Characterization of *Bacillus subtilis* Colony Biofilms via Mass Spectrometry and Fluorescence Imaging

Tong Si,[1, ‡] Bin Li,[2,3, ‡] Ke Zhang,[2] Yiran Xu,[4] Huimin Zhao,[1,2,4,5,*]
and Jonathan V. Sweedler[2,3,5,*]

[1]Department of Chemical and Biomolecular Engineering and Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

[2]Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

[3]Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

[4]Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

[5]Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

SUPPORTING INFORMATION

Contents

Additional methods.

Figure S1. Colony morphology of *B. subtilis* strains on MSgg and ISP2 media.

Figure S2. Representative MALDI LIFT ToF/ToF mass spectra of SKF and SDP.

Figure S3. Cell growth of the *B. subtilis* NCIB3610 strain and its mutants.

Table S1. Strains used in this study.

Table S2. Selected metabolites assigned in *B. subtilis* biofilms by MALDI ToF MS analysis of intact cells.

Supporting references.

ADDITIONAL METHODS

Intact-cell MALDI-MS and LIFT TOF/TOF

After cultivation, a square section (7 mm × 7 mm) containing the biofilms of the wild type *B. subtilis* NCIB3610 strain was transferred to a 1.7 mL Eppendorf tube and suspended in 500 µL of acetonitrile (ACN)-water solution containing 0.1% trifluoroacetic acid (TFA) (1:1, v/v). The suspension was further subjected to a 15 min sonication. Cell residues were separated by centrifugation (5 min, 10,000 rpm, 4 °C), and the supernatant was transferred to a 1.7 mL Eppendorf tube and dried under vacuum in a rotary flash evaporator (MiVac, GeneVac, UK). The samples were reconstituted in 100 µL of ACN-water solution containing 0.1% TFA (1:1, v/v). For MALDI MS analysis, 1 µL of the extracts was mixed with 2 µL of DHB solution (50 mg mL^{-1} in ACN-water with 0.1% TFA, 7:3, v/v) and spotted onto a MTP 384 polished steel target (Bruker Daltonics, Billerica, MA). MALDI LIFT TOF/TOF mass spectra were obtained in positive reflection mode on the same MALDI mass spectrometer described in the main text. For each spot, 1000 laser shots fired at 1000 Hz were collected. The characteristic fragments of those ions and tandem MS spectra of SKF and SDP are shown in **Table S2** and **Figure S2**.

Cell Growth Measurement

Cell growth was monitored by measuring optical density at a wavelength of 600 nm (OD_{600}) of cell culture using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Three freshly streaked *B. subtilis* colonies (biological triplicates) on a luria broth (LB) agar plate were inoculated separately into 3 mL of LB liquid media for cultivation at 37 °C and 250 rpm. After 20 h, cells were re-inoculated into 3 mL of fresh LB liquid media, and initial cell density was adjusted to $OD_{600} = 0.2$ for each culture. Cell density was then measured at indicated time intervals. After 12 h, 1 µL of cell culture from each replicate was spotted onto MSgg plates supplemented with 1.5% agar and allowed to grow under static conditions at 37 °C for 24 h. Colony biofilms were harvested from the MSgg agar surface and placed in 1 mL of PBS. Biofilms were dispersed with 12 pulses of mild sonication with 1 s duration and 30% amplitude on a Fisher Scientific Sonic Dismembrator Model 500. The cell suspension was then subjected to OD_{600} measurement. For colony biofilms, cell growth was calculated as cell density equivalents, assuming biofilms were suspended as single cells in 1 mL of liquid culture.

