Supplementary Methods

**Efficient *in vivo* Synthesis of Lasso Peptide Pseudomycoidin Proceeds in the Absence of both the Leader and the Leader Peptidase**

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**Bacterial strains and growth conditions.**

*Bacillus pseudomycoides* DSM 12442 strain was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). *E. coli* DH5*α* was used for routine cloning and *E. coli* BL21(DE3) was used for the protein expression as well as a heterologous host for the *psm* cluster expression. For the MALDI MS analysis of the lasso peptide production *E.coli* BL21(DE3) cells harboring corresponding sets of plasmids were grown at 30 °C on M9 minimal agar supplemented with 0.2% lactose and 0.2% arabinose as a sole carbohydrate source, 0.01% thiamine and appropriate antibiotics (50 µg/ml for ampicillin, 50 µg/l kanamycin, 34 µg/ml chloramphenicol). For the MALDI MS analysis of the pseudomicoidin production in the *B. subtilis* 168 cells harboring the *psm* genes under inducible promoters were grown at 37 °C on M9 minimal agar supplemented with 0.4% glycerol, 50 µg/ml L-Tryptophan, 5 µg/ml for erythromycin, and 10 µg/l chloramphenicol. For protein purification the *E.coli* BL21 (DE3) cells were grown at 37 °C in 2YT medium containing 50 µg/ml kanamycin to OD600 of ~0.6. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by growth at 18 °C for 16 h.

**Cloning, mutagenesis, and protein purification.**

Cloning of the *psmCAKB1B2ND* gene cluster, its deletion variants and the separate genes for the expression in *E.coli*. Primers used for cloning are listed in Table S1.

The fragment of the *psm* cluster spanning the *psmC* and *psmA* genes was PCR-amplified and inserted into pCA24 plasmid1 harboring T5*lac* promoter using the Gibson assembly kit (NEB, USA). PCR fragments containing either *psmKB1B2ND* or *psmB1B2ND* gene sets were inserted into pCA24 compatible pBAD30 plasmid between the SacI and SalI sites. The *psmN* and *psmB1B2* deletions were introduced into pBAD-*psmKB1B2ND* plasmid containing arabinose-inducible promoter using the Gibson assembly kit and the corresponding primer set. For the ectopic complementation experiment, the *psmN* and *psmK* genes were cloned into the pCA24 and pBAD30 compatible pColA-Duet plasmid between BamHI and XhoI to get T7*lac* promoter driven expression of the His6-tagged proteins. The overlap–extension PCR2 was carried out to create the D134A and D136A double mutant of the PsmN in the pColA-*psmN* plasmid. The D158A and D159A double mutant of PsmK was obtained using the same technique and pColA-*psmK* plasmid as a template. The *psmB1* gene was subcloned into pET28 plasmid (Novagen) between NdeI and XhoI. The *psmB2* gene was introduced into pET22MBP plasmid3 between BamHI and XhoI.

The combinations of the compatible Duet plasmids (Novagen) containing T7*lac* inducible promoters were used to produce the lasso peptide for the lasso topology study. The *psmC* gene was cloned into the second cassette of the pET-Duet plasmid between the NdeI and XhoI. The PCR product containing the *psmA* was digested with BspHI and SacI, and inserted between NcoI and SacI into the first multiple cloning site of the pET-Duet-*psmC* vector. The double-stranded DNA fragment encoding for the core part of the peptide, PsmAc, was obtained by annealing of the corresponding oligonucleotides and inserted between the NcoI and SacI sites of the pET-Duet-*psmC*. The *psmD* was cloned into the pColA-Duet second expression cassette between NdeI and XhoI. The L21F mutantation of the *psmA* and *psmA*c was obtained by PCR amplification with the T7\_for and PsmA\_RMut primers. The PCR product was digested with NcoI and SaI restriction endonucleases and inserted into the pET-Duet-*psmC* vector linearized with the same enzymes. The *psmB1*and *psmB2* genes were amplified as a single DNA fragment and cloned into pColA-Duet-*psmD* plasmid between NcoI and BamHI. The *psmB1* only was introduced into the same position in the pColA-DUET-*psmD*.

To create *psmCAD* expressing *B. subtilis* strain the psmCA genes the PCR fragment which contains *psmC* and *psmA* genes was inserted into pHT-01 shuttle vector (MoBiTec, GmbH) harboring inducible P*grac*-*lac* promoter between BamHI and SpeI. The expression cassette containing *psmCA* operon flanked by P*grac*-*lac* promoter and the terminator was PCR-amplified using pHT-*psmCA* vector template and inserted into EcoRI site of the pBS2E integrating plasmid4. The resulting pBS2E-psmCA was transformed into *B. subtilis* 168 strain as described in Ref. 5. Integration-positive clones were selected on erythromycin-containing LB plates and checked by PCR for the insert. The *psmD* gene was cloned into the pHT-01 vector between BamHI and SpeI. The pHT-psmD vector was transformed into *B. subtilis* 168 *amy::psmCA* strain and selected on LB agar supplemented with erythromycin and chloramphenicol.

