Supporting Information

Characterization of an unusual gene cluster for a ribosomally-synthesized and post-translationally modified peptide

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Materials

All Chemicals were purchased from Sigma-Aldrich, Roche Biosciences or Fisher Scientific unless noted otherwise. All oligonucleotides were purchased from Integrated DNA Technologies and are shown in Table S1. Restriction endonucleases, DNA polymerases, dNTP solutions and a Gibson Assembly® Cloning Kit were purchased from New England Biolabs. Endoproteinase Glu-C was purchased from Roche Applied Science. Gel extraction, plasmid miniprep, and PCR purification kits were purchased from QIAGEN. Genomic DNA isolation kit (UltraClean® Microbial DNA isolation kit) was purchased from MO BIO Laboratories, Inc. Media components for bacterial cultures were purchased from Fisher Scientific. Protein Calibration Standard I and Peptide Calibration Standard II for MALDI-TOF MS were purchased from Bruker.

Supplemental methods

General methods

For peptide residue numbering, positive residue numbers are used for amino acids in the core peptide counting forwards from the leader peptide cleavage site. Negative numbers are used for amino acids in the leader peptide counting backwards from the cleavage site. All polymerase chain reactions (PCR) were carried out on an automated thermocycler (C1000™, Bio-Rad).

Gibson assembly reaction solutions were made based on a published protocol.[1] DNA sequencing was performed by the Biotechnology Center at the University of Illinois at Urbana-Champaign (UIUC) and ACGT, Inc. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were carried out at the Mass Spectrometry Facility of UIUC on an UltrafleXtreme mass spectrometer (Bruker Daltonics). For MALDI-TOF MS analysis, samples were desalted using ZipTipC18 (Millipore) and spotted onto a MALDI target plate with a matrix solution containing 35 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 3:2 MeCN/H_2O with 0.1% trifluoroacetic acid (TFA) or 15 mg/mL sinapinic acid in 3:2 MeCN/H_2O with 0.1% TFA. Peptides were desalted by using a C4 solid-phase extraction (SPE) column and further purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Delta 600 instrument (Waters) equipped with a Phenomenex C18 column at a flow rate of 8 mL/min. For RP-HPLC, solvent A was 0.1% TFA in H_2O and solvent B was 4:1 MeCN/H_2O containing 0.086% TFA. An elution gradient from 0% solvent B to 100% solvent B over 45 min was used unless specified otherwise. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-Q/TOF-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).

Strains and plasmids

Lachnospiraceae bacterium C6A11 was obtained from Dr. William Kelly (AgResearch, New Zealand). *E. coli* DH5α and *E. coli* NEB® Turbo was used as host for cloning and plasmid propagation and *E. coli* BL21 (DE3) was used as host for expression of proteins and peptides. Co-expression vectors pRSFDuet-1 and pETDuet-1 were obtained from Novagen.

Culturing *Lachnospiraceae bacterium* C6A11 and genomic DNA isolation

Anaerobic culture of *Lachnospiraceae bacterium* C6A11 was performed in the same way as described for *Ruminococcus flavefaceins* FD-1.[2] Liquid RM02 and agar *L. bacterium* C6A11 slants were kindly provided by Dr. William Kelly's laboratory and the liquid cultures were transferred to fresh media and allowed to incubate for 4-5 days. Upon establishment of an active *L. bacterium* C6A11 culture, the supernatant and cells were monitored for any potential products and gDNA was isolated using a MoBio kit, as per the manufacturer's instructions.

Cloning and expression of LahA substrates

All LahA substrates were cloned, expressed, and purified in this study in the way described previously.[3]

Construction of pRSFDuet-1 derivatives for co-expression with untagged LahM1/M2 and His_6-LahAs

The gene encoding $LahS_B$ harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 2 was first amplified via PCR using genomic DNA of *Lachnospiraceae bacterium* C6A11 as template and primers LahM1/M2-mcsII-up and LahM1/M2-mcsII-dn (Table S1). Subsequently, the PCR fragment was cloned into the multiple cloning site 2 of the pRSFDuet-1 vector (without His tag) linearized by *Nde*I using Gibson assembly to generate the pRSFDuet-1/LahM1/M2-2 vectors. The genes encoding LahAs harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 1 were then amplified using corresponding primers (Table S1). Then the corresponding fragments were cloned into the multiple cloning site 1 (MCS1) of the pRSFDuet-1/LahM1/M2-2 vectors (thus providing the LahA peptides with a His tag) linearized by *Eco*RI using Gibson assembly to generate pRSFDuet-1/His-LahAs/LahM1 and pRSFDuet-1/His-LahAs/LahM2.

Construction of pRSFDuet-1 derivatives for co-expression of $LahS_B$ and His_6-LahAs

The gene encoding $LahS_B$ harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 2 was first amplified via PCR using genomic DNA of *Lachnospiraceae bacterium* C6A11 as template and primers $LahS_B$-mcsII-up and $LahS_B$-mcsII-dn (Table S1). Subsequently the PCR fragment was cloned into the multiple cloning site 2 of the pRSFDuet-1 vector (without His tag) linearized by *Nde*I using Gibson assembly to generate the pRSFDuet-1/$LahS_B$-2 vector. The genes encoding LahAs harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 1 were then amplified using corresponding primers (Table S1). Then the corresponding fragments were cloned into the multiple cloning site 1 (MCS1) of the pRSFDuet-1/$LahS_B$-2 vector (thus providing the LahA peptides with a His tag) linearized by *Eco*RI using Gibson assembly to generate pRSFDuet-1/His-LahAs/$LahS_B$.

