Supplementary information

Application of bacterial cytological profiling to crude natural product extracts reveals the antibacterial arsenal of *Bacillus subtilis*

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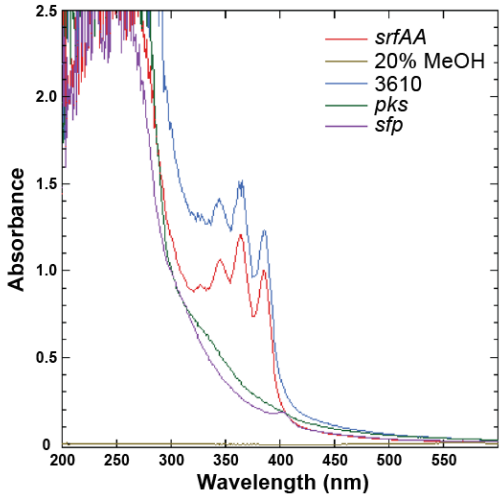
Supplementary figures

a



b

Strain	Bacillaene[1,2]
3610	2.9 mmol
sfp	<0.05 mmol
pks	<0.05 mmol
srfAA	2.3 mmol
ppsB	2.6 mmol
albA	2.6 mmol

[1] estimated extinction coefficient at 363 nm (e_{363nm}) is $80 mM^{-1}cm^{-1}$

[2] mmol in 10 ml extract

Figure S1 Bacillaene signature spectrum and amount found in each extract

(a) Absorption spectrum of *B. subtilis* ethanolic crude extracts assayed for bacillaene (b) Estimated bacillaene content in each crude extract based on 363 nm absorbance

