	GAAATGAC CTG AATCCTGA A ACAACTCCTGCTACAACC TCTT
LicA2-V–4L_QC_RP	TCAGGATT CAG GTCATTTC C TCCTACCAAA GCTTTCAATT CCTC
LicA2-V–4N_QC_FP	GAAATGACAACAATCCTGAAACAACTCCTGCTACAACCTCTT
LicA2-V–4N_QC_RP	CAGGATTGTTGTCATTTCCTCCTACCAAAGCTTTCAATTCC
LicA2-V–4F_QC_FP	GAAATGAC TTC AATCCTGA A ACAACTCCTGCTACAACC TCTT
LicA2-V–4F_QC_RP	TCAGGATT GAA GTCATTTC C TCCTACCAAA GCTTTCAATT CCTC
LicA2-D–5A_QC_FP	AGGAAAT GCC GTCAATC CT GAA ACAACTCCTGCTACAACC
LicA2-D–5A_QC_RP	GATTGAC GGC ATTTCC T CC TACCAAA GCTTTCAATT CCTCTTC
LicA2-D–5K_QC_FP	GGAGGAAAT AAA GTCAATCCT GAA ACAACTCCTGCTACAACCTCT
LicA2-D–5K_QC_RP	AGGATTGAC TTT ATTTCC TCC TACCAAA GCTTTCAATT CCTCTTCG
LicA2-D-5V_QC_FP	GAGGAAATGTGGTCAATCCTGAAACAACTCCTGCTACAACC
LicA2-D-5V_QC_RP	GATTGACCACATTTCCTCCTACCAAAGCTTTCAATTCCTCTTC
9945M2-Gib-FseI-FP	CT CAATTGGATA TCGGCCGG CCAC ATGACTGTTGCAAAAATGAAG
9945M2-Gib-KpnI-RP	TTTACCAGA CTCGAGGGTA CC TTAACATACG GCTCCTCCCT T
9945A2-Gib-BamHI-FP	ACCA TCATCACCAC AGCCAG GATCCG ATGTCACATCGTGAAATGGCT

9945A2-Gib-HindIII-RP	TTAAGCAT TATGCGGCCG CA AGCTT TTAGCAATCA GATGTACATG C
9945LanP42-Gib-BamHI-FP	TTAAGCAT TATGCGGCCG CA AGCTT TTAGCAATCA GATGTACATG C
9945LanP42-Gib-BamHI-FP	TTAAGCAT TATGCGGCCG CA AGCTT TTAGCAATCA GATGTACATG C

Table S3. Sequences of the synthetic genes for the protease from *B. cereus* VD156 optimized for expression in *E. coli.* The sequence shown in red is predicted to be a secretion signal and was removed in the expression constructs. Also shown is the sequence of the LanA3 precursor peptide. The observed cleavage occurred after the Arg shown in blue (Fig. S20).

Name	Accession number	Sequence (The predicted protease cleavage site is indicated in blue)
Strain: *B. cereus* VD156		
LanA3	EJR72967.1	MKNISEKSVGLSMKKLDTTEMEKIYGASGVDTRTHPTVVVVSRASSKFCVTVAASALLSYNMNKC
	optimized nucleotide sequence	ATGAAAAATA TTTCAGAGAA ATCAGTAGGG CTTAGTATGA AGAAGCTGGA TACCACCGAA ATGGAAAAAA TCTATGGCGC GTCTGGTGTA GACACGCGTA CGCATCCCAC AGTCGTCGTA GTGAGCCGTG CCAGCTCAAA ATTTTGCGTC ACCGTAGCTG CATCTGCACT GCTCTCGTAT AACATGAATA AGTGTTAA
LanP	EJR72593.1	MYRFKKYCLSIISFILIISFFPNNTNATQIAYYSILIRDNTDFNTVLDKLSKDNQEVVYSIPEVNLIQVKG EKGKIISGIGLESIEEINPSTGEFKTYTPNINDQKVLDNKAIWDVQWDIKRITNNGESYKLHSPSGKVS VALIDSGYPENHPDIKSISIQKSKNLVPKGGYKGNEENETGNIHQLTDRTGHGTSVLSQVNADGLMK GVAPGMPVNMYRVFGESSAEGSWIIKGIIEAAKDKNDVINISAGSYLLKNGTYSDGSGNNRAEIKA YEKAIHYANKKGSIVVSALGNDSININIYSELLSILNSKIKDEGKSATGIVQDIPAQLAQVVSVASTGMD SKVSSFSNYGKNIIDFTAPGGDIKLLNKFGADVWMAEEMFKKEMILVAHPQGGYYFNYGNSLATPK VSGALALVIDKYGYKNKPNKAINHLKRNTNAENEIDLYKALQE
	optimized nucleotide sequence	ATGTATCGCT TCAAGAAATA TTGCCTCAGC ATTATCTCGT TCATTCTGAT CATCAGTTTC TTCCCGAACA ATACCAACGC GACTCAAATC GCCTATTACT CAATTCTGAT CCGGGATAAC ACTGACTTCA ACACAGTACT GGACAAATTG AGTAAGGATA ACCAGGAGGT GGTCTATAGC ATCCCGGAAG TCAATCTGAT TCAAGTCAAA GGCGAAAAAG GTAAGATTAT TAGTGGTATC GGTTTAGAGA GTATTGAAGA GATTAACCCG TCAACAGGCG AATTCAAAAC CTACACCCCG AATATTAATG ACCAAAAAGT GCTGGATAAC AAAGCCATCT GGGACGTCCA GTGGGATATC AAACGCATTA CGAACAACGG CGAAAGTTAT AAATTACACT CTCCGAGCGG GAAAGTGAGT GTTGCACTGA TTGATAGCGG TTATCCGGAA AACCACCCAG ATATCAAATC AATCTCCATC CAGAAAAGCA AGAACCTTGT TCCGAAGGGG GGCTATAAGG GAAATGAAGA AAACGAAACC GGTAACATTC ACCAGCTGAC GGATCGCACT GGTCACGGGA CCAGCGTGCT GTCTCAAGTG AACGCCGATG GTTTAATGAA AGGAGTTGCT CCTGGCATGC CTGTGAACAT GTATCGTGTC TTCGGCGAAT CATCGGCAGA GGGGAGTTGG ATCATCAAAG GAATTATCGA GGCCGCCAAG GATAAGAATG ATGTTATCAA CATTTCAGCG GGCAGCTATT TACTGAAGAA CGGCACCTAT TCTGACGGAA GCGGCAACAA TCGGGCAGAA ATCAAAGCAT ACGAAAAGGC GATCCATTAT GCGAACAAAA AAGGAAGCAT TGTTGTGAGC GCTCTGGGCA ACGACAGCAT TAACATTAAT ATCTACTCCG AACTGCTGAG CATCCTGAAT AGCAAAATTA AAGATGAAGG CAAAAGTGCC ACGGGCATCG TGCAAGACAT CCCGGCTCAG CTGGCACAAG TTGTGTCTGT CGCGTCGACG GGCATGGATA GCAAAGTGTC AAGCTTTTCG AACTACGGAA AAAACATTAT TGATTTTACC GCCCCGGGAG GGGATATTAA ACTTCTCAAT AAATTCGGCG CTGATGTTTG GATGGCTGAA

GAAATGTTCA AGAAGGAGAT GATTTTGGTC GCTCACCCGC AGGGTGGCTA CTACTTCAAT TACGGTAACT CCTTAGCTAC GCCCAAGGTC AGCGGTGCCC TCGCTCTCGT GATCGATAAA TATGGTTACA AAAATAAACC CAACAAAGCT ATTAATCATC TGAAACGTAA TACTAACGCG GAGAACGAAA TTGATTTGTA CAAAGCACTC CAAGAGTAA

Table S4. Class II LanP proteins and their predicted secretion signal peptide sequences. Secretion signal peptide sequences are predicted using an online tool PrediSi.[11]

Name	Organism	Accession number	Signal peptide (residue)
LicP	*B. licheniformis* ATCC 14580	AAU42937.1	1-24
CylA	*Enterococcus faecalis*	AFJ74725.1	1-24
CerP	*Bacillus cereus* Q1	ACM15351.1	1-36
CrnP	*Carnobacterium maltaromaticum*	AHF21241.1	1-30
LanP	*B. licheniformis* 9945A	AGN34600.1	1-37
LanP	*Bacillus cereus* FRI-35	AFQ13336.1	1-25
LanP	*Kyrpidia tusciae* DSM 2912	ADG07479.1	1-31
LanP	*Enterococcus caccae* ATCC BAA-1240	EOL44526.1	1-28
LanP	*Bacillus cereus* VPC1401	YP_004050051.1	1-31
LanP	*Bacillus bombysepticus*	AHX21587.1	1-31
LanP	*Bacillus thuringiensis* DB27	CDN38711.1	1-36
LanP	*Planomicrobium glaciei* CHR43	ETP67278.1	1-30
LanP	*Bacillus cereus* VD045	EJR29324.1	1-27
LanP	*Bacillus cereus* VD156	EJR72593.1	1-27

Figure S1. (a) Lanthionine synthetases and proteases involved in the biosynthesis of class I and class II lanthipeptides. **(b)** The biosynthetic gene cluster of lichenicidin and the cleavage events employed during lichenicidin maturation.