Construction of pET28a derivative for expression of His_6-MBP-$LahS_B$/M1/M2

The gene encoding $LahS_B$ /M1/M2 sequence with homologous arms to pET28a-MBP[4] multiple cloning site 1 was first amplified using genomic DNA of *Lachnospiraceae bacterium* C6A11 as template and corresponding primers (Table S1) and cloned into the multiple cloning site of the pET28a-MBP vector linearized by *Bam*HI using Gibson assembly to generate pETMBP-$LahS_B$, pETMBP-LahM1 and pETMBP-LahM2.

Co-expression of LahM1/M2 and LahAs in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) cells were transformed with pRSFDuet-1/His-LahAs/LahM1 and pRSFDuet-1/His-LahAs/LahM2 and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin. A single colony was picked and grown in 15 mL of TB amended with 10 mM $MgCl_2$, 50 mg/L kanamycin at 37 °C for 15 h, and the resulting culture was used to inoculate 1.5 L of TB medium containing 50 mg/L kanamycin amended with 10 mM $MgCl_2$. Cells were cultured at 37 °C until the OD_{600} reached 0.6 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.7 mM. The cells were cultured at 18 °C for another 18 h before harvesting.

Expression of His-MBP-LahM1 and His-MBP-LahM2 in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) cells were transformed with pETMBP-LahM1 or pETMBP-LahM2 and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin. A single colony was picked and grown in 15 mL of LB with 50 mg/L kanamycin at 37 °C for 15 h, and the resulting culture was used to inoculate 3 L of LB medium containing 50 mg/L kanamycin. Cells were cultured at 37 °C until the OD_{600} reached 0.6 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.3 mM. The cells were cultured at 16 °C for another 18 h before harvesting.

Co-expression of $LahS_B$ and LahAs in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) cells were transformed with pRSFDuet-LahAs-$LahS_B$ and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin. A single colony was picked and grown in 15 mL of TB amended with 10 mM $MgCl_2$ and 50 mg/L kanamycin at 37 °C for 15 h, and the resulting culture was used to inoculate 1.5 L of TB medium containing 50 mg/L kanamycin amended with 10 mM $MgCl_2$. Cells were cultured at 37 °C until the OD_{600} reached 0.6 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.7 mM. The cells were cultured at 18 °C for another 18 h before harvesting.

Purification of modified His_6-LahAs

The cell pellets were 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 using a high pressure homogenizer (Avestin, Inc.). The sample was centrifuged at 23,700×g for 30 min, and the

supernatant was kept. The pellets were 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 lysed again. The insoluble portion was removed by centrifugation at 23,700 g for 30 min, and the soluble portion was kept. Both soluble portions were combined and passed through 0.45-µm syringe filters (Fisherbrand®), and the His_6-tagged modified peptides were purified by immobilized metal affinity chromatography (IMAC) as previously described.[5] The eluted fractions were desalted by preparative reversed phase (RP) HPLC using a Waters Delta-pak C4 column (15 µm; 300 Å; 25 mm × 100 mm). The desalted peptides were lyophilized and stored at -20 °C.

Expression of His-MBP-LahS$_B$ in *E. coli* BL21 (DE3)

E. coli BL21 (DE3) cells were transformed with pETMBP-LahS$_B$ and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin. A single colony was picked and grown in 15 mL of LB with 50 mg/L kanamycin at 37 °C for 15 h, and the resulting culture was used to inoculate 3 L of LB medium. Cells were cultured at 37 °C until the OD_{600} reached 0.6 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.3 mM. The cells were cultured at 18 °C for another 18 h before harvesting.

Purification of His-MBP-LahM1 and His-MBP-LahM2

All steps were performed at 4 °C in a cold room or on ice. The cell pellets were resuspended in buffer A (20 mM Tris, 1 M NaCl, pH 7.8 at 25 °C) and lysed using a high pressure homogenizer (Avestin, Inc.). The sample was centrifuged at 23,700×g for 30 min. The supernatant was passed through 0.45-µm syringe filters (Fisherbrand®) and loaded onto a 5 mL HisTrap IMAC column pre-charged with Ni^{2+} and equilibrated with buffer A. The column was attached to an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare) and further washed with up to 25% buffer B (20 mM Tris, 1 M NaCl, 500 mM imidazole, pH 7.8 at 25 °C) in buffer A at a flow rate of 1.5 mL/min. Then the protein was eluted using a gradient of 25-100% buffer B. UV absorbance at 280 nm was monitored and fractions were collected and analyzed by SDS-PAGE (Bio-Rad). The fractions containing the desired proteins were combined and exchanged back to buffer A using a PD-10 desalting column (GE Healthcare) and subsequently concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore). Protein aliquots were frozen in liquid nitrogen and stored at -80 °C. The protein purification procedure was monitored by SDS-PAGE (Figure S3).

Size exclusion chromatography (SEC) of His-MBP-LahM1 and His-MBP-LahM2

Fractions containing the highest purity protein, as judged by Coomassie-stained SDS-PAGE (Figure S3), were pooled and further purified by size exclusion chromatography (Superdex Hiload 200 16/60, GE Healthcare) in 1 M NaCl, 20 mM Tris, pH 7.5 buffer. The purified protein was concentrated using Amicon Ultra-4 centrifugal filters (30 kDa molecular weight cut-off, Millipore) and stored in liquid nitrogen until needed. The final concentration was quantified by Bradford analysis (Thermo Scientific).