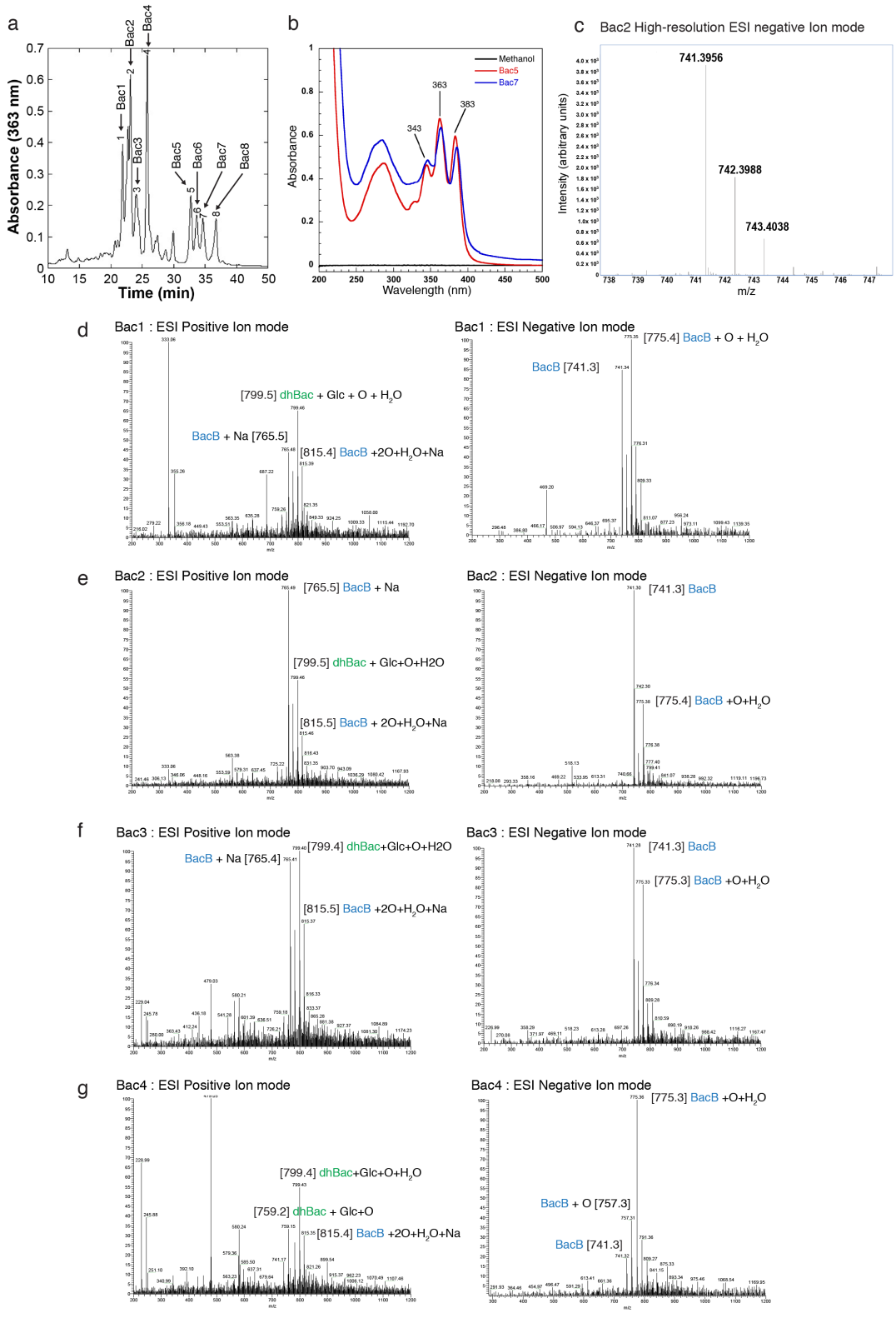

a
Bac2
Bac4
Bac1
Bac3
Bac5
Bac6
Bac7
Bac8
Absorbance (363 nm)
Time (min)
b
Methanol
Bac5
Bac7
363
383
343
Absorbance
Wavelength (nm)
c
Bac2 High-resolution ESI negative Ion mode
741.3956
742.3988
743.4038
Intensity (arbitrary units)
m/z
d
Bac1 : ESI Positive Ion mode
[799.5] dhBac + Glc + O + H2O
BacB + Na [765.5]
[815.4] BacB +2O+H2O+Na
Bac1 : ESI Negative Ion mode
[775.4] BacB + O + H2O
BacB [741.3]
e
Bac2 : ESI Positive Ion mode
[765.5] BacB + Na
[799.5] dhBac + Glc+O+H2O
[815.5] BacB + 2O+H2O+Na
Bac2 : ESI Negative Ion mode
[741.3] BacB
[775.4] BacB +O+H2O
f
Bac3 : ESI Positive Ion mode
[799.4] dhBac+Glc+O+H2O
BacB + Na [765.4]
[815.5] BacB +2O+H2O+Na
Bac3 : ESI Negative Ion mode
[741.3] BacB
[775.3] BacB +O+H2O
g
Bac4 : ESI Positive Ion mode
[799.4] dhBac+Glc+O+H2O
[759.2] dhBac + Glc+O
[815.4] BacB +2O+H2O+Na
Bac4 : ESI Negative Ion mode
[775.3] BacB +O+H2O
BacB + O [757.3]
BacB [741.3]

Figure S2

Figure S2 Bacillaene B dominated other bacillaene species in the early HPLC fractions (Bac1-Bac4 fractions) (a) HPLC separation of bacillaenes from *srfAA* SepPak fraction 5 showing 8 different major peaks (Bac1-Bac8) (b) Absorption spectrum of HPLC purified bacillaene fraction 5 and fraction 7 (c) High-resolution ESI-MS analysis in the negative ion mode of bacillaene fraction 2 (d) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 1 from the bacillaene purification (e) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 2 from the bacillaene purification (f) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 3 from the bacillaene purification (g) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 4 from the bacillaene purification

