Supplemental methods

Instrumentation

High resolution mass spectrometry (HR-MS) analysis was performed using a Q-Exactive™ Focus Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher). The UV-vis spectrum was recorded on a Lamda750S spectrometer (PerkinElmer). Polymerase chain reaction was performed on a Bio-Rad T100TM Thermal Cycler.

Chemicals and Biochemicals

All chemicals and biochemicals were purchased from commercial sources and used without further purification unless otherwise specified. Antibiotics (kanamycin and ampicillin) and culture media were from Sinopharm Chemical Reagent Co. Ltd (China). Triphenyltetrazolium chloride (TTC) and Endoproteinase Glu-C was purchased from Sangon Biotech Co. Ltd. Enzymes (e.g. restriction enzymes, DNA polymerase, et al) were from Takara Biotechnology (Dalian, China), Vazyme Biotech (Nanjing, China) or New England Biolabs (Beijing, China). Molecular biology kits were from CWbio Co. Ltd (Beijing, China). Ni-NTA resins were from Smart lifesciences (Changzhou, China, Ni Smart Beads 6FF: SA036100) or from GE Healthcare, USA. Primers were synthesized at Genewiz Co. Ltd (Suzhou, China).

Molecular Biology

For the preparation of pETDuet-SboA-AlbA, the gene sequences coding for SboA and AlbA were cloned from *Bacillus subtilis* DB-5 and constructed separately into the EcoRI/HindIII restriction site of Multiple Cloning Site-1 (MCS1) and NdeI/XhoI restriction site of Multiple Cloning Site-2 (MCS2). For the preparation of pRSFDuet-AlbE-AlbF, the gene sequences coding for AlbE and AlbF were cloned from *Bacillus subtilis* DB-5 and constructed separately into the NcoI/HindIII restriction site of MCS1 and NdeI/XhoI restriction site of MCS2. For the preparation of pRSFDuet-AlbE or pRSFDuet-AlbF, the gene sequences coding for AlbE or AlbF were cloned from *Bacillus subtilis* DB-5 and constructed into the NcoI/HindIII restriction site of MCS1. The correct recombinant plasmids of each cloning step were verified by sequencing service offered by Sangon Biotech Co. Ltd.

Expression and Purification of the 3t-sboA product

For 3t-sboA expression, *E. coli* BL21 (DE3) cells were transformed with pETDuet-SboA-AlbA. For AlbE and AlbF co-expression, *E. coli* BL21 (DE3) cells were transformed with pETDuet-SboA-

AlbA and pRSFDuet-AlbE-AlbF. Cells were grown for 18-20 hr on LB agar plates containing 100 μg/mL ampicillin (for pETDuet) or additional 50 μg/mL kanamycin (for co-expression) at 37 °C. Single colonies were used to inoculate 10 mL of LB containing the same concentration of antibiotics and grown at 37 °C for 14–16 h. This culture was used to inoculate 1 L of LB (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) supplemented with the same concentration of antibiotics for about 4 h. Protein expression was then induced with the addition of 0.2 mg/L isopropyl β-D-1-thiogalactopyranoside (IPTG). Expression was allowed to proceed for 16-18 h at 18 °C (at 80 rpm). Cells were harvested by centrifugation at 4,500 × g for 15 min, washed with phosphate-buffered saline (PBS buffer, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The cells were subsequently subjected to peptide purification, or were flash-frozen and stored at -80 °C upon further use.

The cells were re-suspended in Ni-NTA lysis buffer (20mM Na_2HPO_4, 4M GuHCl, 200mM NaCl, pH=8) and were then lysed by sonication (every 3s sonication at 55% intensity followed by 20s interval) for 55 min on ice. Insoluble debris was removed by centrifugation at 13000 × g for 55 min at 4 °C. The supernatant containing desired product was then applied to a lysis-buffer pre-equilibrated Ni-NTA resin (3 mL of resin per L of initial cell culture). The column was washed with 2 column volumes (CV) of Ni-NTA lysis buffer followed by 2 CV of Ni-NTA wash buffer (20mM Na_2HPO_4, 4M GuHCl, 200mM NaCl, 50mM imidazole, pH=8). The peptides were eluted using Ni-NTA elution buffer (20mM Na_2HPO_4, 4M GuHCl, 200mM NaCl, 400mM imidazole, pH=8). The eluent obtained was desalted three times in pure water within a cutoff=3500Da dialysis membrane. Finally, the product was lyophilized and dissolved in desalting buffer (20mM Tris, 25mM NaCl, 10% glycerol, pH=7~8) for LC-MS analysis.