Purification of His-MBP-LahS$_B$

All steps were performed at 4 °C in a cold room or on ice. The cell pellets were resuspended in buffer A (20 mM Tris, 1 M NaCl, pH 7.8 at 25 °C) and lysed using a high pressure homogenizer (Avestin, Inc.). The sample was centrifuged at 23,700×g for 30 min. The supernatant was passed through 0.45-µm syringe filters (Fisherbrand®) and loaded onto a 5 mL HisTrap IMAC column pre-charged with Ni^{2+} and equilibrated with buffer A. The column was attached to an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare) and further washed with up to 25% buffer B (20 mM Tris, 1 M NaCl, 500 mM imidazole, pH 7.8 at 25 °C) in buffer A at a flow rate of 1.5 mL/min. Then the protein was eluted using a gradient of 25-100% buffer B over 45

min. UV absorbance at 280 nm was monitored and fractions were collected and analyzed by SDS-PAGE (Bio-Rad). The fractions containing the desired proteins were combined and exchanged back to buffer A using a PD-10 desalting column (GE Healthcare) and subsequently concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore). Protein aliquots were frozen in liquid nitrogen and stored at -80 °C. The protein purification was monitored by SDS-PAGE (Figure S4).

Peptide Synthesis

A 15-mer peptide corresponding to the C-terminus of LahA3 was synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) performed on a CEM Liberty Microwave Peptide Synthesizer (CEM Corporation), following the instrument's standard wash procedures. The synthesis was performed in 0.1 mmol scale, with the use of Fmoc-Val-Wang resin (Novabiochem, Merck) that has a loading capacity of 0.69 mmol/g. Fmoc-amino acids (0.2 M in dimethylformamide, DMF) were coupled using HCTU (0.5 M in DMF) as coupling reagent and *N*,*N*-diisopropylethylamine (2 M in 1-methyl-2-pyrrolidinone) as activator base. Fmoc deprotection was accomplished using 20% piperidine in DMF. Peptides were then cleaved from the resin by mixing with a solution of 95:2.5:2.5 trifluoroacetic acid (TFA):triisopropylsilane (TIPS):H_2O for 2 h at room temperature. The solution was filtered into 10 mL of cold diethyl ether to precipitate the peptides. The sample was centrifuged (4,500×g) for 10 min and washed twice with 10 mL of cold diethyl ether. The pelleted crude peptide was dried, redissolved in 20% acetonitrile with 0.1% TFA, and purified by preparative RP-HPLC on a Vydac C18 column (10 μm particle size, 300 Å pore size, 2.2×25 cm). A water-acetonitrile solvent system with 0.1% TFA was used. Solvent B (acetonitrile with 0.1% TFA) was programmed to increase from 2 to 100% in 45 min. The detector was set at 220 nm and the flow rate was 10 mL/min. The collected fractions were monitored by MALDI-TOF MS. Purified peptides were lyophilized and stored at -20 °C.

Supporting Tables and Figures

Table S1. Primers used in this study

Primer Name	Primer Sequences (5‘ – 3‘)
Primers for cloning *lahA*s	See Ref. 3
LahM1-up-MCSII	TAGTTAAGTATAAGAAGGAGATATACATATGAAGCAG AATGAATTCTACGATTATATAT
LchM1-dn-MCSII	TTCGCAGCAGCGGTTTCTTTACCAGATTATGTTAATT CCTCCTTAGTTTTTTTTATTGC
LahM2-up-MCSII	TAGTTAAGTATAAGAAGGAGATATACATATGAACTTA ACACTGAAGCAATGGCTGGAGC
LchM2-dn-MCSII	AATTTCGCAGCAGCGGTTTCTTTACCAGATTACTTC GACAGCCATCCCAAAAATAAAACC
$LahS_B$-up-MCSII	TAGTTAAGTATAAGAAGGAGATATACATGAGAAATAT AAGCTTATGGAGAAAGAGAT
$LchS_B$-dn-MCSII	AATTTCGCAGCAGCGGTTTCTTTACCAGATTACCTC TGTTTATTGCAAATACCTGT
LahM1-up-MBP	AACCTGTACTTCCAATCCGGATCCCTCGAGAAGCA GAATGAATTCTACGATTATATATCC
LahM1-dn-MBP	GTGGTGGTGGTGCTCGAGTGCGGCCGTTATGTTAA TTCCTCCTTAGTTTTTTTTATTGC
LahM2-up-MBP	CCTGTACTTCCAATCCGGATCCCTCGAGATGAACTT AACACTGAAGCAATGGCTGGAGC
LahM2-dn-MBP	GTGGTGGTGGTGCTCGAGTGCGGCCGTTACTTCG ACAGCCATCCCAAAAATAAAACC
$LahS_B$-up-MBP	CCTGTACTTCCAATCCGGATCCCTCGAGGAGAAATA TAAGCTTATGGAGAAAGAGATT
$LahS_B$-dn-MBP	GTGGTGGTGGTGCTCGAGTGCGGCCGTTACCTCT GTTTATTGCAAATACCTGTTA

Table S2. List of masses of the ions shown in Figure 3.

species	Calculated masses	Observed masses	Errors (ppm)
unmodified His-LahA3, $[M+H]^+$	12479.50 (average mass)	12479.8	20
2-fold dehydrated His-LahA3, $[M+H]^+$	12443.50 (average mass)	12443.7	18
Tandem MS fragments			
Parent ion: 2-fold dehydrated LahA3 core peptide, $[M+H]^+$	2704.35976	2704.3705	4.0
y5	404.2140	404.2208	16.8
y6	491.2461	491.2509	9.8
y8	633.3203	633.3250	7.5
y9	732.3887	732.3969	11.2
b7	790.3466	790.3651	23.5
y10	845.4728	845.4785	6.8
b8	903.4306	903.4377	7.9
b9	1016.5147	1016.5179	3.2
b10	1147.5552	1147.5641	7.8
y12	985.5419	985.5480	6.2
y14	1155.6474	1155.6593	10.3
b11	1262.5821	1262.5962	11.2
y15	1302.7158	1302.7282	9.5
b12	1319.6036	1319.6143	8.1
b13	1402.6513	1402.6619	7.6
b14	1549.7197	1549.7339	9.2
y19	1688.8524	1688.8614	5.3
b15	1662.8037	1662.8160	7.4
b17	1788.8572	1788.8755	10.2
y20	1801.9365	1801.9556	10.6
y25	2389.1803	2389.1975	7.2
b25	2459.2222	2459.2328	4.3
b26	2516.2437	2516.2643	8.2
b27	2587.2808	2587.3046	9.2
b28	2686.3492	2686.3607	4.3