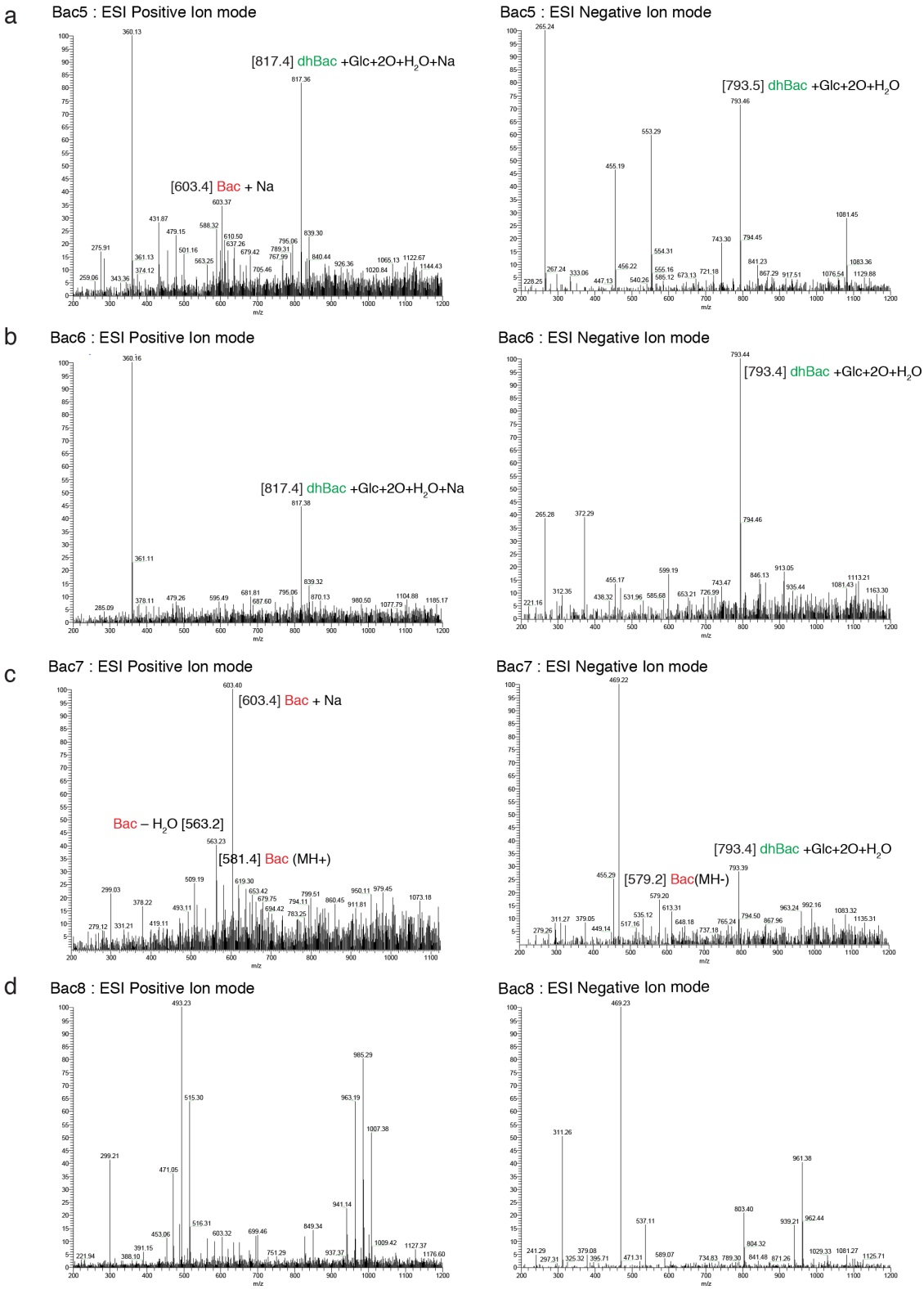

a
Bac5 : ESI Positive Ion mode
[817.4] dhBac +Glc+2O+H2O+Na
[603.4] Bac + Na
Bac5 : ESI Negative Ion mode
[793.5] dhBac +Glc+2O+H2O
b
Bac6 : ESI Positive Ion mode
[817.4] dhBac +Glc+2O+H2O+Na
Bac6 : ESI Negative Ion mode
[793.4] dhBac +Glc+2O+H2O
c
Bac7 : ESI Positive Ion mode
[603.4] Bac + Na
Bac – H2O [563.2]
[581.4] Bac (MH+)
Bac7 : ESI Negative Ion mode
[793.4] dhBac +Glc+2O+H2O
[579.2] Bac(MH-)
d
Bac8 : ESI Positive Ion mode
Bac8 : ESI Negative Ion mode
m/z

Figure S3

Figure S3 Bacillaene found in the later fractions of HPLC (Bac5 and Bac7)

(a) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 5 from the bacillaene purification (b) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 6 from the bacillaene purification (c) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 7 from the bacillaene purification (d) ESI-MS analysis in the positive (left) and negative (right) ion mode of fraction 8 from the bacillaene purification