Purification of co-expression product

Co-expression product extraction was performed similarly to previous report in subtilosin A.[1] A 30% (v/v) volume of n-butanol was added to the fermentation supernatant. The mixture was shaken for 2 hours and allowed to stand overnight. The butanol layer was collected and distilled in vacuo. Residues were then dissolved in methanol and loaded onto a YMC*GEL ODS-A-HG packing column (12 nm S-50μm). The column was first prewashed with 10% aqueous acetonitrile before the product was eluted with 50% aqueous acetonitrile. The product was then concentrated by vacuum distillation, and the residue was dissolved with 2mL water. After centrifugation at 2,000 × g for 10 min, the supernatant was collected and subjected to LC-MS analysis.

LC-MS analysis

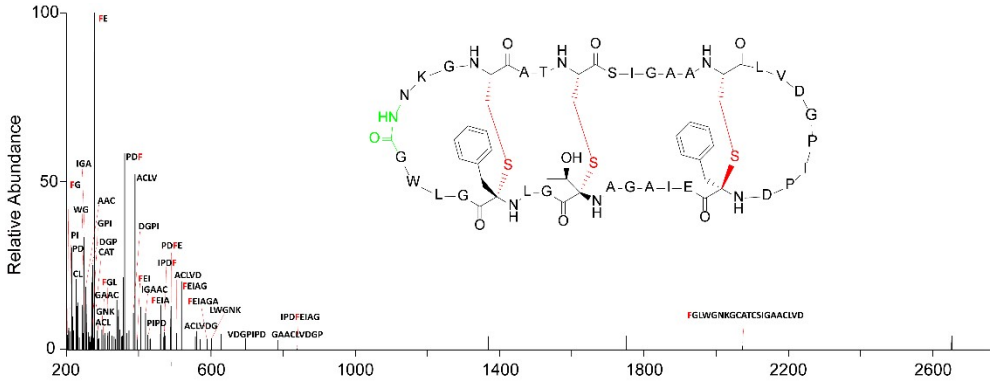
The product was injected onto a reverse phase Phenomenex Aeris PEPTIDE XB-C18 column (250 x 10 mm, 5 µm). The gradient was operated at 0.3 ml/min with solvent A (0.1% FA in MilliQ water) and solvent B (MeCN) under the following condition: t = 0 min, 2%B; t = 2, 2%B; t = 2.5, 40%B; t = 10, 60%B; t = 10.5, 95%B; t = 11, 95%B; t = 12, 2%B; t = 13, 2%B.

Minimum inhibitory concentration (MIC)

The MIC assay was performed according to a modified colorimetric method reported by Murakami et al.[2] To determine the minimum inhibitory concentration (MIC) of subtilosin A_{OC}, 10 µL of tested strains were inoculated into LB medium and subcultured until the optical density at 600 nm (OD_{600}) reached 0.5. The culture was then diluted to match the turbidity of a 0.5 McFarland standard, resulting in a bacterial concentration of approximately 1 × 10^8 cells/mL. This solution (working medium) was further diluted to achieve a working concentration of 1 × 10^7 cells/mL.

In the first column of a 96-well plate, 20 µL of antibiotics (i.e. subtilosin A or subtilosin A_{OC}) was added to 180 µL of working medium. A twofold serial dilution of the samples was performed from the first to the next well in each row. Subsequently, 100 µL of the working medium was added to each well, bringing the total volume to 200 µL. The plate was incubated at at 35 °C overnight, and 0.05% (w/v) TTC solution was then added followed by 2 more hours of incubation, and subsequent reading in a spectrophotometer to check the MIC values.