Table S3. List of masses of the ions shown in Figure 4.

species	Calculated masses	Observed masses	Errors (ppm)
unmodified His-LahA5, $[M+H]^+$	12759.03 (average mass)	12759.2	12
3-fold dehydrated His-LahA5, $[M+H]^+$	12705.03 (average mass)	12705.3	17
mono-phosporylated His-LahA5, $[M+H]^+$	12839.01 (average mass)	12839.2154	16
mono-phosphorylated and 3-fold dehydrated LahA5 core peptide, $[M+H]^+$	12785.01 (average mass)	12785.2913	22
Tandem MS fragments			
Parent ion: 3-fold dehydrated LahA5 core peptide, $[M+H]^+$	2079.1836	2079.2009	8.3
y1	150.0584	150.0598	9.6
y2	221.0955	221.0977	10.2
b9	242.1500	242.1527	11.5
y3	308.1275	308.1299	7.6
y5	508.2436	508.2479	8.4
b13	594.3716	594.3747	5.3
b14	695.4193	695.4241	6.9
b15	778.4669	778.4726	7.3
y6	621.3277	621.3330	8.5
b16	877.5354	877.5435	9.3
b17	948.5725	948.5788	6.7
b18	1017.6045	1017.6149	10.2
b19	1116.6729	1116.6858	11.5
b20	1187.7100	1187.7212	9.4
b21	1258.7471	1258.7557	6.8
b24	1571.9473	1571.9523	3.2
y17	1598.9034	1598.9138	6.5
b25	1658.9793	1658.9931	8.3
b26	1772.0634	1772.0795	9.1
y20	1838.0409	1838.0597	10.2
b27	1859.0954	1859.1186	12.5
b28	1930.1325	1930.1588	13.6
b29	2061.1730	2061.1959	11.1

Table S4. List of masses of the ions shown in Figure 5A-D.

species	Calculated masses	Observed masses	Errors (ppm)
unmodified His-LahA5, $[M+H]^+$	12759.03 (average mass)	12759.4	26
mono-methylated His-LahA5, $[M+H]^+$	12773.03 (average mass)	12773.3	19
3-fold dehydrated LahA5 core peptide, $[M+H]^+$	2079.1836	2079.2025	9.1
3-fold dehydrated and mono-methylated LahA5 core peptide, $[M+H]^+$	2093.1836	2093.1932	4.6

Table S5. List of masses of the ions shown in Figure 5E.

species	Calculated masses	Observed masses	Errors (ppm)
Parent ions, $[M+H]^+$	2093.1836	2093.1903	3.2
y1	164.0584	164.0619	21.3
b3	242.15	242.1519	7.8
y5	522.2436	522.2498	11.9
y6	635.3277	635.3308	4.9
b7	594.3716	594.3794	13.1
b8	695.4193	695.4227	4.9
b9	778.467	778.4702	4.1
b10	877.5354	877.5412	6.6
b11	948.5725	948.5803	8.2
b12	1017.6045	1017.6128	8.2
y12	1145.6184	1145.6309	10.9
y13	1216.6555	1216.6758	16.7
b21	1859.0954	1859.1124	9.1
b22	1930.1325	1930.1499	9.0

Table S6. Data Collection, phasing, refinement statistics.

	Native	SeMet
Data collection		
Space Group	$P3_2 21$	$P3_2 21$
Cell: a, b, c (Å)	115.8, 115.8, 71.3	116.6, 116.4, 71.5
Resolution (Å)[1]	100.2 – 2.01 (2.04 – 2.01)	30.0 – 2.7 (2.75 – 2.7)
Total reflections	695657	175179
Unique reflections	34750 (1825)	14123 (714)
R_{merge} (%)[1]	7.5 (130.4)	12.5 (139.5)
CC1/2	0.999	
I/σ(I)	20.8 (2.3)	21.4 (2.1)
Completeness (%)	94.1 (99.4)	99.9 (100)
Redundancy	20.0 (21.3)	12.4 (12.6)
Refinement		
Resolution (Å)	25.0 – 2.01	
No. reflections	33046	
R_{work} / R_{free}[2]	20.9/24.4	
Number of atoms		
Protein	2391	
Ligand	26	
Solvent	83	
B-factors		
Protein	70.2	
Ligand	72.7	
Solvent	63.6	
R.M.S. deviations		
Bond lengths (Å)	0.010	
Bond angles (°)	1.52	

1. Highest resolution shell is shown in parentheses. 2. R-work = $\Sigma(|F_{obs}|-k|F_{calc}|)/\Sigma|F_{obs}|$ 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.

Figure S1. Sequence alignment of LctM, CylM, LahM1 and LahM2. Conserved residues Lys159, Asn247, Glu261 in the dehydratase domain are highlighted.