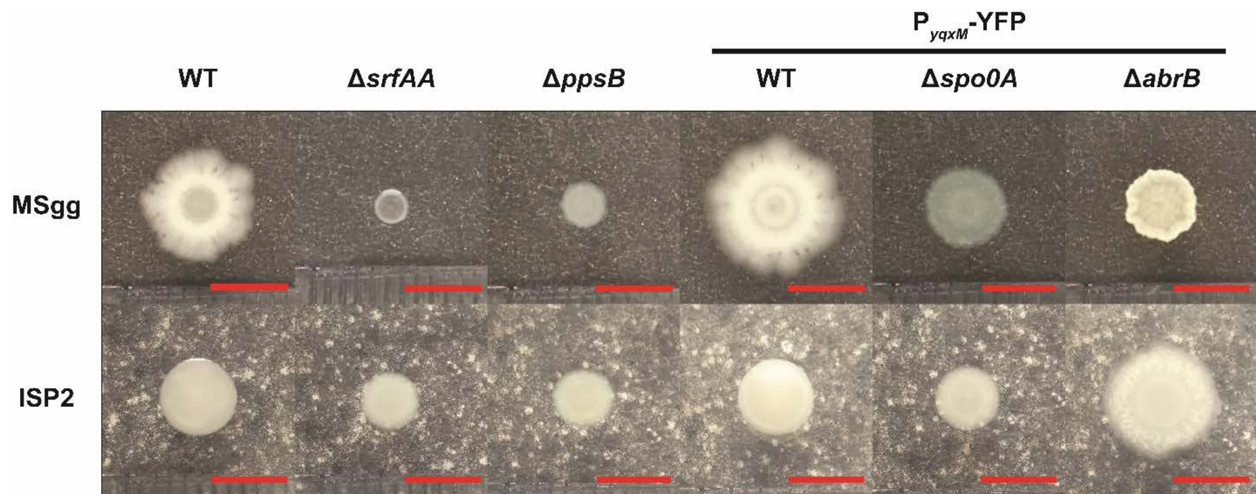

Figure S1. Colony morphology of *B. subtilis* strains on MSgg and ISP2 media. Pictures were taken after incubation at 37 °C for 24 h on solid agar media. Scale bars = 5 mm.