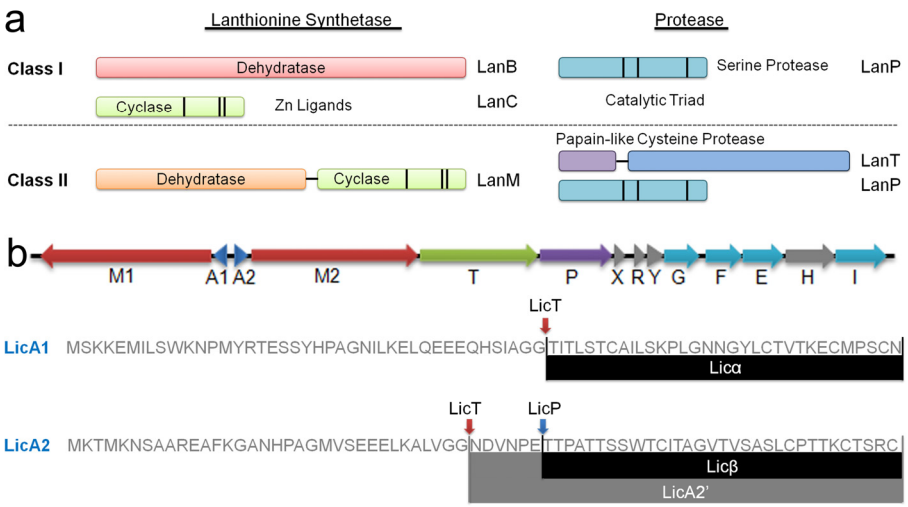


Figure S2. SDS-PAGE image of soluble His_6-LicP-25-433-S376A.

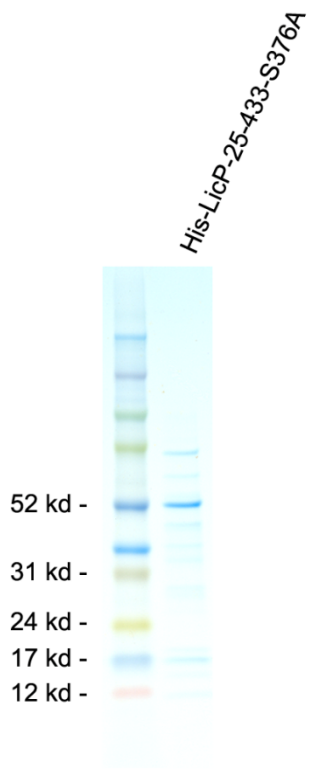


Figure S3. Investigation whether LicP cleavage is intra- or intermolecular. To differentiate whether the self-cleavage of LicP occurred intramolecularly or intermolecularly, His_6-LicP-25-433 (consisting of a complex of His_6-LicP-25-100 and LicP-101-433) was incubated with His_6-LicP-25-433-S376A. The reaction was monitored by SDS-PAGE to determine if wild type LicP catalyzes the proteolytic cleavage of His_6-LicP-25-433-S376A. When incubated separately, His_6-LicP-25-433 and His_6-LicP-25-433-S376A did not show any changes throughout the 19-hour incubation period, whereas the full length protein His_6-LicP-25-433-S376A was consumed gradually when incubated with His_6-LicP-25-433, suggesting that cleavage of LicP can take place intermolecularly. Proteins were supplied at a final concentration of 0.1 mg/mL each.

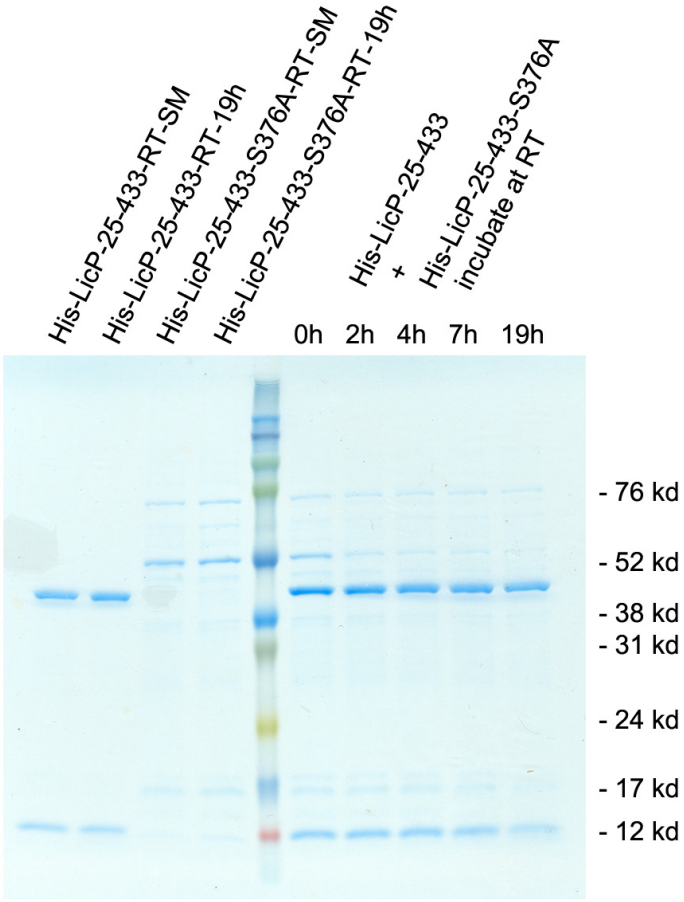


Figure S4. SDS-PAGE (a) and MALDI-TOF MS (b) analysis of His_6-LicP-25-433-E100A. His_6-LicP-25-102-E100A, calculated M: 10,096, average; observed $M+H^+$: 10,099, average. LicP-103-433, calculated M: 37,219, average; observed $M+H^+$: 37,207, average.

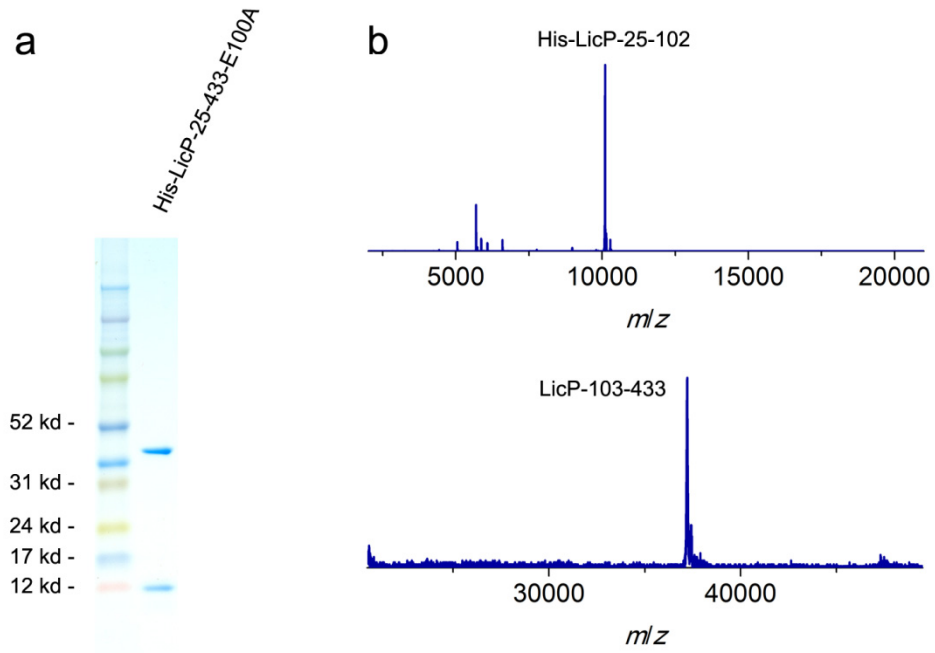


Figure S5. MALDI-TOF mass spectra of linear LicA2 (a) and LicM2-modified LicA2 (b). LicA2, calculated M: 8,930, average; observed $M+H^+$: 8,929, average. LicM2-modified LicA2, calculated $M-12H_2O$: 8,714, average; observed $M-12H_2O+H^+$: 8,713, average. Gluconoylation at the N terminus of LicA2 was introduced when expressing the peptide in *E. coli* BL21(DE3), resulting in a +178 Da peak in addition to the peak with the desired mass.[11]