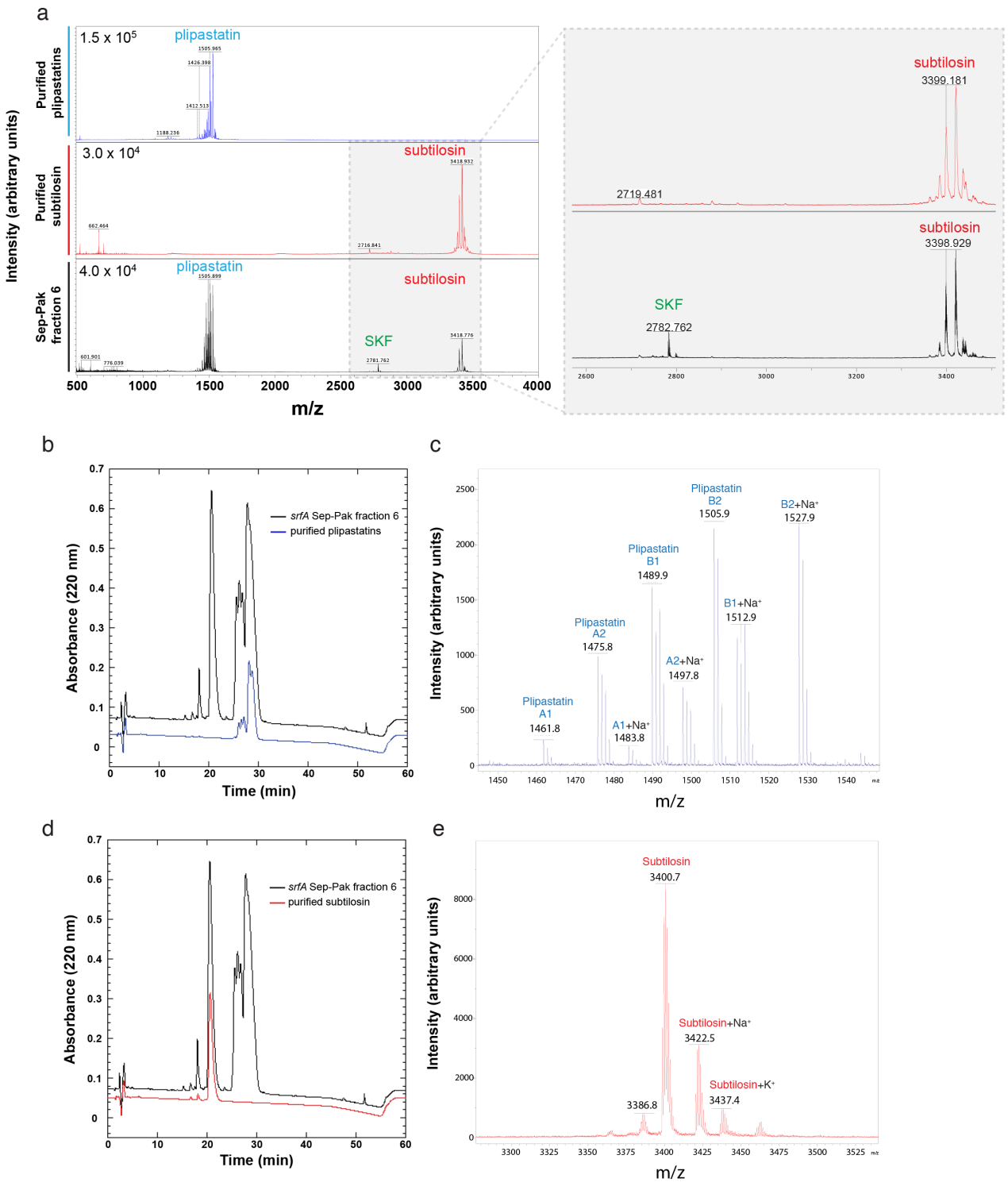

a
Purified plipastatins
1.5 x 10^5
plipastatin
Purified subtilosin
3.0 x 10^4
subtilosin
Sep-Pak fraction 6
4.0 x 10^4
plipastatin
subtilosin
SKF
Intensity (arbitrary units)
m/z
subtilosin
3399.181
2719.481
subtilosin
3398.929
SKF
2782.762
b
srfA Sep-Pak fraction 6
purified plipastatins
Absorbance (220 nm)
Time (min)
c
Plipastatin B2
1505.9
B2+Na+
1527.9
Plipastatin B1
1489.9
B1+Na+
1512.9
Plipastatin A2
1475.8
A2+Na+
1497.8
Plipastatin A1
1461.8
A1+Na+
1483.8
Intensity (arbitrary units)
m/z
d
srfA Sep-Pak fraction 6
purified subtilosin
Absorbance (220 nm)
Time (min)
e
Subtilosin
3400.7
Subtilosin+Na+
3422.5
Subtilosin+K+
3437.4
3386.8
Intensity (arbitrary units)
m/z