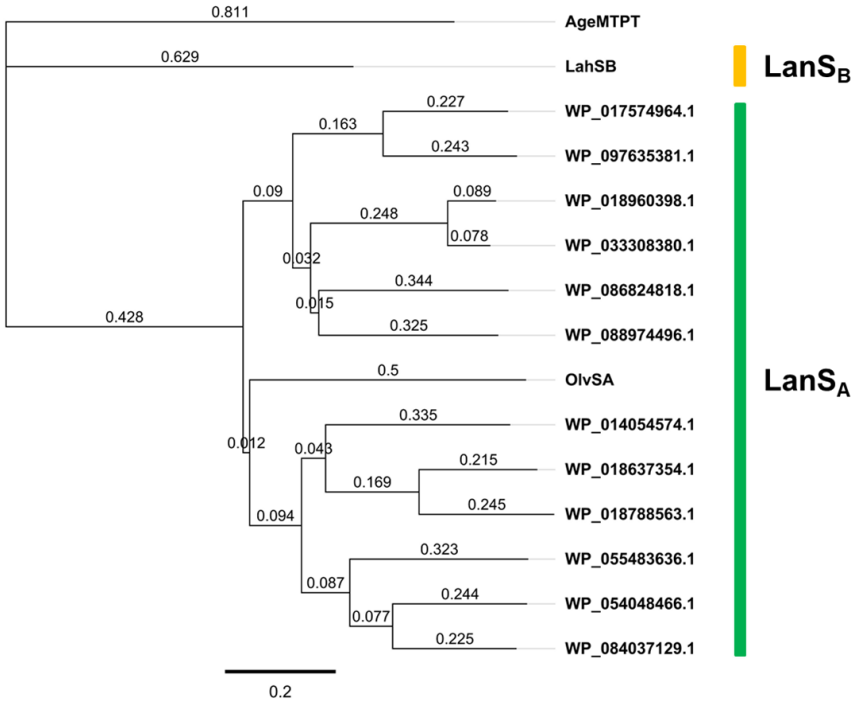

Figure S2. Protein-based phylogenetic tree of the sequences of LahS$_B$, OlvS$_A$,[6] AgeMTPT,[7] and 12 other LanS$_A$ homologues.

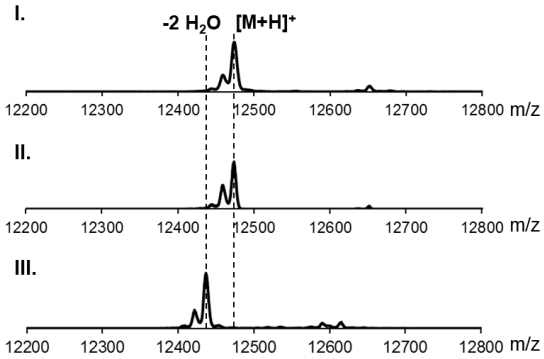

Figure S3. MALDI-TOF-MS results of *in vivo* co-expression of His-LahA3 peptide with LahM1 and LahM2. I) negative control: His-LahA3; II) His-LahA3 co-expressed with LahM2; III) His-LahA3 co-expressed with LahM1. The small shoulder peaks appearing to the left of each mass peak correspond to the laser-induced deamination of the parent peak. Deamination occurs as a consequence of analyzing large peptides using MALDI-TOF MS in reflective mode (see Extended Data Figure 6 in reference [8]).

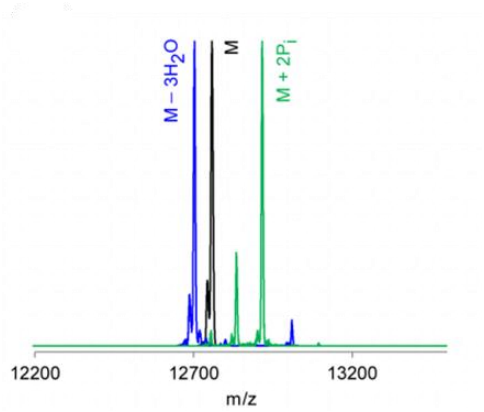

Figure S4. MALDI-TOF-MS results of co-expression of His-LahA5 peptide with LahM1 and LahM2 in *E. coli*. black line: His-LahA5; blue line: His-LahA5 co-expressed with LahM1; green line: His-LahA5 co-expressed with LahM2. The small shoulder peaks appearing to the left of each mass peak correspond to the laser-induced deamination of the parent peak. Deamination occurs as a consequence of analyzing large peptides using MALDI-TOF MS in reflective mode (see Extended Data Figure 6 in reference [8]).