Figure S1. HR-MS/MS analysis of subtilosin A produced by *Bacillus subtilis* DB-5.



Ion	Sequence	Calc.	Obs.	Error	Note
31-32	FG	203.0815	203.0809	-2.95	-2H
18-19	PI/IP	211.1441	211.1434	-3.32	-
20-21	PD	213.0870	213.0863	-3.29	-
13-14	CL	217.1005	217.0995	-4.61	-
9-11/24-26	IGA/IAG	242.1499	242.1490	-3.72	-
34-35	WG	244.1081	244.1073	-3.28	-
11-13	AAC	246.0907	246.0897	-4.06	-
17-19	GPI	268.1656	268.1647	-3.36	-
16-18	DGP	270.1084	270.1072	-4.44	-
22-23	FE	275.1026	275.1013	-4.73	-2H
4-6/5-7	CAT/ATC	276.1013	276.1009	-1.45	-
12-14	ACL	288.1376	288.1364	-4.16	-
35-2/1-3	GNK/NKG	300.1666	300.1655	-3.66	
10-13	GAAC	303.1122	303.1115	-2.31	-
29-31/30-32/31-33/	GLF /LFG/FGL	316.1656	316.1644	-3.80	-2H
20-22	PDF	358.1397	358.1382	-4.19	-2H
16-19	DGPI	383.1925	383.1910	-3.91	-
12-15	ACLV	387.2061	387.2046	-3.87	-
22-24	FEI	388.1867	388.1849	-4.64	-2H
9-13/10-14	IGAAC/GAACL	416.1962	416.1943	-4.57	-
18-21	PIPD	423.2238	423.2251	3.07	-
22-25	FEIA	459.2238	459.2224	-3.05	-2H
19-22	IPDF	471.2238	471.2218	-4.24	-2H
20-23	PDFE	487.1823	487.1802	-4.31	-2H
12-16	ACLVD	502.2330	502.2347	3.38	-
22-26	FEIAG	516.2453	516.2429	-4.65	-2H
12-17	ACLVDG	559.2545	559.2551	1.07	-
22-27	FEIAGA	587.2824	587.2799	-4.26	-2H
33-2	LWGNK	599.3300	599.3276	-4.00	-
15-21	VDGPIPD	694.3404	694.3383	-3.02	-
10-18	GAACLVDGP	784.3658	784.3655	-0.38	-
19-26	IPDFEIAG	841.4090	841.4057	-3.92	-2H
31-16	FGLWGNKGCATCSIGAACL VD	2065.9292	2065.9190	-4.94	-2H

Figure S2. Sequence alignments of AlbA from different subtilosin A biosynthetic gene clusters (BGCs), including BGCs from *Bacillus subtilis* DB-5, *Bacillus subtilis* 168, *Bacillus inaquosorum* KCTC 13429, *Bacillus tequilensis* EA-CB00015, and *Bacillus halotolerance* F41-3. All sequences are retrieved from Protein Data Bank or National Center for Biotechnology information database.