Figure S4

Figure S4 Plipastatin and subtilosin purification

(a) MALDI mass spectrometry analysis (positive mode) of purified plipastatins, purified subtilosin from *B. subtilis srfAA* SepPak fraction 6 (b) HPLC purification of plipastatins from *B. subtilis srfAA* SepPak fraction 6.(c) MALDI-TOF mass spectrometry analysis (positive mode) of HPLC purified and reconstituted *B. subtilis* plipastatins A1, A2, B1 and B2 (d) HPLC purification of subtilosin from *B. subtilis srfAA* SepPak fraction 6. (e) MALDI-TOF mass spectrometry analysis (positive mode) of HPLC purified subtilosin

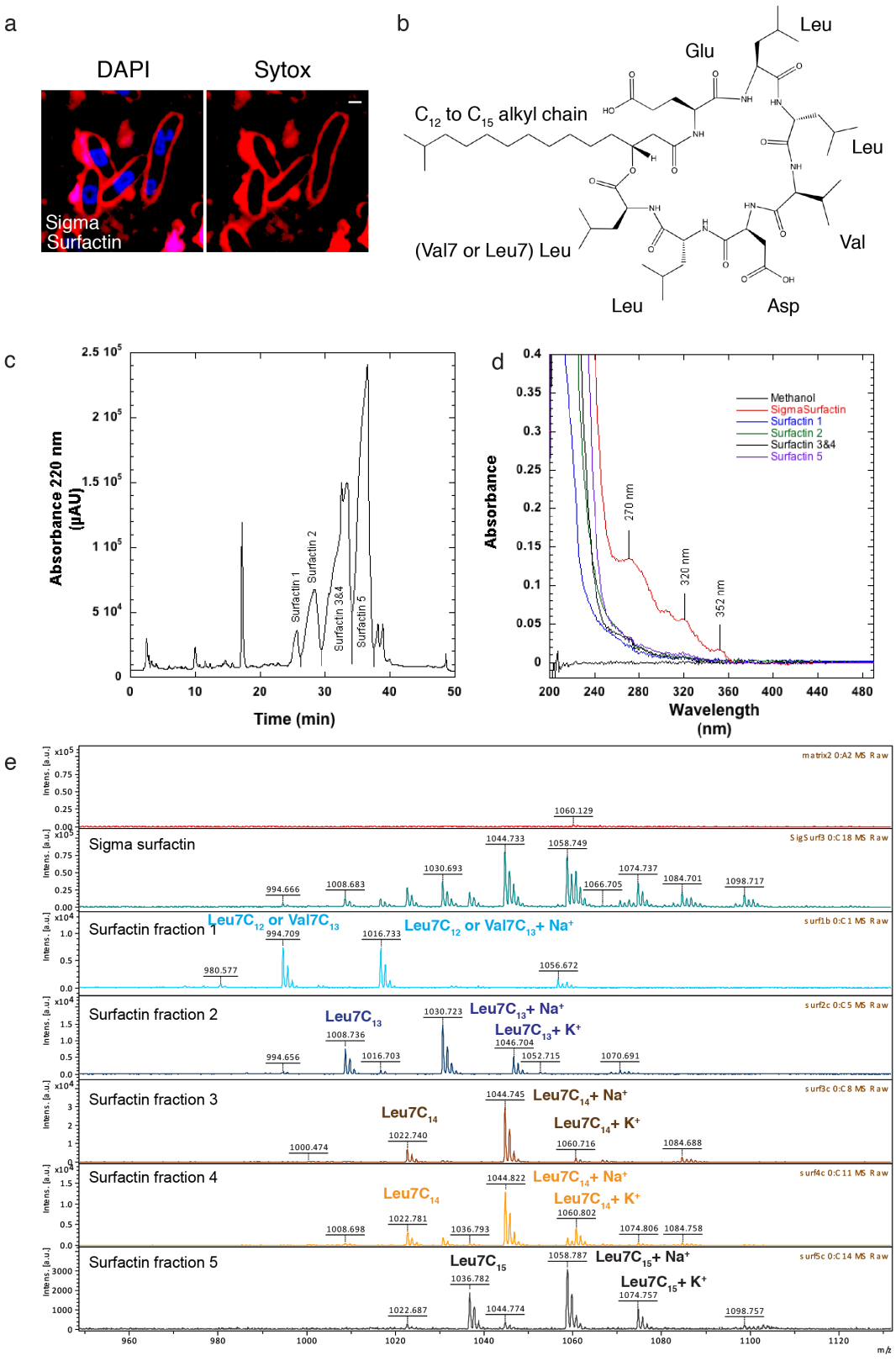

a
DAPI
Sytox
Sigma
Surfactin
b
Leu
Glu
C12 to C15 alkyl chain
Leu
(Val7 or Leu7) Leu
Val
Leu
Asp
c
Absorbance 220 nm (µAU)
Surfactin 1
Surfactin 2
Surfactin 3&4
Surfactin 5
Time (min)
d
Absorbance
Methanol
SigmaSurfactin
Surfactin 1
Surfactin 2
Surfactin 3&4
Surfactin 5
270 nm
320 nm
352 nm
Wavelength (nm)
e
Sigma surfactin
Surfactin fraction 1
Surfactin fraction 2
Surfactin fraction 3
Surfactin fraction 4
Surfactin fraction 5
Leu7C12 or Val7C13
Leu7C12 or Val7C13+ Na+
Leu7C13
Leu7C13+ Na+
Leu7C13+ K+
Leu7C14
Leu7C14+ Na+
Leu7C14+ K+
Leu7C15
Leu7C15+ Na+
Leu7C15+ K+
m/z

Figure S5

Figure S5 Surfactin purification

(a) *E. coli* cells treated for 2 hours with Sigma commercial surfactin 576μM. *E. coli* cells were stained with FM4-64 (Red), DAPI (Blue) and SYTOX-Green (Green). Scale bar, 1 μm. (b) Surfactin chemical structure and annotation (c) HPLC purification of commercial *B. subtilis* surfactin (Sigma S3523) (d) UV-Visible absorption spectrum of commercial surfactin and HPLC purified surfactins assayed for bacillaene contamination. (e) MALDI-TOF mass spectrometry analysis (positive mode) of commercial surfactin and HPLC purified surfactin fractions.

