### Supplemental methods

## Instrumentation

High resolution mass spectrometry (HR-MS) analysis was performed using a Q-Exactive<sup>TM</sup> 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 pRSFDuet-AlbF, the gene sequences coding for 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  $\mu$ g/mL ampicillin (for pETDuet) or additional 50  $\mu$ g/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  $\beta$ -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<sub>2</sub>HPO<sub>4</sub>, and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>). 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<sub>2</sub>HPO<sub>4</sub>, 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<sub>2</sub>HPO<sub>4</sub>, 4M GuHCl, 200mM NaCl, 50mM imidazole, pH=8). The peptides were eluted using Ni-NTA elution buffer (20mM Na<sub>2</sub>HPO<sub>4</sub>, 4M GuHCl, 200mM Na<sub>2</sub>HPO<sub>4</sub>, 4M GuHCl, 200mM NaCl, 50mM imidazole, pH=8). The peptides were eluted using Ni-NTA elution buffer (20mM Na<sub>2</sub>HPO<sub>4</sub>, 4M GuHCl, 200mM Na<sub>2</sub>HPO<sub>4</sub>, 40 GuHCl, 200mM Na<sub>2</sub>, 40 GuHCl, 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.<sup>1</sup> 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  $\mu$ m). 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.<sup>2</sup> 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<sub>600</sub>) 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  $1 \times 10^{8}$  cells/mL. This solution (working medium) was further diluted to achieve a working concentration of  $1 \times 10^{7}$  cells/mL.

In the first column of a 96-well plate, 20  $\mu$ L of antibiotics (i.e. subtilosin A or subtilosin A<sub>OC</sub>) was added to 180  $\mu$ L of working medium. A twofold serial dilution of the samples was performed from the first to the next well in each row. Subsequently, 100  $\mu$ L of the working medium was added to each well, bringing the total volume to 200  $\mu$ L. 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.

FG PI/IP	203.0815	203.0809	2 05	211
PI/IP		200.0000	-2.95	-2H
,	211.1441	211.1434	-3.32	-
PD	213.0870	213.0863	-3.29	-
CL	217.1005	217.0995	-4.61	-
IGA/IAG	242.1499	242.1490	-3.72	-
WG	244.1081	244.1073	-3.28	-
AAC	246.0907	246.0897	-4.06	-
GPI	268.1656	268.1647	-3.36	-
DGP	270.1084	270.1072	-4.44	-
FE	275.1026	275.1013	-4.73	-2H
CAT/ATC	276.1013	276.1009	-1.45	-
ACL	288.1376	288.1364	-4.16	-
GNK/NKG	300.1666	300.1655	-3.66	
GAAC	303.1122	303.1115	-2.31	-
GL <mark>F</mark> /L <mark>F</mark> G/FGL	316.1656	316.1644	-3.80	-2H
PDF	358.1397	358.1382	-4.19	-2H
DGPI	383.1925	383.1910	-3.91	-
ACLV	387.2061	387.2046	-3.87	-
FEI	388.1867	388.1849	-4.64	-2H
IGAAC/GAACL	416.1962	416.1943	-4.57	-
PIPD	423.2238	423.2251	3.07	-
FEIA	459.2238	459.2224	-3.05	-2H
IPD <mark>F</mark>	471.2238	471.2218	-4.24	-2H
PDFE	487.1823	487.1802	-4.31	-2H
ACLVD	502.2330	502.2347	3.38	-
FEIAG	516.2453	516.2429	-4.65	-2H
ACLVDG	559.2545	559.2551	1.07	-
FEIAGA	587.2824	587.2799	-4.26	-2H
LWGNK	599.3300	599.3276	-4.00	-
VDGPIPD	694.3404	694.3383	-3.02	-
GAACLVDGP	784.3658	784.3655	-0.38	-
<b>IPD</b> FEIAG	841.4090	841.4057	-3.92	-2H
FGLWGNKGCATCSIGAACL	2065.9292	2065.9190	-4.94	-2H
	PD CL IGA/IAG WG AAC GPI DGP FE CAT/ATC ACL GNK/NKG GAAC GLF /LFG/FGL PDF DGPI ACLV FEI IGAAC/GAACL PIPD FEIA IPDF PDFE ACLVD FEIA IPDF PDFE ACLVD FEIAG ACLVDG FEIAG ACLVDG FEIAGA LWGNK VDGPIPD GAACLVDGP IPDFEIAG FGLWGNKGCATCSIGAACL VD	PD 213.0870   CL 217.1005   IGA/IAG 242.1499   WG 244.1081   AAC 246.0907   GPI 268.1656   DGP 270.1084   FE 275.1026   CAT/ATC 276.1013   ACL 288.1376   GNK/NKG 300.1666   GAAC 303.1122   GLF /LFG/FGL 316.1656   PDF 358.1397   DGPI 383.1925   ACLV 387.2061   FEI 388.1867   IGAAC/GAACL 416.1962   PIPD 423.2238   FEIA 459.2238   IPDF 471.2238   PDFE 487.1823   ACLVD 502.2330   FEIAG 516.2453   ACLVD 502.2330   FEIAGA 587.2824   LWGNK 599.3300   VDGPIPD 694.3404   GAACLVDGP 784.3658   IPDFEIAG 841.4090	PD   211.1441   211.14434     PD   213.0870   213.0863     CL   217.1005   217.0995     IGA/IAG   242.1499   242.1490     WG   244.1081   244.1073     AAC   246.0907   246.0897     GPI   268.1656   268.1647     DGP   270.1084   270.1072     FE   275.1026   275.1013     CAT/ATC   276.1013   276.1009     ACL   288.1376   288.1364     GNK/NKG   300.1666   300.1655     GAAC   303.1122   303.1115     GLF /LFG/FGL   316.1656   316.1644     PDF   358.1397   358.1382     DGPI   383.1925   383.1910     ACLV   387.2061   387.2046     FEI   388.1867   388.1849     IGAAC/GAACL   416.1962   416.1943     PIPD   423.2238   423.2251     FEIA   459.2238   459.2224     IPDF   471.2238   471.2218	PD   211.1441   211.1404   5.52     PD   213.0870   213.0863   -3.29     CL   217.1005   217.0995   -4.61     IGA/IAG   242.1499   242.1490   -3.72     WG   244.1081   244.1073   -3.28     AAC   246.0907   246.0897   -4.06     GPI   268.1656   268.1647   -3.36     DGP   270.1084   270.1072   -4.44     FE   275.1026   275.1013   -4.73     CAT/ATC   276.1013   276.1009   -1.45     ACL   288.1376   288.1364   -4.16     GNK/NKG   300.1666   300.1655   -3.66     GAAC   303.1122   303.1115   -2.31     GLF /LFG/FGL   316.1656   316.1644   -3.80     PDF   358.1397   358.1382   -4.19     DGPI   383.1925   383.1910   -3.91     ACLV   387.2061   387.2046   -3.87     FEI   388.1867   388