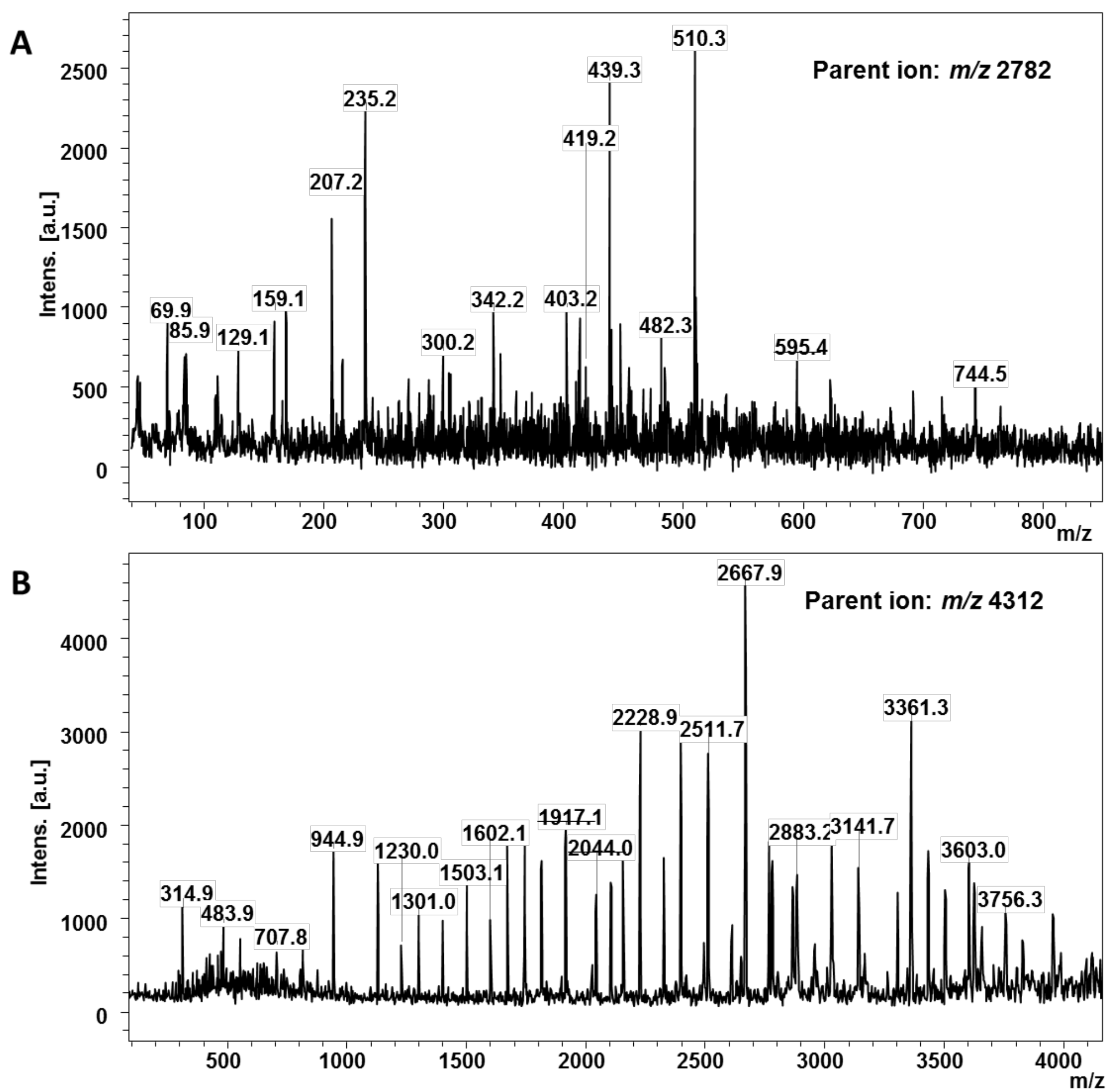

Figure S2. Representative MALDI LIFT TOF/TOF mass spectra of (A) SKF and (B) SDP.

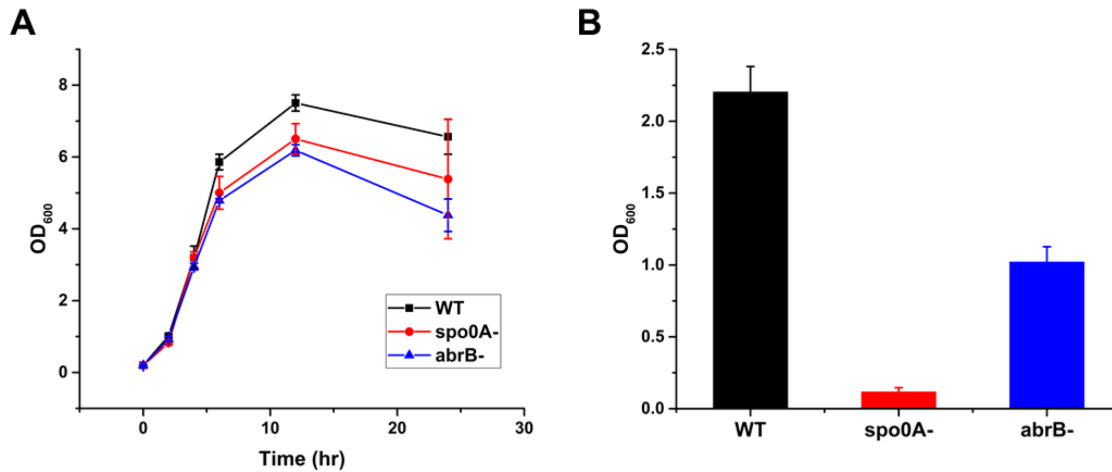

Figure S3. Cell growth of the *B. subtilis* NCIB3610 strain and its mutants. (A) *B. subtilis* strains were cultivated in LB liquid media with an initial cell density of $OD_{600} = 0.2$. (B) *B. subtilis* biofilms were developed from 1 μL of overnight cell cultures spotted on MSgg agar. After 24 h, biofilms were harvested and dispersed in 1 mL of PBS using sonication before cell density measurement. For biofilms, the equivalent values of initial cell density were estimated as $OD_{600} = 0.0075 \pm 0.0002$ (WT), 0.0065 ± 0.0004 (Δ*spo0A*), and 0.0062 ± 0.0002 (Δ*abrB*), assuming that 1 μL of the cell cultures were added in 1 mL of liquid media. Error bars indicate the SDs of three biological replicates. *P* values were calculated using the independent two-tailed, two-sample *t*-test for equal sample sizes and equal variance: (**A**) 0.023 (spo0A-/WT) and 0.0011 (abrB-/WT) at 12 h for liquid media when cell cultures were spotted onto MSgg agar media; and (**B**) 3.6×10^{-5} (spo0A-/WT) and 5.8×10^{-4} (abrB-/WT) at 24 h for agar media.

Table S1. Strains used in this study.

Strain name	Genotype	Sources
Donor laboratory strains		
1S143	PY79 spo0A::kan	BGSC*
1A935	168 Cm pheA1 trpC2 abrB::cat	BGSC
BK03480	168 trpC2 srfAA::erm	BGSC
BK18330	168 trpC2 ppsB::erm	BGSC
NCIB3610		
3A1	Wild type isolate	BGSC
3A1_ΔsrfAA	3A1 srfAA::erm	This study
3A1_ΔppsB	3A1 ppsB::erm	This study
Reporter strain (ZK3779)		
ZK3779_WT	NCIB3610 amyE::P_{srfAA}-yfp** (spec), lacA::P_{yqxM}-cfp (mls)	Ref[1]
ZK3779_Δspo0A	ZK3779_WT spo0A::kan	This study
ZK3779_ΔabrB	ZK3779_WT abrB::cat	This study
SPP1 phage		BGSC

*BGSC: Bacillus Genetic Stock Center, Columbus, OH

**As only a tiny fraction of the cell population was actively expressed from the P_{srfAA} promoter[1], the YFP fluorescence was too weak for the fluorescence stereoscope, and therefore not studied in this work.