Proteins, fused to His and MBP N-terminal affinity tags were purified using Talon CellThru Co2+-chelating resin (Takara-Clontech) and Amylose (NEB, USA) resin, respectively, as described in Ref. 3. Purity was checked by SDS PAGE and the concentration was measured using Bradford assay (BioRad, USA).

**Pseudomycoidin purification.**

The overnight cultures of *E. coli* BL21(DE3) cells harboring vectors with the required combinations of the *psm* genes were inoculated into fresh LB medium supplemented with 0.4% glucose, and appropriate antibiotics and grown at 37C until OD600 reached 0.6. Cells were harvested by centrifugation and resuspended in 4L of M9 minimal medium contained 10 µg/ml thiamine, appropriate antibiotics. For the cells containing pET-DUET/pColA-DUET vector systems 0.4% glycerol was used as a carbon source and 0.2 μM IPTG was used as inducer of protein expression. For the cells containing pCA/pBAD/pColA-DUET expression vectors combination, a mixture of 0.2% arabinose and lactose were used as a carbon source and as inducers. Cells allowed to grow at 20°C for 10-18 hours then harvested by centrifugation. Culture medium was supplemented with TFA to 0.1% and fractionated using C18 gravity column (Sep-Pak C18, 55-105 μm particle size). The column was washed with 20% acetonitrile/0.1% TFA. Pseudomycoidin-containing fraction was eluted with 30% acetonitrile/0.1% TFA, dried in rotary vacuum evaporator and dissolved in MQ water. Reverse-phase HPLC on C18 Luna semi-preparative column (Phenomenex Luna 5 mm) was performed using gradient elution from 15 to 56% ACN/0.1% TFA during 40 minutes and UV detection at 210nm. Fractions, containing pseudomicoidin forms were collected and repurified using C18 Eclipse column (Agilent Eclipse Plus C18 5m 4.6\*250 mm) and elution 15-50% gradient of acetonitrile in Na phosphate buffer pH 7.0. Fractions containing pseudomycoidin forms were identified using MALDI MS.

**Biochemical reactions.**

For the proteolytic activity test of PsmB1B2 enzyme, 25 µM synthetic PsmA peptide (Genscript, USA) was incubated in the reaction buffer A (20 mM Tris HCl, pH 7.5, 5mM MgSO4, 100 mM NaCl, 1 mM DTT, 1mM ATP) in the presence of either 1µM His6-PsmB1, or 1µM MBP-PsmB2 proteins, or the combination of thereof at 30 °C for 16h.

Endoproteolytic digestion of the pseudomycoidin was performed with 0.1µg/µl GluC from *Staphylococcus aureus* V8 (Sigma-Aldrich) in 50 mM ammonium bicarbonate, pH 7.8 at 37 °C for 3h. Carboxypeptidase Y treatment was carried out as described in Ref. 6. When indicated, samples of the lasso peptides were heated at 95 °C for 2 h a BioRad T100 Thermal Cycler followed by HPLC separation as described above.

**MALDI MS and MS/MS analysis.**

MALDI-TOF MS analysis was performed on UltrafleXetreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Germany) equipped with Nd laser. Samples for analysis were prepared as described earlier.7 Due to high instability of the phosphorylated forms of pseudomycoidin the MH+ molecular ions were measured in linear mode; the accuracy of average mass peak measurement was within 1 Da. Spectra of fragmentation were obtained in LIFT mode, the accuracy of daughter ions measurement was within 1 Da range. Mass-spectra were processed with the use of FlexAnalysis 3.2 software (Bruker Daltonik, Germany) and analyzed manually.

High-resolution mass spectra were recorded on a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Varian 902-MS) equipped with a 9.4 T magnet (FTMS) in positive MALDI mode. Calibration was performed using a ProteoMass Peptide MALDI-MS Calibration Kit (Sigma-Aldrich).

**NMR spectroscopy**

Samples contained ~3mM peptide in 200µl buffer (100mM phosphate pH 6.5 25mM NaCl). High-resolution NMR spectra were recorded on a 800MHz Bruker Avance III spectrometer equipped with a cryogenically cooled QCI probe head. Spectra were acquired at 293K, as matrices of 4k x 400 complex points. The TOCSY experiment was acquired with 4 scans per increment, with a mixing time of 69ms, whereas the NOESY spectra were acquired with 16 scans per increment, with a 200ms mixing time. Spectra were zero-filled to 8k x 1k before apodization with a shifted squared sine bell function, and Fourier transformed with the Bruker Topspin3.5pl7 software. Analysis of the spectra was done manually within the same software package.