Supplementary tables

Table S1 Strains used in this study

Strains	Genotype	References
Bacillus subtilis		
3610	undomesticated parent of 168	Previous study[1]
PSK0178	*Δpks::spc*	Previous study[2]
PSK0060	*srfAA::mls*	Previous study[3]
PSK0417	*ΔalbA::kan*	Previous study[4]
EG220-1	*sfp::mls*	Previous studies[1,3]
PSK0156	*ppsB::spc*	Previous study[3]
Escherichia coli		
NR698	*lptD4213*	Previous study[5]

Table S2. Bacillaene fractions from HPLC of *srfAA* SepPak 5

Fraction (% of Total Bacillaenes)	Bacillaene concentration (μM) Assumes E_{363}~80 mM^{-1}	Killing activity*	Minimal Cytological Concentration (μM)
Fraction 1 : Bac1 (9.5%)	300	-	NA
Fraction 2 : Bac2 (26%)	353	+	20
Fraction 3 : Bac3 (11%)	188	-	NA
Fraction 4 : Bac4 (18%)	158	-	NA
Fraction 5 : Bac5 (7%)	61	-	NA
Fraction 6 : Bac6 (6%)	74	-	NA
Fraction 7 : Bac7 (6%)	68	+	2
Fraction 8 : Bac8 (6%)	44	-	NA

* Determined based on killing spot test

Table S3 Minimal inhibitory concentration of purified natural products from *B. subtilis*

Molecules	Minimal inhibitory concentration (μM)
Bacillaene	NA
Bacillaene B	NA
Plipastatin	>128
Subtilosin	8
Surfactin	>512
SKF	NA

Supplementary references

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3. Straight, P. D., Willey, J. M. & Kolter, R. Interactions between Streptomyces coelicolor and Bacillus subtilis: Role of Surfactants in Raising Aerial Structures. *J. Bacteriol.* **188,** 4918–4925 (2006).
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5. Ruiz, N., Falcone, B., Kahne, D. & Silhavy, T. J. Chemical Conditionality: A GeneticStrategy to Probe Organelle Assembly. *Cell* **121,** 307–317 (2005).