**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.

DB-5 168 KCTC EA-CB0015 F41-3	L LFIEQMFF MFIEQMFF MFIEQMFF MFIEQMFF	10 FINESVRVE EINESVRVE YINESVRVE FINESVRVE	20 QLPEGGVLE1 QLPEGGVLE1 QLPEGGVLE1 QLPEGGVLE1 QLPEGGVLE1	30 CDYLRDNVSIS CDYLRDNVSIS CDYLRDNVSIS CDYLRDNVSIS CDYMRDNVSIS	40 SDFEYLDLNKT SDFEYLDLNKT SDFEYLDLNKT SDFEYLDLNKT SDFEYLDLNKT	50 AYELCMRMD0 AYELCMRMD0 AYELCMRMD0 AYELCMRMD0 AYELCMLMD0	60 SQKT SQKT SQKT SQKT SQRT
DB-5 168 KCTC EA-CB0015 F41-3	AEQILAEC AEQILAEC AKQILAEC AEQILEEC AEQILEEC	70 CAVYDESPE CAVYDESPE CAVYDESPE CAVYDESPE CAVYDESPE CAKYDESPE	80 DHKDWYYDMI DHKDWYYDMI DHKDWYYDMI DHKDWYYDMI DHKDWYYDMI	90 NMLONKQVIQ NMLENKQVIQ NMLENKQVIQ SMLENKQVIQ	100 LEONRASRHTI LEONRASRHTI VGRASRHTI LEONRARPHKI	110 TTSGSNEFP TTSGSNEFP TTSGSNEFP ATSGSNEFP	120 APLH APLH MPLH MPLH MPLH
DB-5 168 KCTC EA-CB0015 F41-3	ATFELTH ATFELTH ATFELTH ATFELTH ATFELTH ATFELTH	30 CNLKCAHCY CNLKCAHCY CNLKCAHCY CNLKCAHCY	140 LLESSPEALC (LESSPEALC (LESSPEALC) (LESSPEALC) (LESSPEALC)	150 VSIEQFKKT VSIEQFKKT VSIEQFKKT VSIEQFKKT VSIEQFKKT	160 ADMLEDNGVLT ADMLEDNGVLT ADMLEDNGVLT ADMLEDNGVLT ADMLEDNGVLT	170 CEITGGEIF CEITGGEIF CEITGGEIF CEITGGEIF	180 HPN HPN HPN HPN HPN
DB-5 168 KCTC EA-CB0015 F41-3	ANEILDYV ANEILDYV ANEILDYV ANEILDYV ANEILDYV ANEILEYV	90 CKKFKKVAV CKKFKKVAV CKFKKVAV LKKFKKVAV	200 /LTNGTLMRKE /LTNGTLMRKE /LTNGTLMRKE /LTNGTLMRKE	210 SLELKTYKC SLELLKTYKC SLELLKTYKC SLELKAYKC	220 2KIIVGISLDS 2KIIVGISLDS 2KIIVGISLDS 2KIIVGISLDS 2KIIVGISLDS	230 VNSEVHDSFI VNSEVHDSFI VNSEVHDSFI VNSEVHDSFI VOSEVHDSFI	240 RGRK RGRK RGRK RGRK RGRK
DB-5 168 KCTC EA-CB0015 F41-3	GSFAQTCK GSFAQTCK GSFAQTCK GSFAQTCK GSFAQTCK	TIKLLSDHO TIKLLSDHO TIKLLSDHO TIKLLSDHO TIKLLSDHO	260 IFVRVAMSVE IFVRVAMSVE IFVRVAMSVE IFVRVAMSVE	270 FEKNMWEIHDM FEKNMWEIHDM FEKNMWEIHDM FEKNMWEIHDM	280 HAOKVRDLGAK HAOKVRDLGAK HAOKVRDLGAK HAOKVRDLGAK	290 AFSYNWVDDI AFSYNWVDDI AFSYNWVDDI AFSYNWVDDI	300 FGRG FGRG FGRG FGRG FGRG
DB-5 168 KCTC EA-CB0015 F41-3	RD IVHPTY RD IVHPTY RD IVHPTY RD I HPTY RD IVHPTY RD IVHPTY	DAEQHRKFN DAEQHRKFN NAEQHRKFN DAEQHRKFN DAEQHRKFN	920 BYEQNVIDE BYEQNVIDE BYEQNVIDE AYEQNVIDE BYEQNVIDE	330 KDLIPIIPY KDLIPIIPY KDLIPIIPY KDLIPIIPY	340 ERKRAAN CGAG ERKRAAN CGAG ERKRAAN CGAG ERKRAAN CGAG	950 WKSIVISPF WKSIVISPF WKSIVISPF WKSIVISPF	360 SEVR SEVR SEVR SEVR SEVR
DB-5 168 KCTC EA-CB0015 F41-3	PCALFPKE PCALFPKE PCALFPKE PCALFPKE PCALFPKE	70 FSLGNIFHI FSLGNIFHI FSLGNIFHI FSLGNIFHI	980 SYESIFNSPI SYESIFNSPI SYESIFNSPI SYESIFDSPI	390 VHKLWGAQAE VHKLWGAQAE VHKLWGAQAE VHKLWGAQAE	400 PRFSEHCMKDK PRFSEHCMKDK PRFSEHCMKDK PRFSEHCMKDK PRFSEHCMKDK	410 CPFSGYCGG CPFSGYCGG CPFSGYCGG CPFSGYCGG	420 YLK YLK YLK YLK YLK
DB-5 168 KCTC EA-CB0015 F41-3	GLN SN KY F GLN SN KY F GLN SN KY F GLN SN KY F GLN SN KY F	ISO IRKNICSWAH IRKNICSWAH IRKNICSWAH IRKNICSWAH	440 (NEQLEDVVQI (NEQLEDVVQI (NEQLEDVVQI (NEQLEDVVQI (NEQLEDVVQI				