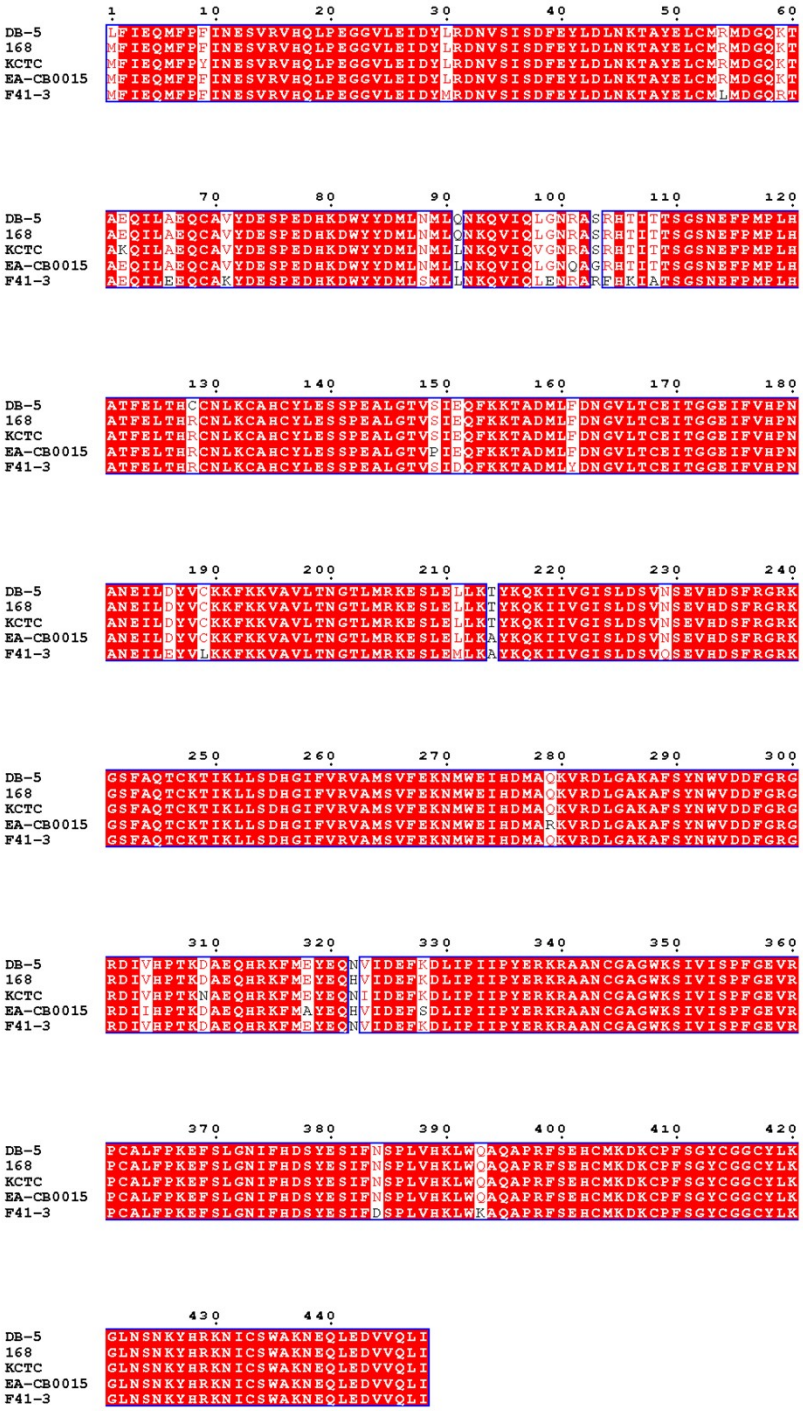


Figure S3. Sequence alignments of AlbE from different subtilosin A BGCs, including BGCs from *Bacillus subtilis* DB-5, *Bacillus subtilis* 168, *Bacillus inaquosorum* KCTC 13429, *Bacillus tequilensis* EA-CB00015, and *Bacillus halotolerance* F41-3. All sequences are retrieved from Protein Data Bank or National Center for Biotechnology information database.