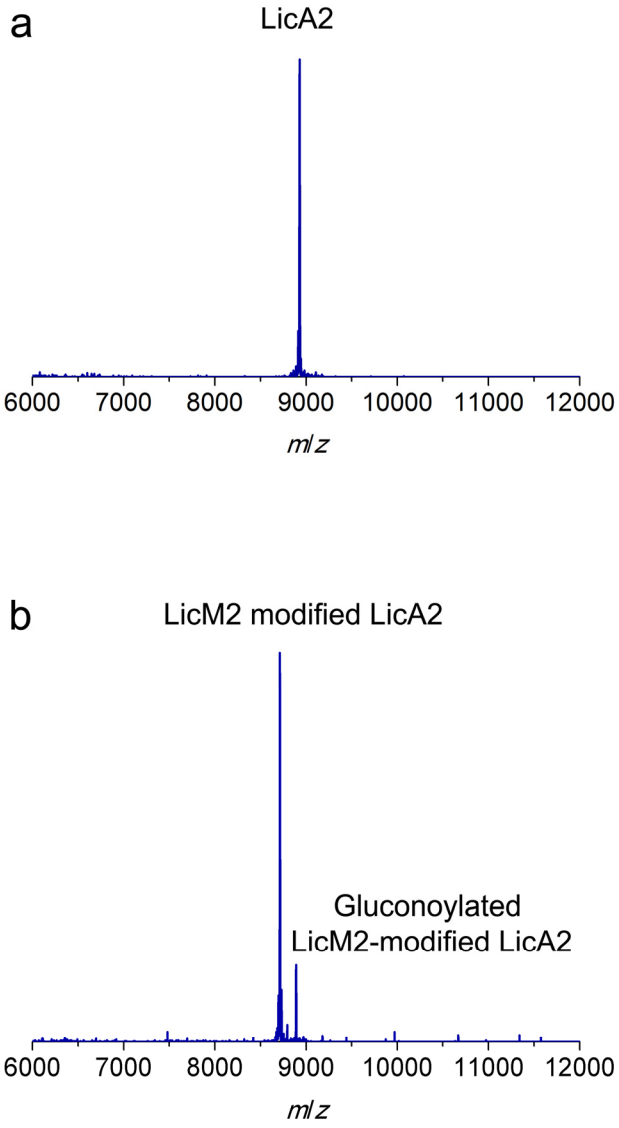


Figure S6. MALDI-TOF mass spectra for LicM2 modified LicA2 (a) and linear LicA2 (b) incubated with LicP. Licβ, calculated M: 3,019.4, monoisotopic; observed $M+H^+$: 3,020.6, monoisotopic. LicA2 leader peptide, calculated M: 5,711, average; observed $M+H^+$: 5,711, average (a), 5,713, average (b). Linear LicA2 core peptide was not observed presumably due to poor solubility and/or ionization efficiency.

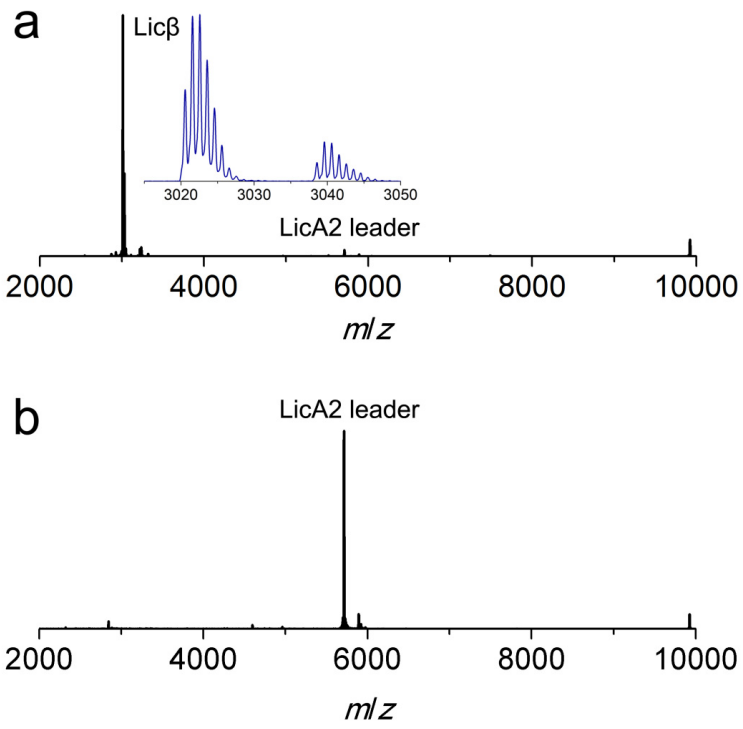
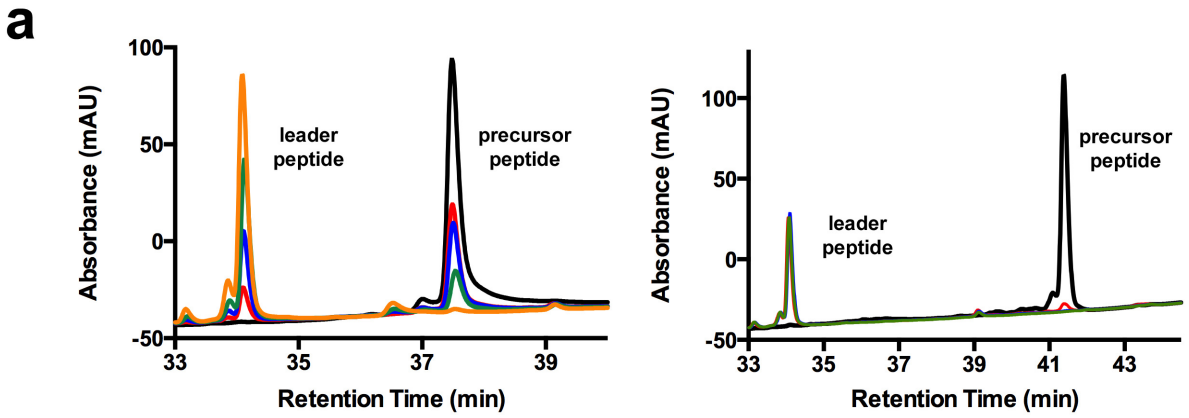
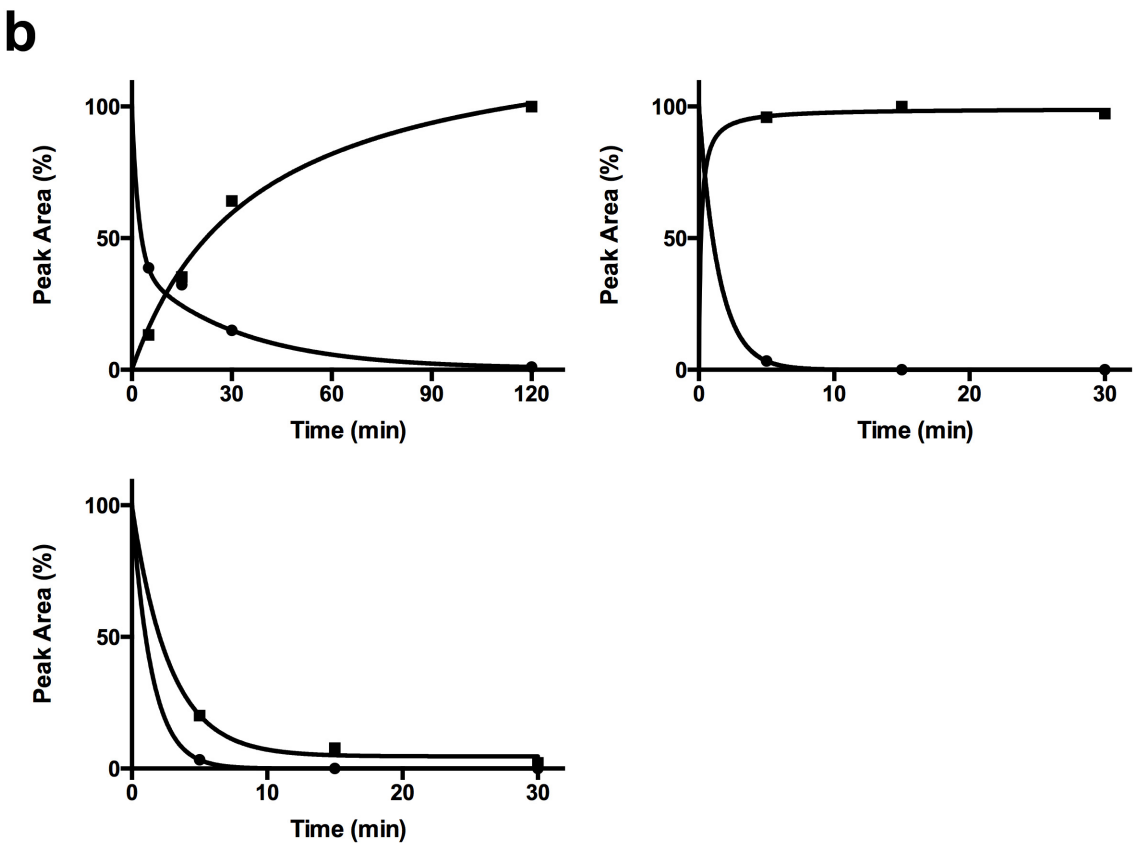
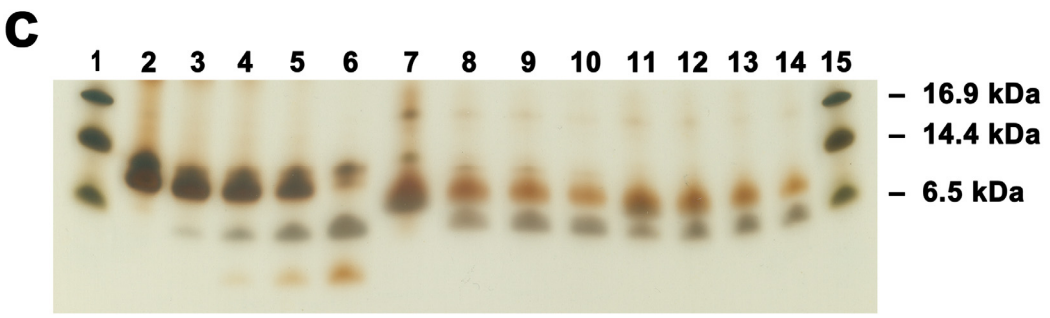