**References.**

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**Table S1. Primes used in the study.**

|  |  |
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| Primer name | Sequence 5’-3’ |
| Psm\_CA\_FBgl | TTATAAGATCTTGGGAGGAAAAAAATGAGTGCAA |
| Psm\_CA\_RSal | TTATAGTCGACATAAATCTCATTTAAATATTAGTGCATT |
| PsmK\_FSac | TTATAGAGCTCGGAGGAAAAAAATGATACATACTGTAAAGAAAGAGAT |
| PsmB1\_FSAC | GAGGAGGAAAAATAATGAGTAGTAAACAA |
| PsmD\_RSAL | TTATAGTCGACTTAAATGTTTACTTTGGCCTGT |
| deletion\_N\_F | ATGAGAAATATTTTATATTTTACAAAGCAGTTATAC |
| deletion\_N\_R | ATAACTGCTTTGTAAAATATAAAATATTTCTCATCTCATTCTCCTTCAAGATTTTT |
| deletion\_B\_F | AAAAATCTTGAAGGAGAATGAGATG |
| deletion\_B\_R | GTATACATCTCATTCTCCTTCAAGATTTTTTTATTTTTCCTCCTCTTTTAAAGACTCTAG |
| psmN\_FB(BamhI) | TTATAGGATCCGATGTATACCGGTTGTAATCTTGATT |
| psmN\_RX (XhoI) | TTATATCTCGAGTTACTTCTCCTTACGGTACCTCATTCC |
| psmp\_F\_bamHi | ATGGATCCATGATACATACTGTAAAGAAAGAG |
| psmp\_R\_xhoi | ATAATTCTCGAGTTATTTTTCCTCCTCTTTTAAAGACTC |
| psmN\_D134-6\_F | TCTTCGAACATCAGGGGCGCTGGCGGCCCTTGTTCCTATAGA |
| psmN\_D134-6\_R | TCTATAGGAACAAGGGCCGCCAGCGCCCCTGATGTTCGAAGA |
| psmP\_D158-9\_F | GCTATCAGCTTTTAAGCGCGGCGGTAATTGCTGTGTCTCTT |
| psmP\_D158-9\_R | AAGAGACACAGCAATTACCGCCGCGCTTAAAAGCTGATAGC |
| psmB1\_F\_NdeI | TTATATCATATGAGTAGTAAACAAACAA |
| psmB1\_R\_XhoI | TTATATCTCGAGTCAAAGTTTTTCATCAACCGA |
| psmB2\_F\_BamHI | TTATATGGATCCGATGAATATCATAAATAAAGC |
| psmB2\_R\_XhoI | TTATATCTCGAGTTACCCTATCCTTTTTGC |
| psmC\_NdeI\_F | AATTAACATATGAGTGCAATAACCGGAATTTATC |
| psmC\_XhoI\_R | AATTAACTCGAGTCAAATATATTTTTTGATAAATCGATAGAC |
| psmAс\_NcoI\_F | AATTAACCATGGCTGGACCTGGAAAGAGACTTGTTGACCAAGTTTTTGAAGATGAGGATGAGCAAGGCGCACTTCACCACAGCTAAGAGCTCTTAATT |
| psmAс\_SacI\_R | AATTAAGAGCTCTTAGCTGTGGTGAAGTGCGCCTTGCTCATCCTCATCTTCAAAAACTTGGTCAACAAGTCTCTTTCCAGGTCCAGCCATGGTTAATT |
| PsmA\_RMut | AATTAAGAGCTCTTAGCTGTGGTGAAATGCGCCTTGCTCAT |
| psmD\_NdeI\_F | AATTAACATATGAGAAATATTTTATATTTTACAAAGCAG |
| psmD\_XhoI\_R | AATTAACTCGAGTTAAATGTTTACTTTGGCCTGTTG |
| psmB1\_NcoI\_F | AATTAACCATGGCCATGAGTAGTAAACAAACAATTTCATTACAC |
| psmB2\_R\_BamHI | TTATATGGATCCTTACCCTATCCTTTTTGC |
| psmB1\_BamHI\_R | AATTAAGGATCCTTCTCCTTAGATCTTCAAAGTTTTTCATCAACCGAGATT |
| pHT\_CA\_F\_seq | ATTAAAGGATCCTATGAGTGCAATAACCGGAATT |
| pHT\_CA\_R\_seq | AATATTACTAGTATTAGCTGTGGTGAAGTGCGCC |
| pHT\_Pgrac\_CA\_F | ATTATATGAATTCGGTACCAGCTATTGTAACATAATCG |
| pHT\_Pgrac\_CA\_R | TTATTATGAATTCTGAACATCAAATCGCTTTATTCTTT |
| pHT\_psmD\_BamHI | AATATTGGATCCATGAGAAATATTTTATATTTTACAAAGCAGTTATACT |
| pHT\_psmD\_SpeI\_R | AATATTACTAGTGTTAAATGTTTACTTTGGCCTGTTG |