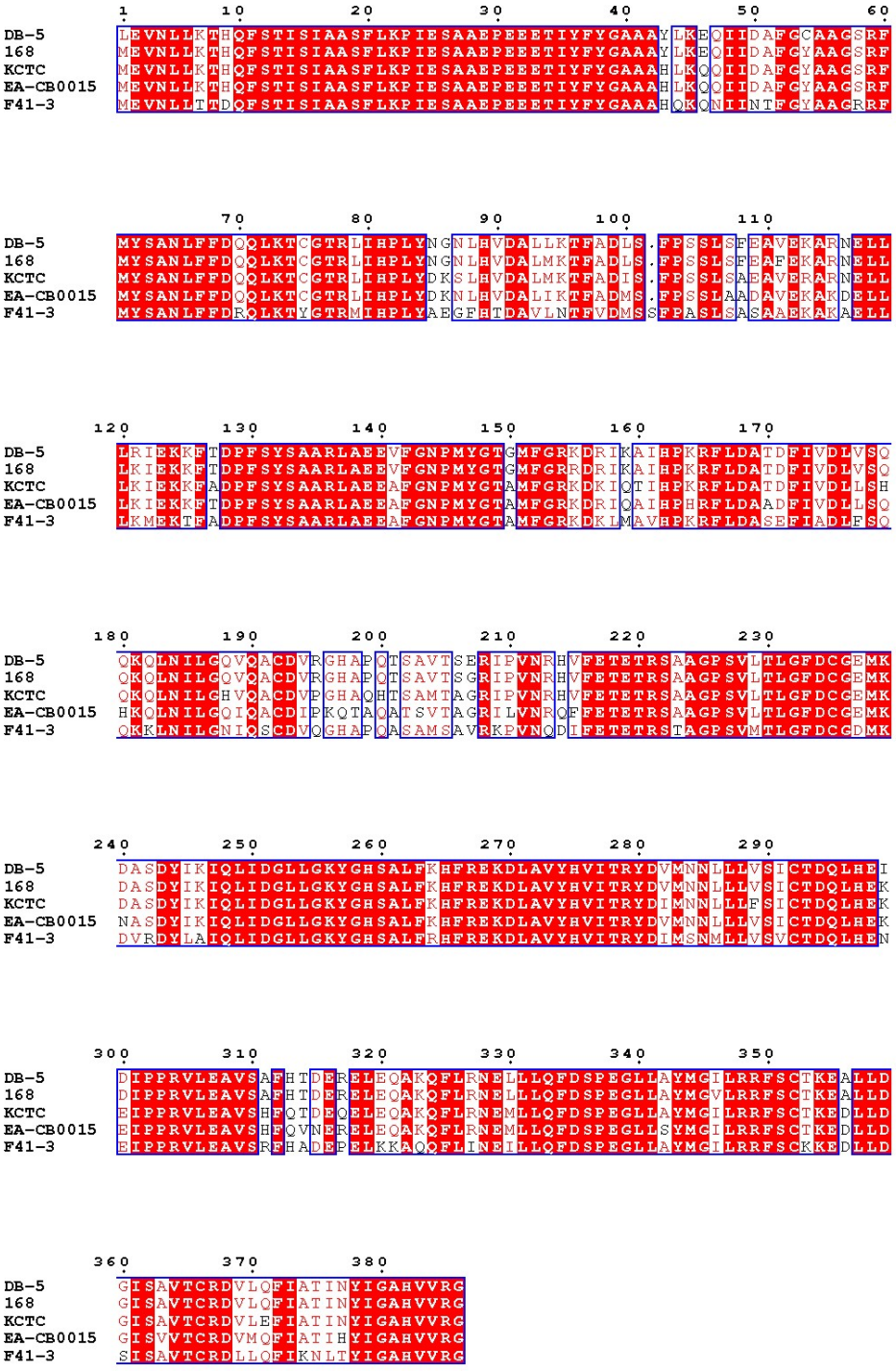


Figure S4. Sequence alignments of AlbF from different subtilosin A BGCs, including BGCs from *Bacillus subtilis* DB-5, *Bacillus subtilis* 168, *Bacillus inaquosorum* KCTC 13429, *Bacillus tequilensis* EA-CB00015, and *Bacillus halotolerance* F41-3. All sequences are retrieved from Protein Data Bank or National Center for Biotechnology information database.