Figure S7. Analysis of the time dependence of LicP activity toward modified and linear LicA2 peptides. Consumption of precursor peptide and appearance of leader peptide were monitored by both HPLC and SDS-PAGE. **(a)** HPLC traces: *Left*: Incubation of 100 μM linear LicA2 with 0.4 μM LicP. *Right*: Incubation of 100 μM modified LicA2 with 0.4 μM LicP. black: 0 min; red: 5 min; blue: 15 min; green: 30 min; orange: 2 h. **(b)** Graphs of different time points (x axis) vs. percentages of precursor peptide remaining and leader peptide formed (y axis) as calculated from peak areas in the HPLC traces: *Top left*: linear LicA2 remaining (circles) and leader peptide formed (squares) with 0.4 μM LicP. *Top right*: modified LicA2 remaining (circles) and leader peptide formed (squares) with 0.4 μM LicP. *Bottom*: modified LicA2 remaining with 0.4 μM (circles) and 0.1 μM LicP (squares). Absorbance was monitored at 220 nm. **(c)** SDS-PAGE analysis of HPLC samples with consecutive coomassie and silver staining. **(d)** Competitive MALDI-TOF MS assay of modified LicA2 and linear G-LicA2 peptides treated with LicP, monitoring the consumption of precursor peptides (*Top*) and expanded-view MALDI-TOF mass spectra of modified LicA2 and linear G-LicA2 peptides treated with or without LicP (*Bottom*). Modified LicA2 and linear G-LicA2 were each supplied with a final concentration of 17 μM. For the purple trace, 2.1 μM of LicP was added and the reaction was incubated at room temperature for 12 h (asterisk); for the other traces, 21 nM LicP was employed. The intensity of the highest peak observed in the region of 8,600-9,000 Da was set to 100%. No signal was observed in this region for the purple trace. Although linear and modified LicA2 were present at the same concentration, at t = 0, the modified peptide is the highest intensity peak because of higher ionization efficiency. At t = 5 min, almost all modified LicA2 has been consumed and the linear LicA2 peak is now set to 100% (the relative increase in intensity of the linear LicA2 peak does not signify that its concentration is increased). Linear LicA2 core peptide was not observed presumably due to poor solubility.







Lanes: **1**: #161-0326 ladder; **2**: linear LicA2; **3**: linear LicA2, 5 min with 0.4 μM LicP; **4**: linear LicA2, 15 min with 0.4 μM LicP; **5**: linear LicA2, 30 min with 0.4 μM LicP; **6**: linear LicA2, 2 h with 0.4 μM LicP; **7**: modified LicA2; **8**: modified LicA2, 5 min with 0.4 μM LicP; **9**: modified LicA2, 15 min with 0.4 μM LicP; **10**: modified LicA2, 30 min with 0.4 μM LicP; **11**: modified LicA2, 5 min with 0.1 μM LicP; **12**: modified LicA2, 15 min with 0.1 μM LicP; **13**: modified LicA2, 30 min with 0.1 μM LicP; **14**: modified LicA2, 2 h with 0.1 μM LicP; **15**: #161-0326 ladder.

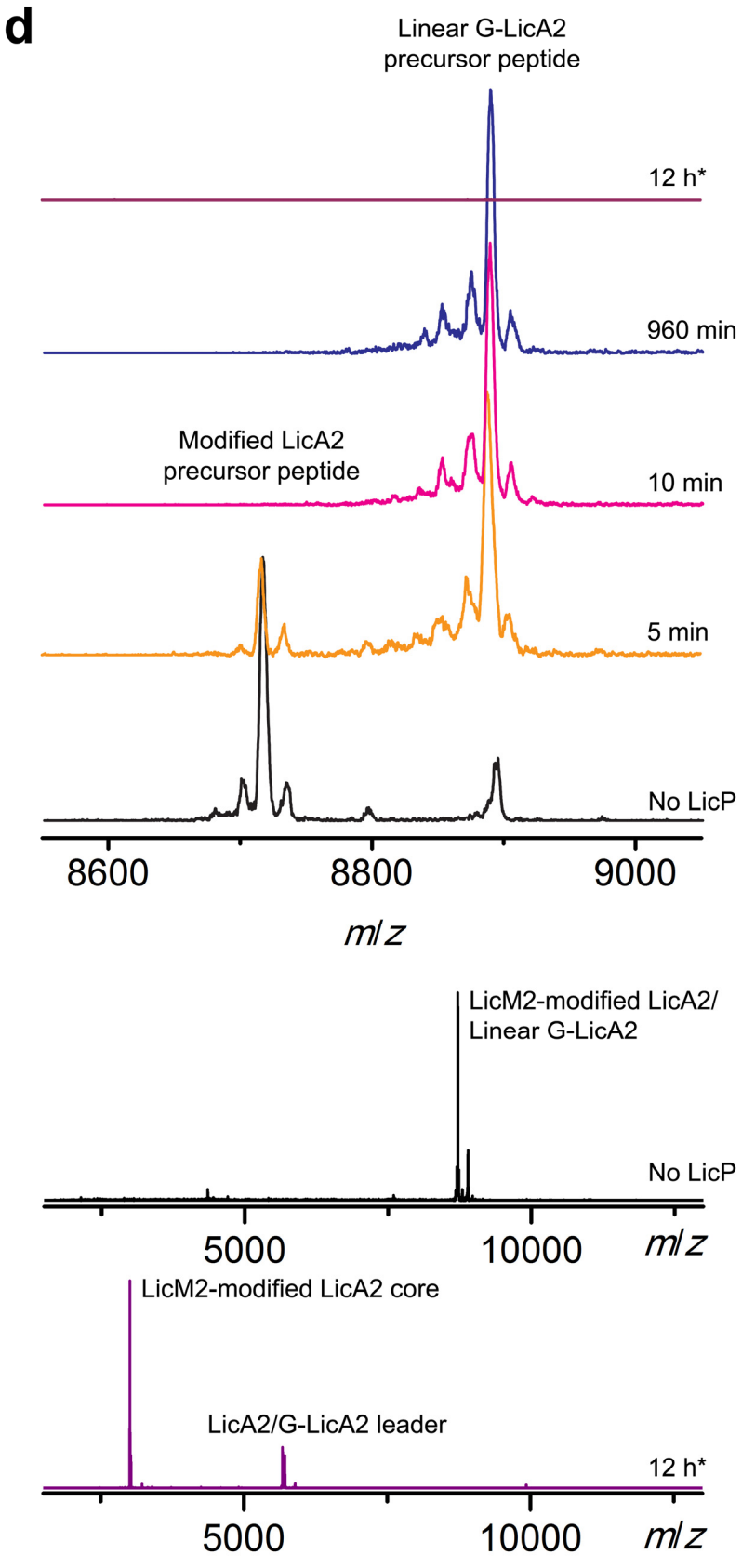

d
Linear G-LicA2
precursor peptide
12 h*
960 min
Modified LicA2
precursor peptide
10 min
5 min
No LicP
8600
8800
9000
m/z
LicM2-modified LicA2/
Linear G-LicA2
No LicP
5000
10000
m/z
LicM2-modified LicA2 core
LicA2/G-LicA2 leader
12 h*
5000
10000
m/z

Figure S8. MALDI-TOF analyses of NDVNPE-ProcA1.7 (a and b) and NDVNPE-NisA (a and c) peptides and their cleaved products when incubated with LicP (d and e). NDVNPE-ProcA1.7, calculated M: 12,244, average; observed $M+H^+$: 12,246, average. NDVNPE-NisA, calculated M: 7,557, average; observed $M+H^+$: 7,558, average. ProcA1.7 core peptide, calculated M: 2,256.1, monoisotopic; observed $M+H^+$: 2,257.6, monoisotopic. NDVNPE-ProcA1.7 leader peptide, calculated M: 10,004, average; observed $M+H^+$: 10,003, average. NisA core peptide, calculated M: 3,495.6, monoisotopic; observed $M+H^+$: 3,496.4, monoisotopic. NDVNPE-NisA leader peptide, calculated M: 4,074.9, monoisotopic; observed $M+H^+$: 4,075.5, monoisotopic. Gluconoylation at the N terminus of NDVNPE-NisA was introduced when expressing the peptide in *E. coli* BL21(DE3), resulting in a +178 Da peak in addition to the peak with the desired mass.[11] The reasons why some peptides are gluconoylated and others not are not known.