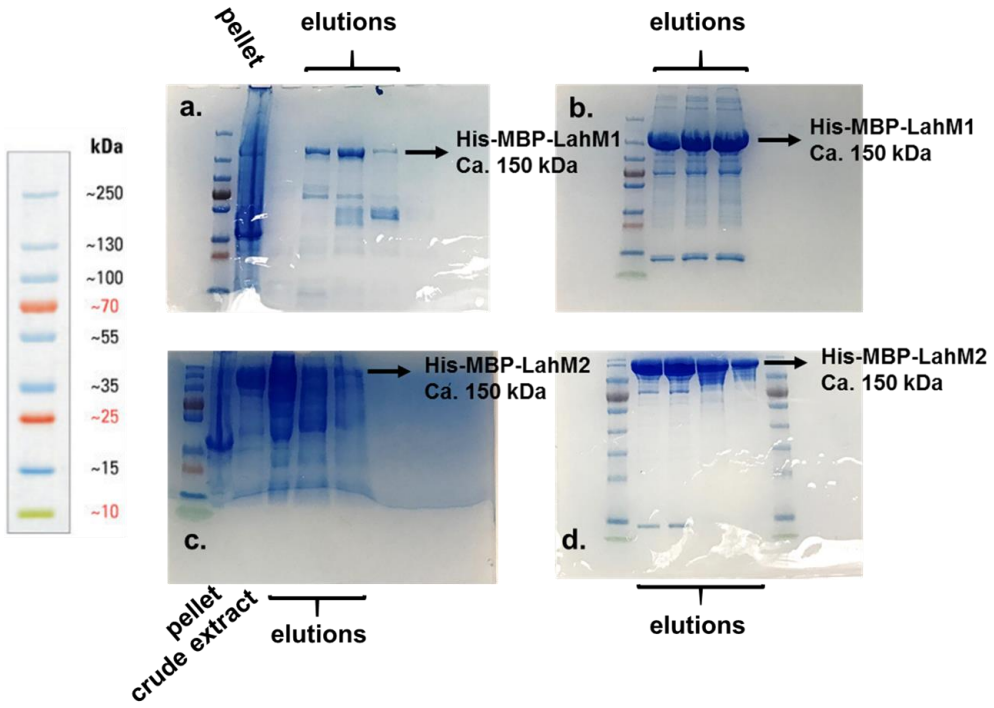

Figure S5. Coomassie-stained SDS-PAGE of purified His-MBP-LahM1 and His-MBP-LahM2. a) IMAC-purification of His-MBP-LahM1; b) SEC-purified His-MBP-LahM1; c) IMAC-purification of His-MBP-LahM2; d) SEC-purified His-MBP-LahM2.

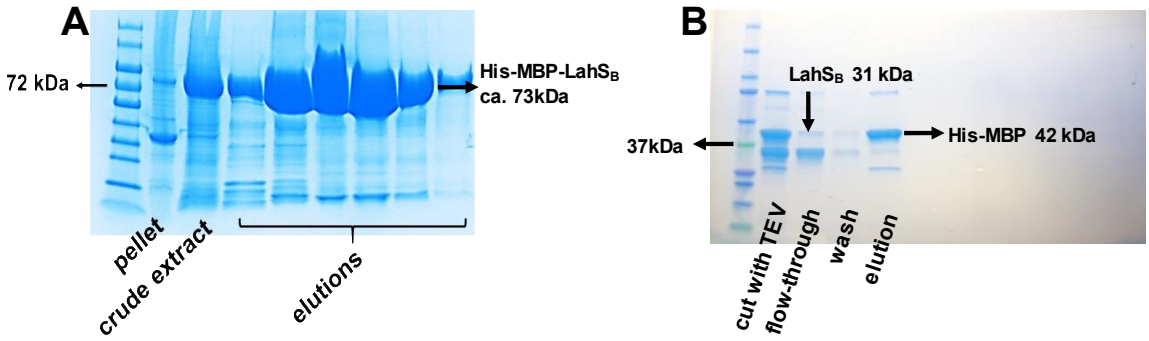

Figure S6. A) Coomassie-stained SDS-PAGE of IMAC purification of His-MBP-LahS_B. B) Coomassie-stained SDS-PAGE of subtractive IMAC purification of tag-free LahS_B.

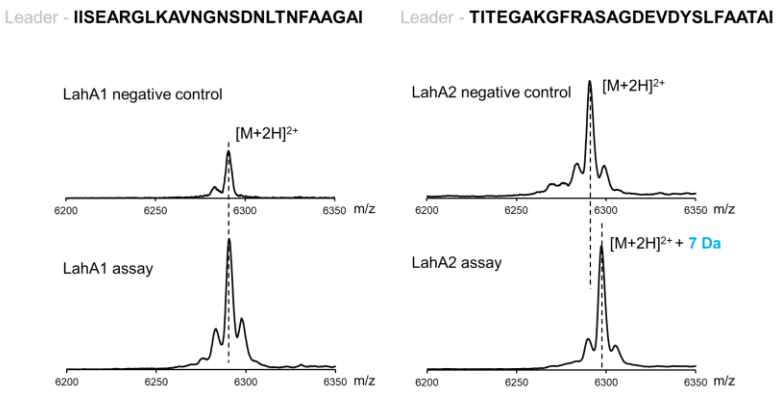

Figure S7. MALDI-TOF MS results of *in vitro* assays using His-MBP-LahS$_B$ with LahA1 and LahA2.

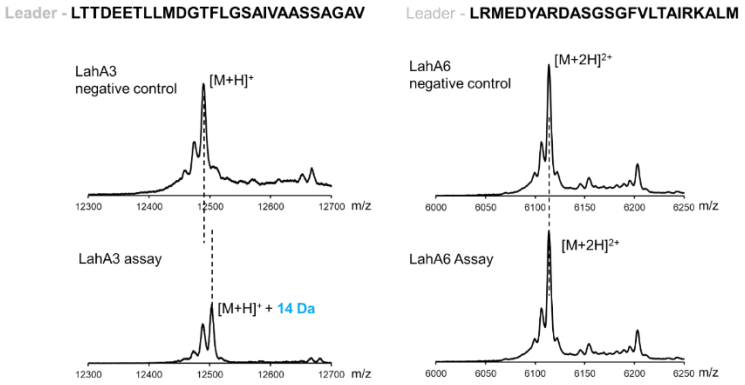

Figure S8. MALDI-TOF MS results of *in vitro* assays using His-MBP-LahS$_B$ with LahA3 and LahA6.