Table S2. Selected metabolites assigned in *B. subtilis* biofilms by MALDI TOF MS analysis of intact cells.

Compounds	Molecular formula	Measured [M + H]+ [theoretical monoisotopic mass, mass error (ppm)]	Measured [M + Na]+ [theoretical monoisotopic mass, mass error (ppm)]	Measured [M + K]+ [theoretical monoisotopic mass, mass error (ppm)]	Fragments (parent ions, ([M+Na]+)	Ref.
surfactin C_{13}	$C_{51}H_{89}N_7O_{13}$	ND	1030.6387 [1030.6411, -2.3]	ND	ND	Ref[2]
surfactin C_{14}	$C_{52}H_{91}N7O_{13}$	ND	1044.6540 [1044.6567, -2.6]	ND	931.5, 800.3, 707.3, 594.2	Ref[2]
surfactin C_{15}	$C_{53}H_{93}N7O_{13}$	ND	1058.6720 [1058.6724, -0.4]	1074.6487 [1074.6463, 2.2]	945.6, 814.4, 707.3, 594.2	Ref[2]
Plipastatin-C_{16}-Ala	$C_{72}H_{110}N_{12}O_{20}$	1463.7903 [1463.8032, -8.8]	1485.7707 [1485.7852, -9.8]	ND	ND	Ref[2]
Plipastatin-C_{17}-Ala	$C_{73}H_{112}N_{12}O_{20}$	1477.7988 [1477.8189, -13.6]	1499.7962 [1499.8008, -3.1]	ND	1130.9, 1016.7	Ref[2]
Plipastatin-C_{16}-Val	$C_{74}H_{114}N_{12}O_{20}$	1491.8203 [1491.8345, -9.5]	1513.8152 [1513.8165, -0.9]	ND	1301.6, 1130.3, 1016.1	Ref[2]
Plipastatin-C_{17}-Val	$C_{75}H_{116}N_{12}O_{20}$	1505.8384 [1505.8502, -7.8]	1527.8301 [1527.8321, -1.3]	1543.8150 [1543.8060, 5.8]	1301.6, 1130.4, 1016.2	Ref[2]
Subtilosin	$C_{152}H_{226}N_{38}O_{45}S_3$	3400.5076 [3400.5799, -21.2]	3422.5111 [3422.5619, -14.8]	3438. 4739 [3438.5358, -18.0]	ND	Ref[2,3]
SKF	$C_{119}H_{192}N_{36}O_{29}S_6$	2782.2572 [2782.3053, -17.3]	ND	ND	*	Ref[4]
SDP	$C_{200}H_{307}N_{47}O_{55}S_2$	4312.1293 [4312.2185, -20.7]	4334.1719 [4334.2004, -6.6]	4350.0520 [4350.1744, -28.1]	*	Ref[4]

ND: not detectable

*: Characteristic fragments of ions at *m/z* 2782 and *m/z* 4312 were shown in **Figure S2**.

Supporting References

(1) Lopez, D.; Vlamakis, H.; Losick, R. Kolter, R. Paracrine signaling in a bacterium. *Genes Dev.* **2009,** *23*, (14), 1631-1638.
(2) Yang, Y. L.; Xu, Y.; Straight, P. Dorrestein, P. C. Translating metabolic exchange with imaging mass spectrometry. *Nat. Chem. Biol.* **2009,** *5*, (12), 885-887.
(3) Marx, R.; Stein, T.; Entian, K. D. Glaser, S. J. Structure of the *Bacillus subtilis* peptide antibiotic subtilosin A determined by 1H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Protein Chem.* **2001,** *20*, (6), 501-506.
(4) Liu, W. T.; Yang, Y. L.; Xu, Y.; Lamsa, A.; Haste, N. M.; Yang, J. Y.; Ng, J.; Gonzalez, D.; Ellermeier, C. D.; Straight, P. D.; Pevzner, P. A.; Pogliano, J.; Nizet, V.; Pogliano, K. Dorrestein, P. C. Imaging mass spectrometry of intraspecies metabolic exchange revealed the cannibalistic factors of *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **2010,** *107*, (37), 16286-16290.