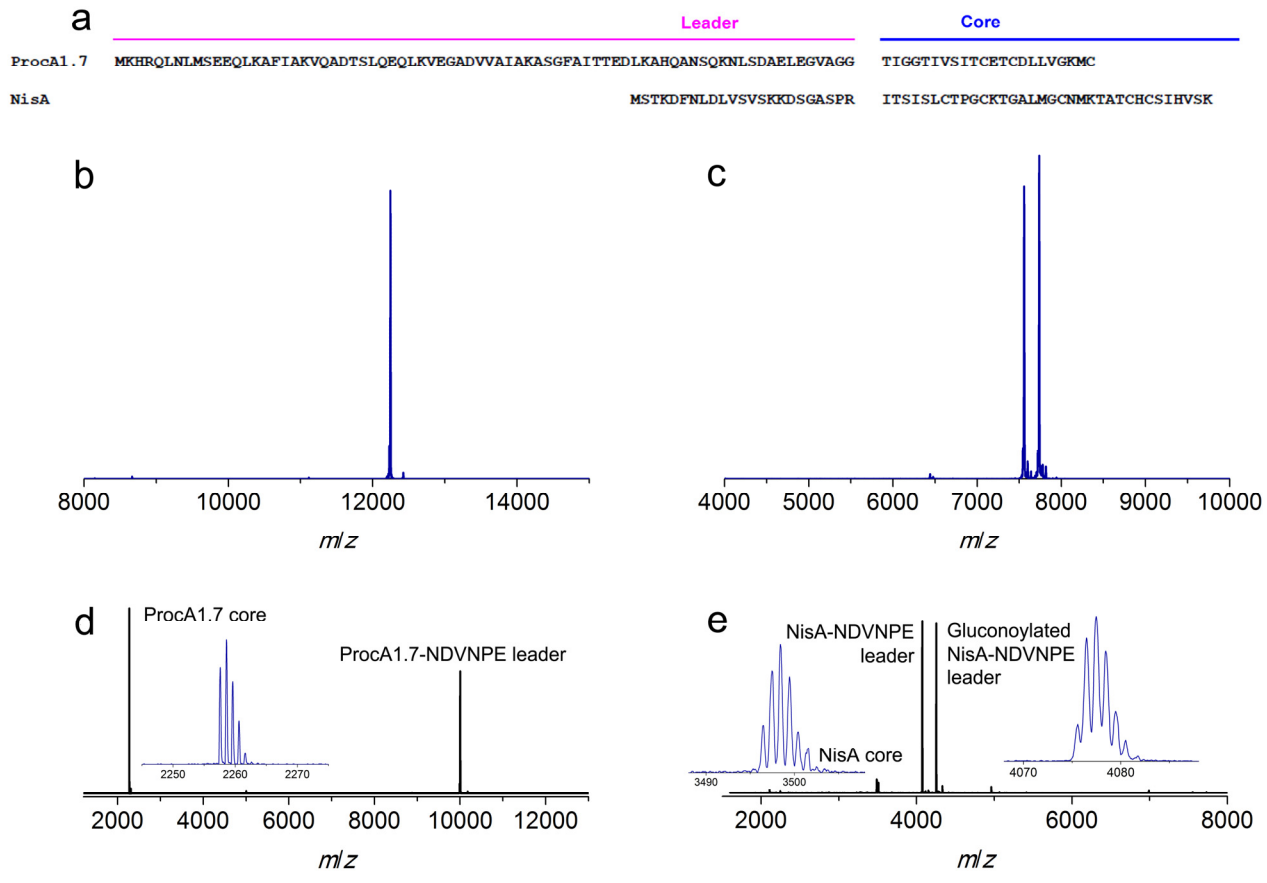


Figure S9. MBP-BamL protein incubated with TEV or LicP. MBP-BamL (50 μM) was incubated with LicP or TEV (final concentration 0.54 μM) at 4 °C and the reactions were quenched at different time points before analysis by SDS-PAGE. O/N: overnight.

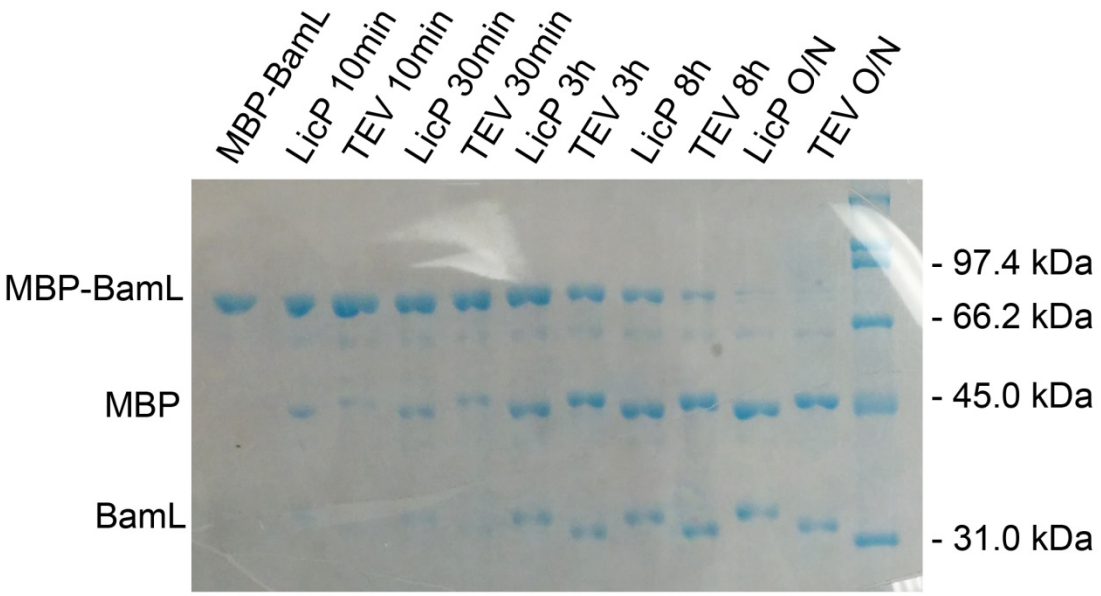


Figure S10. MALDI-TOF mass spectra for NDVNPE-NisA peptides with various P1' substitutions. Gluconoylation at the N terminus of NDVNPE-NisA peptides was introduced when expressing these peptides in *E. coli* BL21(DE3), resulting in a +178 Da peak in addition to the peak of the desired mass.[11] An unidentified modification of +58 Da was installed on NDVNPE-NisA-I1K, but MALDI-TOF MS analysis suggests that the desired species is the major product.

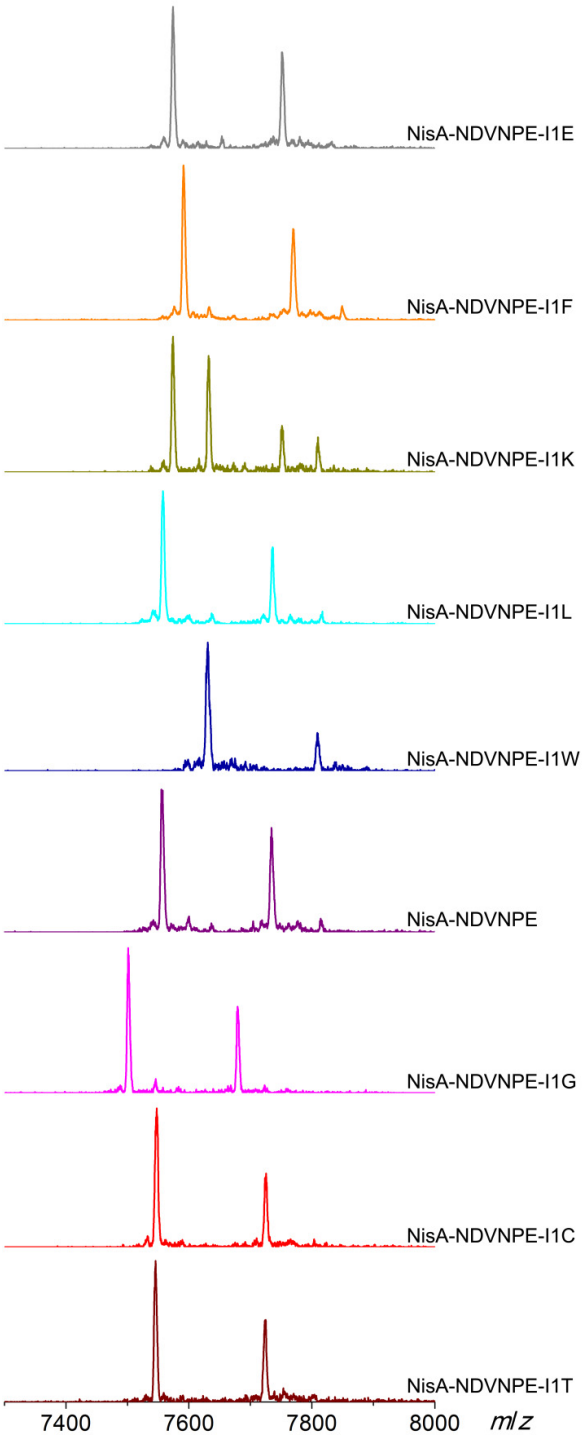


Figure S11. MALDI-TOF mass spectra for NDVNPE-NisA peptides with various P1' substitutions incubated with LicP. For all reactions, 0.5 mg/mL (65 μM) of NisA variants were included. For NDVNPE-NisA-I1T and NDVNPE-NisA-I1C, LicP was supplied at a final concentration of 0.01 mg/mL (210 nM) (enzyme:substrate = 310:1) and the reactions were incubated at room temperature for 20 hours. For other NisA variants, LicP was supplied at a final concentration of 0.1 mg/mL (2.1 μM) (enzyme:substrate = 31:1) and the reactions were incubated at room temperature for 30 hours.