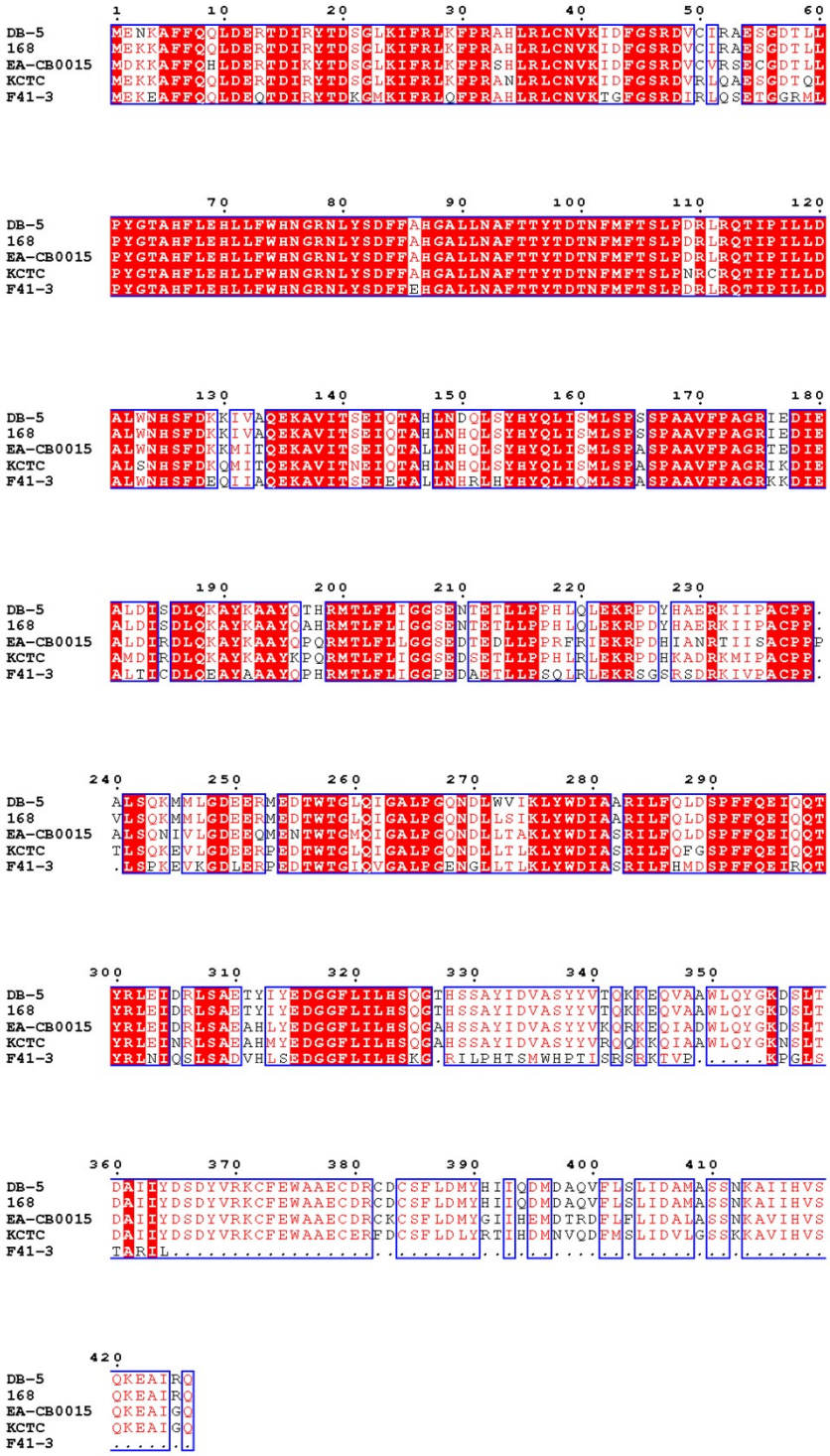


Figure S5. Maps of plasmids for coexpression of subtilosin A in *E. coli*.