**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.



DB-5	GISAVTCRDVLQFIATINYIGAHVVR	G
168	GISAVTCRDVLQFIATINYIGAHVVR	G
KCTC	GISAVTCRDVLEFIATIN YIGAHVVR	G
EA-CB0015	GISVVTCRDVMQFIATIHYIGAHVVR	G
F41-3	SISAVTCRDLLOFIKNLTYIGAHVVR	G

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<sub>OC</sub> produced by in *E. coli*. Ions containing -2H or - 4H modification are shown in red.



У	Sequence	Calculated	Observed	Mass	Error (ppm)
ions		Mass [M +	Mass	Difference	
		H]⁺		(Da)	
y2	WG	262.1186	262.1185	0.0001	0.38
у3	LWG	375.2027	375.2029	0.0002	0.53
у4	GLWG	432.2241	432.2232	0.0009	2.08
у5	<u>F</u> GLWG	579.2926	577.2778	2.0148	1.47
у6	L <u>F</u> GLWG	692.3766	NA	NA	NA
у7	GL <u>F</u> GLWG	749.3981	747.3830	2.0151	0.74
у8	<u>T</u> GL <u>F</u> GLWG	850.4458	846.4096	4.0362	5.79
у9	A <u>T</u> GL <u>F</u> GLWG	921.4829	NA	NA	NA
y10	GA <u>T</u> GL <u>F</u> GLWG	978.5043	974.4732	4.0311	0.21
y11	AGA <u>T</u> GL <u>F</u> 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.

у	Sequence	Calculated	Observed	Mass	Error (ppm)
ions		Mass [M +	Mass	Difference	
		H] <sup>+</sup>		(Da)	
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
у5	<u>F</u> GLWG	579.2926	577.2753	2.0173	2.86
у6	L <u>F</u> GLWG	692.3766	NA	NA	NA
у7	GL <u>F</u> GLWG	749.3981	747.3706	2.0275	15.86
y8	<u>T</u> GL <u>F</u> GLWG	850.4458	846.4063	4.0395	9.69
y9	A <u>T</u> GL <u>F</u> GLWG	921.4829	NA	NA	NA
y10	GA <u>T</u> GL <u>F</u> GLWG	978.5043	974.4659	4.0384	7.29
y11	AGA <u>T</u> GL <u>F</u> GLWG	1049.5415	1045.5199	4.0216	9.28

**Figure S8**. MIC analysis for subtilosin A and subtilosin A<sub>OC</sub>, 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.