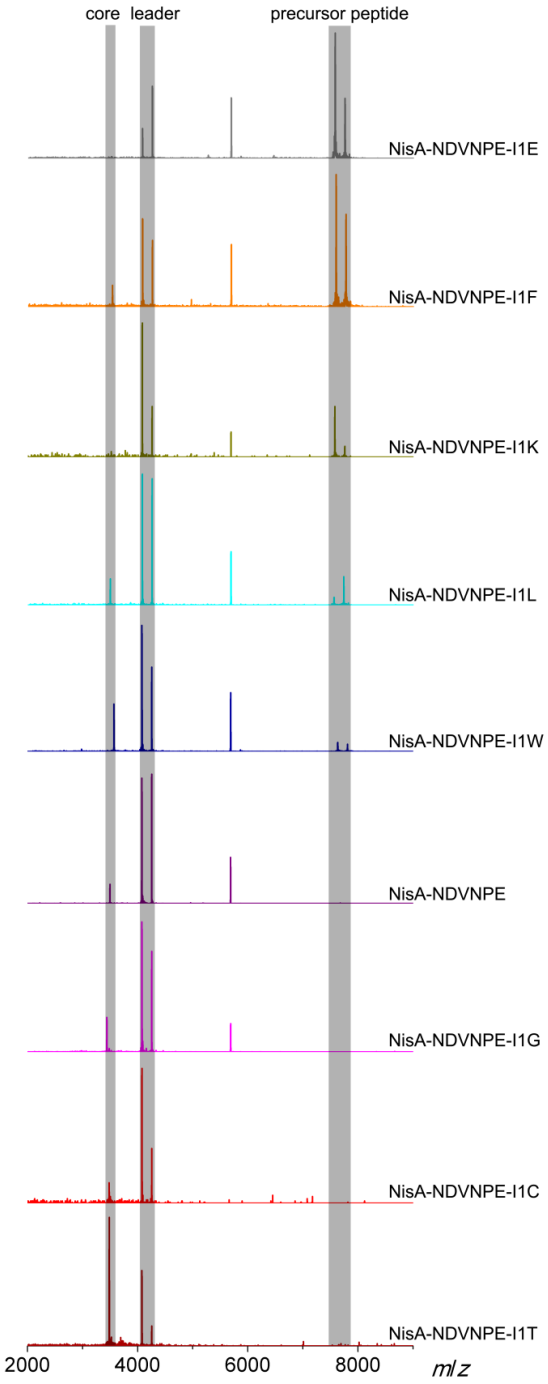


Figure S12. The organization of LicP, including the prodomain, cleavage site, and protease domain. The large distance separating the two ends of the peptides that are formed by protease cleavage is indicated.

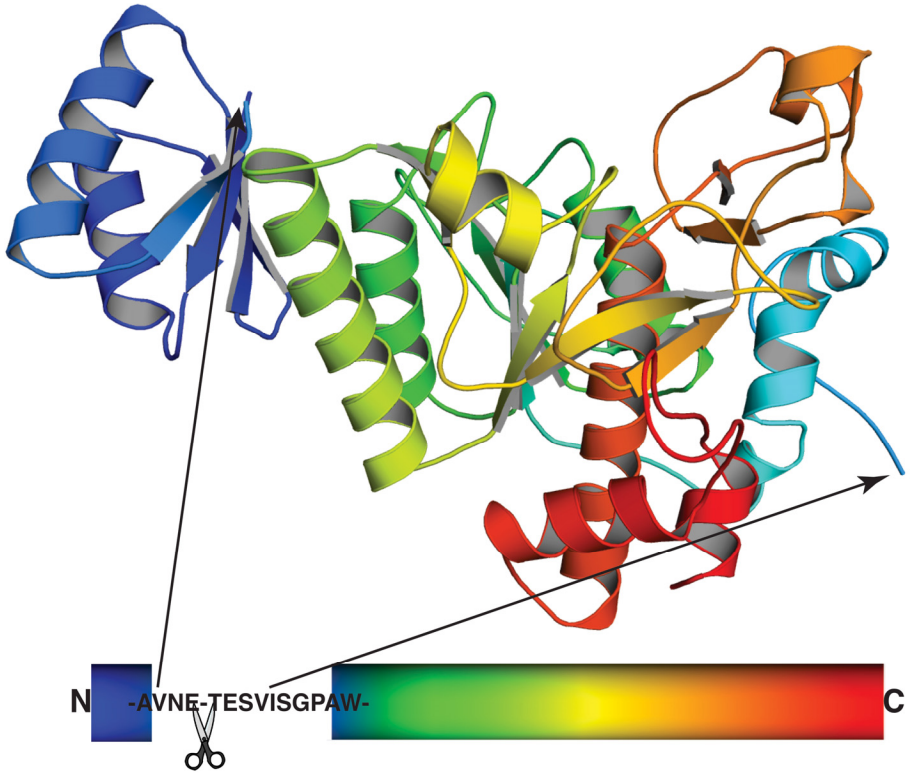


Figure S13. LicP assay with wild type LicA2, LicA2-E–1A, and LicA2-E–1Q.
Linear LicA2 peptides (100 μM) were incubated with 0.4 μM LicP for 30 min, 2 h or 7.5 h. The reactions were quenched with formic acid and the assay was analyzed by SDS-PAGE with consecutive coomassie and silver staining.
Lanes: **1**: #161-0326 ladder; **2**: wild type LicA2; **3**: wild type LicA2, 30 min with LicP; **4**: wild type LicA2, 2 h with LicP; **5**: wild type LicA2, 7.5 h with LicP; **6**: LicP; **7**: LicA2-E–1A; **8**: LicA2-E–1A, 30 min with LicP; **9**: LicA2-E–1A, 2 h with LicP; **10**: LicA2-E–1A, 7.5 h with LicP; **11**: LicA2-E–1Q; **12**: LicA2-E–1Q, 30 min with LicP; **13**: LicA2-E–1Q, 2 h with LicP; **14**: LicA2-E–1Q, 7.5 h with LicP; **15**: #161-0326 ladder.

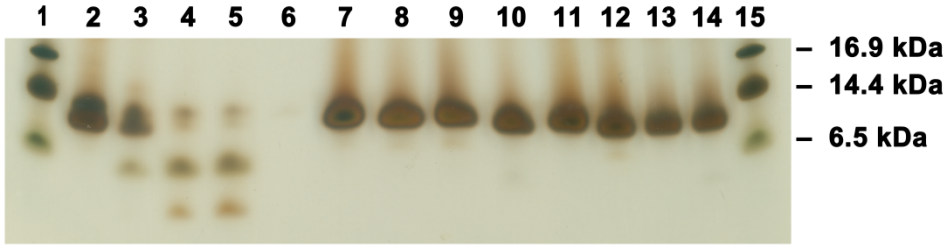


Figure S14. LicP assay with wild type LicA2, LicA2-E–1D, and LicA2-D–5K.
Linear LicA2 peptides (100 μM) were incubated with 0.4 μM LicP for 30 min, 2 h or 7.5 h. Then the reactions were quenched with formic acid and the assay was analyzed by SDS-PAGE with consecutive coomassie and silver staining.
Lanes: **1**: #161-0326 ladder; **2**: wild type LicA2; **3**: wild type LicA2, 30 min with LicP; **4**: wild type LicA2, 2 h with LicP; **5**: wild type LicA2, 7.5 h with LicP; **6**: LicP; **7**: LicA2-E–1D; **8**: LicA2-E–1D, 30 min with LicP; **9**: LicA2-E–1D, 2 h with LicP; **10**: LicA2-E–1D, 7.5 h with LicP; **11**: LicA2-D–5K; **12**: LicA2-D–5K, 30 min with LicP; **13**: LicA2-D–5K, 2 h with LicP; **14**: LicA2-D–5K, 7.5 h with LicP; **15**: #161-0326 ladder.

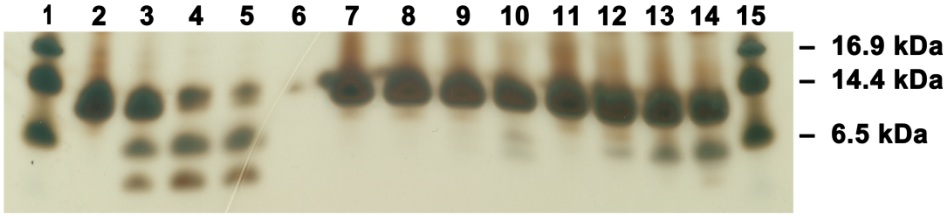


Figure S15. LicP assay with wild type LicA2, LicA2-V–4F, and LicA2-D–5A.
Linear LicA2 peptides (100 μM) were incubated with 0.4 μM LicP for 30 min, 2 h or 7.5 h. The reactions were quenched with formic acid and the assay was analyzed by SDS-PAGE with consecutive coomassie and silver staining.
Lanes: **1**: #161-0326 ladder; **2**: wild type LicA2; **3**: wild type LicA2, 30 min with LicP; **4**: wild type LicA2, 2 h with LicP; **5**: wild type LicA2, 7.5 h with LicP; **6**: LicA2-V–4F; **7**: LicA2-V–4F, 30 min with LicP; **8**: LicA2-V–4F, 2 h with LicP; **9**: LicA2-V–4F, 7.5 h with LicP; **10**: LicA2-D–5A, **11**: LicA2-D–5A, 30 min with LicP; **12**: LicA2-D–5A, 2 h with LicP; **13**: LicA2-D–5A, 7.5 h with LicP; **14**: #161-0326 ladder.