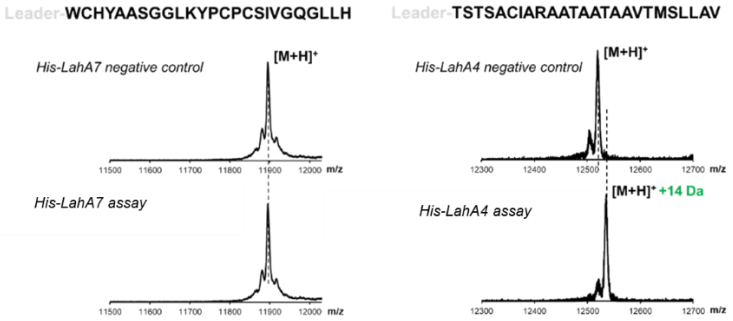

Figure S9. MALDI-TOF MS results of *in vitro* assays of His-MBP-LahS$_B$ with LahA4 and LahA7. The shoulder peaks appearing to the left of each mass peak correspond to the laser-induced deamination of the parent peak. Deamination occurs as a consequence of analyzing large peptides using MALDI-TOF MS in reflective mode (see Extended Data Figure 6 in reference [8])

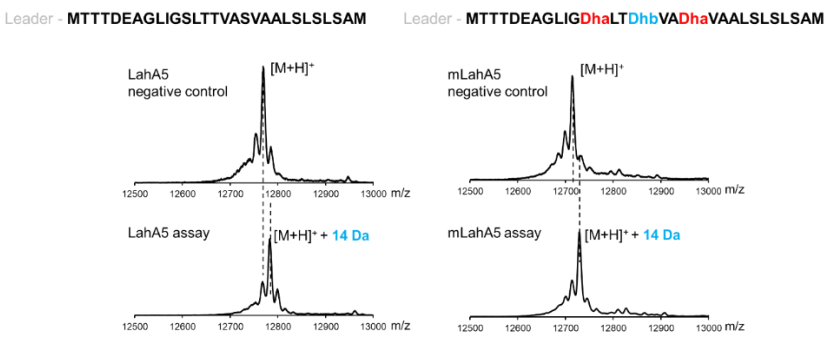

Figure S10. MALDI-TOF MS results of *in vitro* assays using His-MBP-LahS$_B$ against LahA5 and LahM1-modified LahA5.

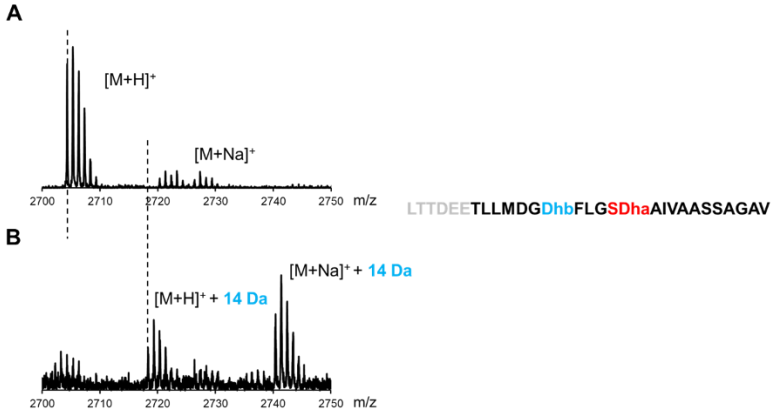

Figure S11. MALDI-TOF MS results of *in vitro* assays using His-MBP-LahS$_B$ against LahT150-treated mLahA3 peptide. (A) LahT150-treated mLahA3 peptide incubated with heat-inactivated enzyme; (B) LahT150-treated LahA3 peptide incubated with His-MBP- LahS$_B$.

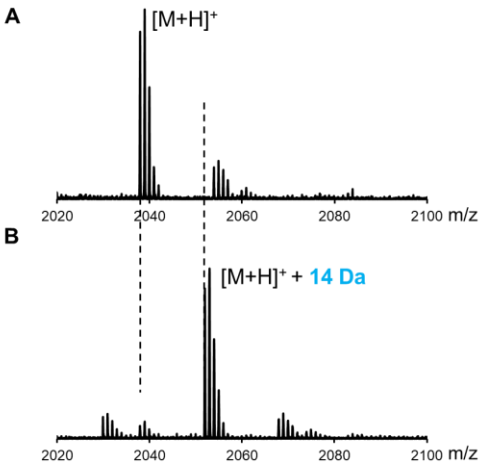

Figure S12. MALDI-TOF MS results of *in vitro* assays using His-MBP- LahS$_B$ against GluC-digested mLahA3 peptide. (A) GluC-modified mLahA3 peptide incubated with heat-inactivated enzyme; (B) GluC-modified LahA3 peptide incubated with His-MBP- LahS$_B$.

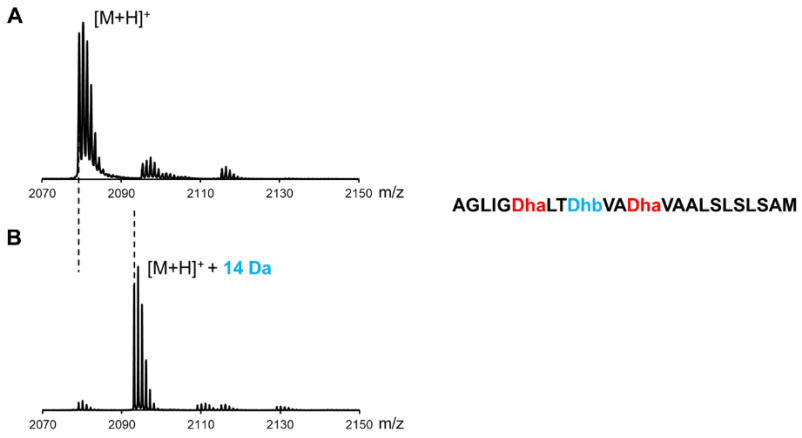

Figure S13. MALDI-TOF MS results of *in vitro* assays using His-MBP- $LahS_B$ against GluC-modified mLahA5 peptide. (A) GluC-modified mLahA5 peptide incubated with heat-inactivated enzyme; (B) GluC-modified LahA5 peptide incubated with His-MBP- $LahS_B$.