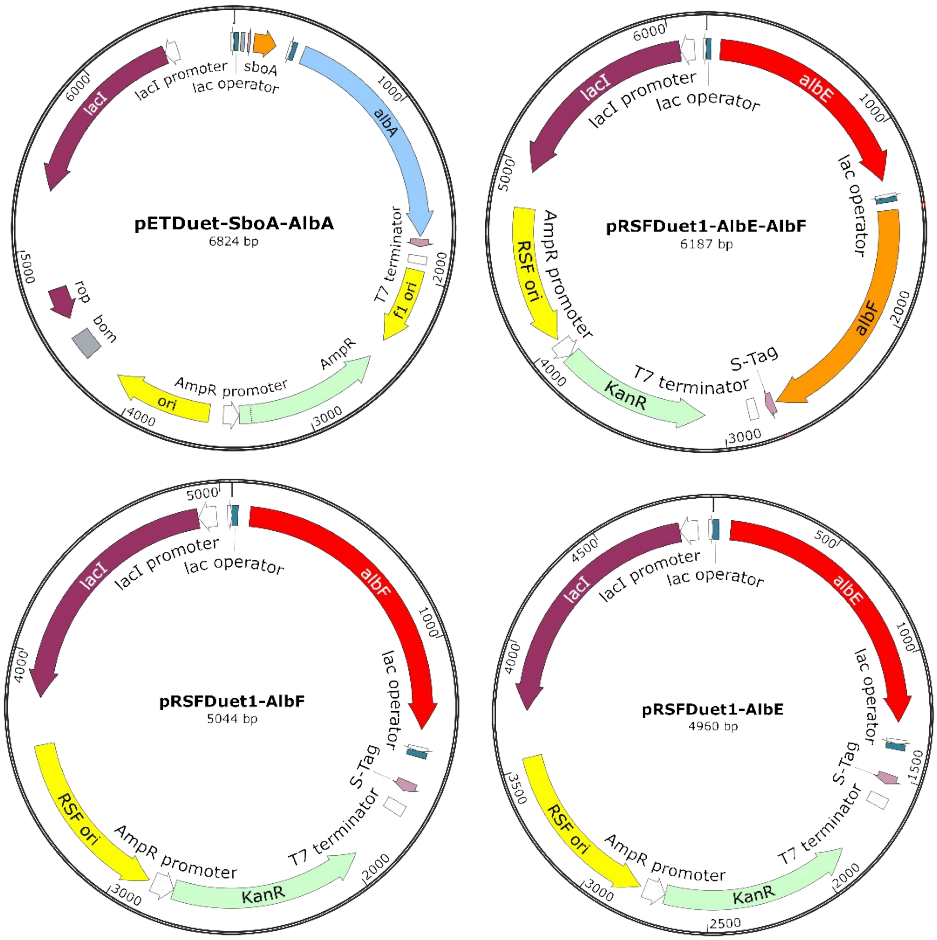
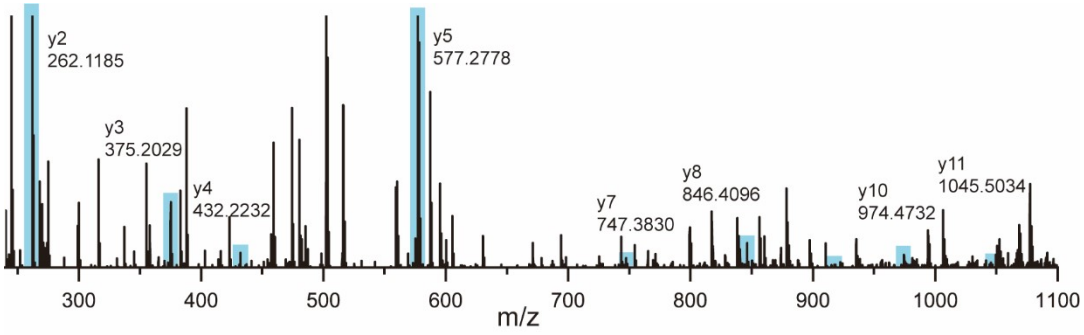


Figure S6. HR-MS/MS analysis of subtilosin A_{OC} produced by in *E. coli.* Ions containing -2H or -4H modification are shown in red.