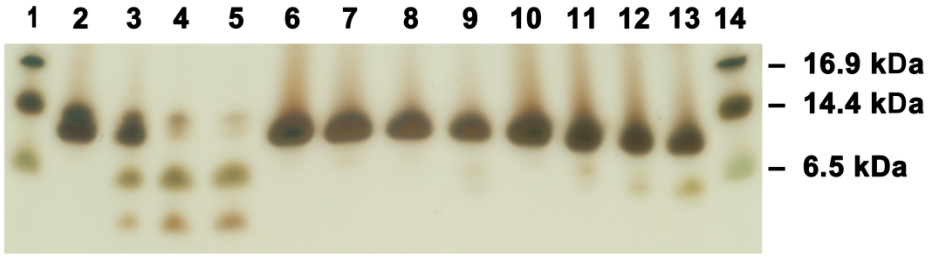


Figure S16. LicP assay with wild type LicA2, LicA2-V–4N, and LicA2-D-5V.
Linear LicA2 peptides (100 μM) were incubated with 0.4 μM LicP for 30 min, 2 h or 7.5 h. The reactions were quenched with formic acid and the assay was analyzed by SDS-PAGE with consecutive coomassie and silver staining.
Lanes: **1**: #161-0326 ladder; **2**: wild type LicA2; **3**: wild type LicA2, 30 min with LicP; **4**: wild type LicA2, 2 h with LicP; **5**: wild type LicA2, 7.5 h with LicP; **6**: LicA2-V–4N; **7**: LicA2-V–4N, 30 min with LicP; **8**: LicA2-V–4N, 2 h with LicP; **9**: LicA2-V–4N, 7.5 h with LicP; **10**: LicA2-D–5V; **11**: LicA2-D–5V, 30 min with LicP; **12**: LicA2-D–5V, 2 h with LicP; **13**: LicA2-D–5V, 7.5 h with LicP; **14**: #161-0326 ladder

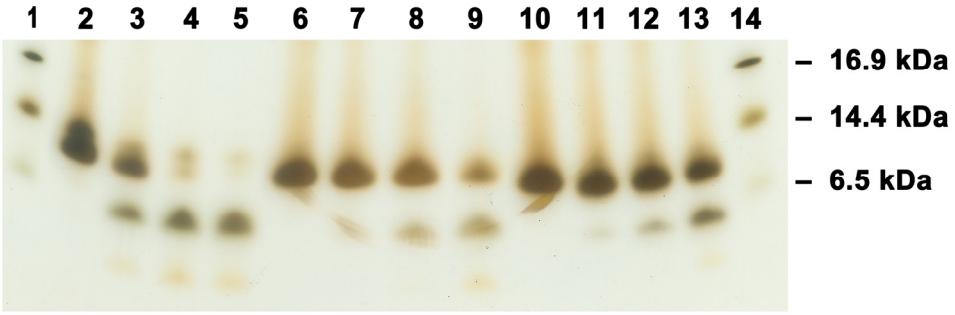


Figure S17. LicP assay with wild type LicA2, LicA2-V–4A, and LicA2-V–4L.
Linear LicA2 peptides (100 μM) were incubated with 0.4 μM LicP for 15 min, 1 h or 2 h. The reactions were quenched with formic acid and the assay was analyzed by SDS-PAGE with consecutive coomassie and silver staining.
Lanes: **1**: #161-0326 ladder; **2**: wild type LicA2; **3**: wild type LicA2, 15 min with LicP; **4**: wild type LicA2, 1 h with LicP; **5**: wild type LicA2, 2 h with LicP; **6**: LicA2-V–4A; **7**: LicA2-V–4A, 15 min with LicP; **8**: LicA2-V–4A, 1 h with LicP; **9**: LicA2-V–4A, 2 h with LicP; **10**: LicA2-V–4L; **11**: LicA2-V–4L, 15 min with LicP; **12**: LicA2-V–4L, 1 h with LicP; **13**: LicA2-V–4L, 2 h with LicP; **14**: #161-0326 ladder.

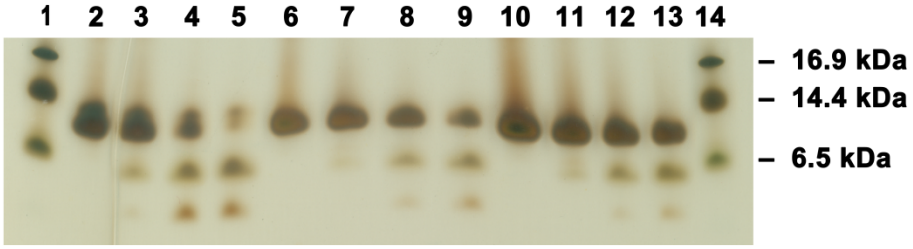


Figure S18. LicP assay with wild type LicA2, LicA2-P–2A, and LicA2-N–3A.
Linear LicA2 peptides (100 μM) were incubated with 0.4 μM LicP for 15 min, 1 h or 2 h. The reactions were quenched with formic acid and the assay was analyzed by SDS-PAGE with consecutive coomassie and silver staining.
Lanes: **1**: #161-0326 ladder; **2**: wild type LicA2; **3**: wild type LicA2, 15 min with LicP; **4**: wild type LicA2, 1 h with LicP; **5**: wild type LicA2, 2 h with LicP; **6**: LicP; **7**: LicA2-P–2A; **8**: LicA2-P–2A, 15 min with LicP; **9**: LicA2-P–2A, 1 h with LicP; **10**: LicA2-P–2A, 2 h with LicP; **11**: LicA2-N–3A; **12**: LicA2-N–3A, 15 min with LicP; **13**: LicA2-N–3A, 1 h with LicP; **14**: LicA2-N–3A, 2 h with LicP; **15**: #161-0326 ladder.

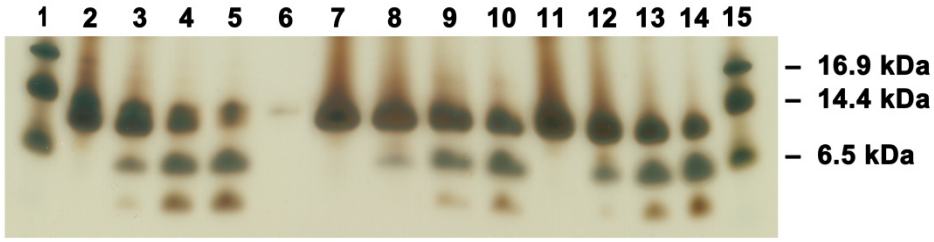
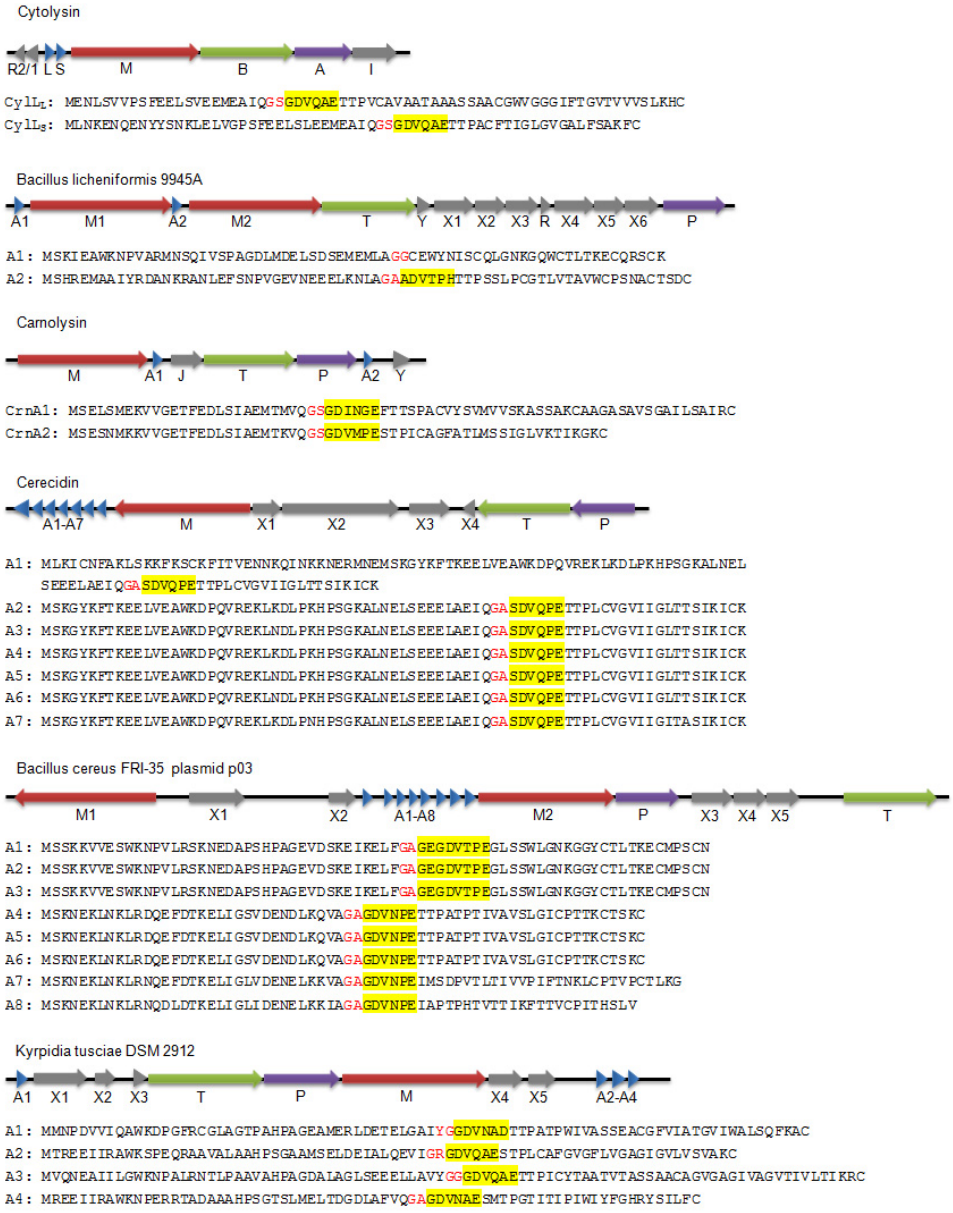
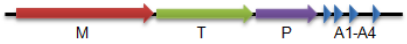