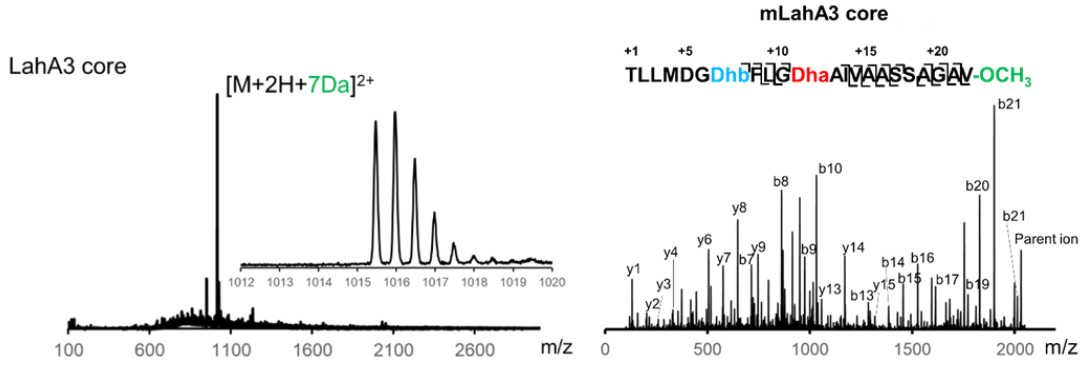

Figure S14. Tandem MS-MS of $LahS_B$-modified mLahA3 core peptide obtained by GluC treatment of mLahA3 peptide.

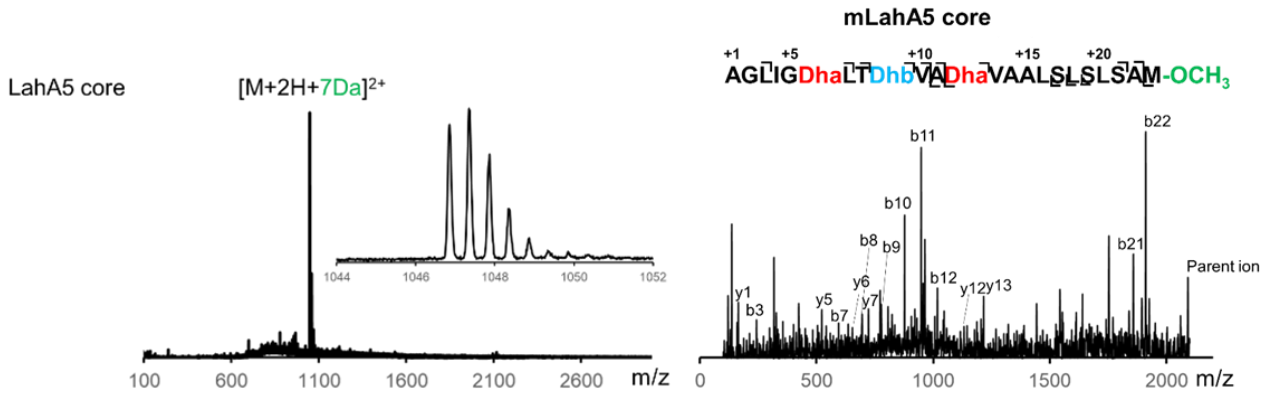

Figure S15. Tandem MS-MS of $LahS_B$-modified mLahA5 core peptide obtained by treatment of mLahA5 with GluC.

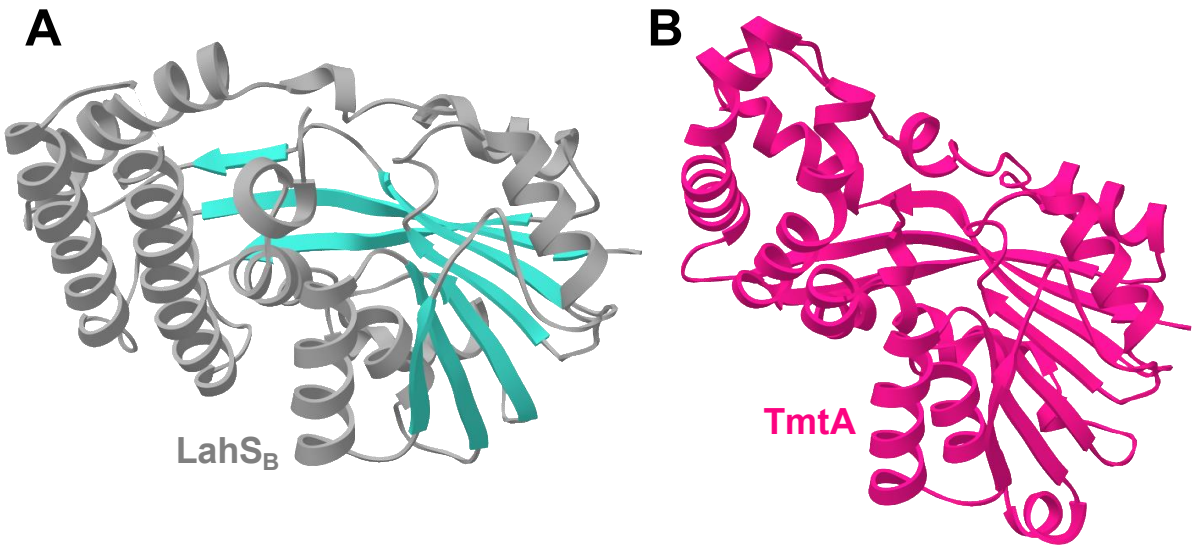

Figure S16. Comparison of the structure of (A) LahS$_B$ (B) and the *S*-methyltransferase TmtA (PDB: 5EGP).

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