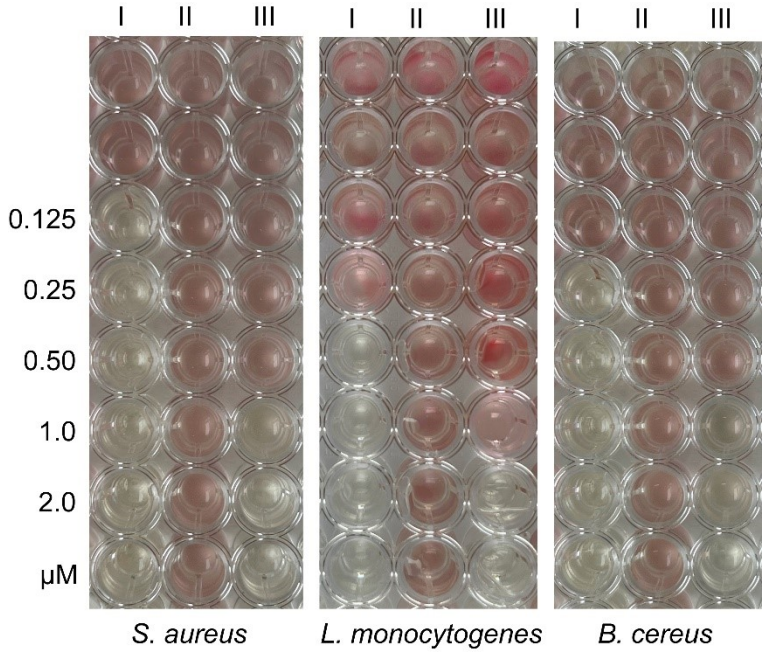


y ions	Sequence	Calculated Mass [M + H]$^+$	Observed Mass	Mass Difference (Da)	Error (ppm)
y2	WG	262.1186	262.1185	0.0001	0.38
y3	LWG	375.2027	375.2029	0.0002	0.53
y4	GLWG	432.2241	432.2232	0.0009	2.08
y5	**F**GLWG	579.2926	577.2778	2.0148	1.47
y6	L**F**GLWG	692.3766	NA	NA	NA
y7	GL**F**GLWG	749.3981	747.3830	2.0151	0.74
y8	**T**GL**F**GLWG	850.4458	846.4096	4.0362	5.79
y9	A**T**GL**F**GLWG	921.4829	NA	NA	NA
y10	GA**T**GL**F**GLWG	978.5043	974.4732	4.0311	0.21
y11	AGA**T**GL**F**GLWG	1049.5415	1045.5034	4.0381	6.50

Figure S7. HR-MS/MS analysis of subtilosin A_{OC} produced by *Bacillus subtilis* DB-5. Ions containing -2H or -4H modification are shown in red.

y ions	Sequence	Calculated Mass $[M + H]^+$	Observed Mass	Mass Difference (Da)	Error (ppm)
y2	WG	262.1186	262.1182	0.0004	1.53
y3	LWG	375.2027	375.2019	0.0008	2.13
y4	GLWG	432.2241	432.2237	0.0004	0.93
y5	**F**GLWG	579.2926	577.2753	2.0173	2.86
y6	L**F**GLWG	692.3766	NA	NA	NA
y7	GL**F**GLWG	749.3981	747.3706	2.0275	15.86
y8	**T**GL**F**GLWG	850.4458	846.4063	4.0395	9.69
y9	A**T**GL**F**GLWG	921.4829	NA	NA	NA
y10	GA**T**GL**F**GLWG	978.5043	974.4659	4.0384	7.29
y11	AGA**T**GL**F**GLWG	1049.5415	1045.5199	4.0216	9.28

Figure S8. MIC analysis for subtilosin A and subtilosin A_{OC}, using *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* as the test strains.



References

1. K. Babasaki, T. Takao, Y. Shimonishi and K. Kurahashi, *J Biochem*, 1985, **98**, 585-603.
2. A. Veiga, M. Toledo, L. S. Rossa, M. Mengarda, N. C. F. Stofella, L. J. Oliveira, A. G. Goncalves and F. S. Murakami, *J Microbiol Methods*, 2019, **162**, 50-61.