Figure S19. Thirteen class II lanthipeptide biosynthetic gene clusters containing LanP genes. Substrate LanA sequences are listed under the genetic pathways and the putative LanP recognition sequences are highlighted. The predicted sites were verified for the proteins from *B. licheniformis* 9945A and *B. cereus* VD156 (see Figure S20). Clusters were annotated using the standard lanthipeptide biosynthesis nomenclature: LanM proteins catalyze the dehydrations and cyclizations, LanA peptides are lanthipeptide precursors, LanT proteins are transporters with a Cys protease domain, LanJ enzymes are dehydrogenases, LanEFGHI are immunity-conferring proteins, and LanR are regulatory proteins. The cytolysin cluster has historically been annotated differently: The substrates are $CylL_L$ and $CylL_S$, CylB is a transporter with a protease domain, and CylA is the class II LanP. Genes with unknown functions are indicated with X. The cluster for *B. cereus* VD156 is located on four different subcontigs and was manually assembled. LanA3 was chosen for expression since it was entirely on one contig unlike some of the other LanAs.

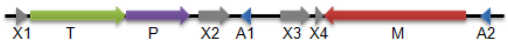


Enterococcus caccae ATCC BAA-1240




```
A1: MEKIVGKAFEELTEEEMKKMQGSGDVQPETTPICGFTIGIGIGALASVKWC
A2: MEKIIGQSFEELSKKEMEAMQGSGDVQAETTPVCAISATVAASSAACAGVGAGIGTGITIVLTFKRC
A3: MEKIIGQSFEELSKKEMEAMQGSGDVQVETTPVCAISATVAASSAACAGVGAGIGTGITIVLTFKRC
A4: MEEIVGKAFEELTEEEMKNMQGSGDVQPETTPLCGFTIGIGIGALVSVKWC
```

Bacillus cereus VPC1401




```
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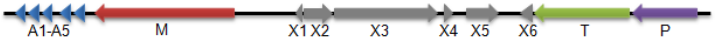
```

Bacillus bombysepticus


```
A1: MNRNQVIEELSVKHPAGAKLVEVSKEELTRISGGGDVQPETTPLCAFGGGVALGLSLSKVYC
A2: MNRNQVIEELAVNHPAGAKLVEVSREELTRVYGGGDVQAETTPMTPTLYLNGITIGLALSKQSC
```

Bacillus thuringiensis DB27




```
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A2: MSKEYKFTKEELVEAWKDPQVREKLKDLPKHPSGKALNELSEEELAEIQGASDVQPETTPLCVGVIIGLTTSIKICK
A3: MSKGYKFTKEELVEAWKDPQVREKLKDLPKHPSGKALNELSEEELAEIQGASDVQPETTPLCVGVIIGLTTSIKICK
A4: MSKGYKFTKEELVEAWKGPQVREKLKDLPKHPSGKALNELSEEELAEIQGASDVQPETTPLCVGVIIGLTTSIKICK
A5: MSREYKFTKEELVEAWKDPQVREKLKDLPKHPSGKALNELSEEELAEIQGASDVQPETTPLCVGVIIGITASIKICK
```

Planomicrobium glaciei CHR43


```
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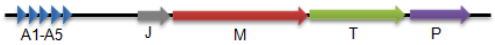
A2: MTKQQIIDAWKNPEIRDGIEKVTPHPSGKAFNELTMDELAEVQGASDVAPETTPACVAAFATGMLFSIKYC
```

Bacillus cereus VD045

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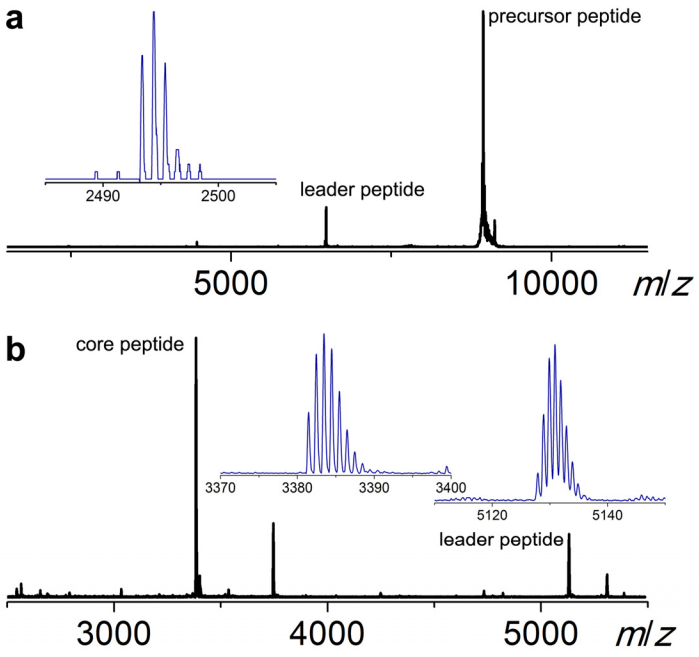
A2: MKNISEKSVGLSMKRLDTTEMEKIYGASGVDTRTSSKACLSGVSAISGLLS
A3: MKNISEKSVGLSMKKLDATEMEKIYGASGVDTRTHPTVVVVSRASSKFCVTVAASAVLSYNMNKC
A4: MKNISEKSVGLSMKKLDTTEMEKIYGASGVDTRTHPTVIVVSRASSKACLSGVSAISGLLSYNKDCIG
A5: MKNISEKSVGLSMKKLDTTEMEKIYGASGVDPRTTPSPLLASFVASYIASAQYRCGKDNKGK
```

Bacillus cereus VD156


```
A1: MRNLKEDVVGLSMKKLNTLEMEKIYGASGVDTRTHSMAIVATTTTRTVGQTGPVTISKVLSLNTICL
A2: MKNISEKSVGLSMKRLDTTEMEKIYGASGVDTRTHPTVIVVSRTSSKACLSGVSAISGLLSYNKDCIG
A3: MKNISEKSVGLSMKKLDTTEMEKIYGASGVDTRTHPTVVVVSRASSKFCVTVAASALLSYNMNKC
A4: MKNISEKSVGLSMKKLDTTEMEKIYGASGVDPRTTPSPLLASFVASYIASAEFRCGKDNKGK
A5: MSTLKENVVGLSMKKLDTTEMEKIYGASGVDPRTTPSPLLASFVASYIASAQYRCGKDNKGK
```

Figure S20. MALDI-TOF mass spectra of two LanA precursor peptides treated with their corresponding LanP proteases that were identified in the genomes of *B. licheniformis* 9945A (a) and *B. cereus* VD156 (b). For the dehydrated and cyclized LanA2 peptide from *B. licheniformis* 9945A, fully modified core peptide is observed after protease treatment (inset): calculated $[M+Na]^+$: 2,493.0, monoisotopic mass; observed $[M+Na]^+$: 2,493.4, monoisotopic mass; the leader peptide is also observed: calculated M: 6,478, average mass; observed $[M+H]^+$: 6,482, average mass. For the LanA3 peptide encoded by the genome of *B. cereus* VD156, the core and leader peptides waere observed after protease treatment. Core peptide: calculated M: 3,382.7, monoisotopic mass; observed $[M+H]^+$: 3,381.8, monoisotopic mass; leader peptide: calculated M: 5,126.5, monoisotopic mass; observed $[M+H]^+$: 5,127.9, monoisotopic mass. The observed proteolytic products are consistent with the cleavage sites predicted in Figure S19.



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