Supporting Information for

Real-time Metabolomics on Living Microorganisms Using Ambient Electrospray Ionization Flow-Probe

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** A demonstration video is also available*

This document contains:

1. Figure S1-S3. Real-time mass spectrometry analysis of living microorganisms using flowprobe prototype: *S. coelicolor* A3(2), *B. subtilis* 3610 and PY79, *P. aeruginosa* PA14
2. Figure S4-S11. MS/MS validation of specialized metabolites investigated
3. Figure 12. MS spectra of microbial colony **1-34**; and Figure 13. NanoDESI and LMJ-SSP results.
4. Experimental section: Instrument methods and preparation procedures of the microbial colonies
5. References

Mass Spectrometry Analysis

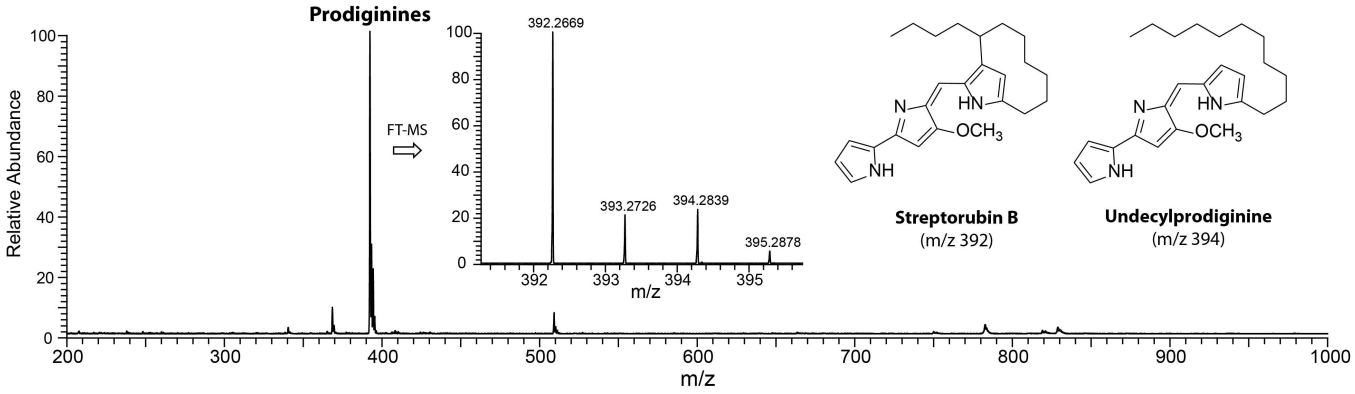


Figure S1. Mass spectrum acquired from the extract of the surface of *S. coelicolor* A3(2) colony. The peaks at *m/z* 392 and 394 are prodiginines as confirmed by fragmentation shown in the supporting information. *Inset*: expanded high-resolution FT-MS spectrum; mass errors: -7 ppm (streptorubin B) and -4 ppm (undecylprodigine). *Right*: molecular structures of cyclic and open ring prodiginines secreted by *S. coelicolor* A3(2).

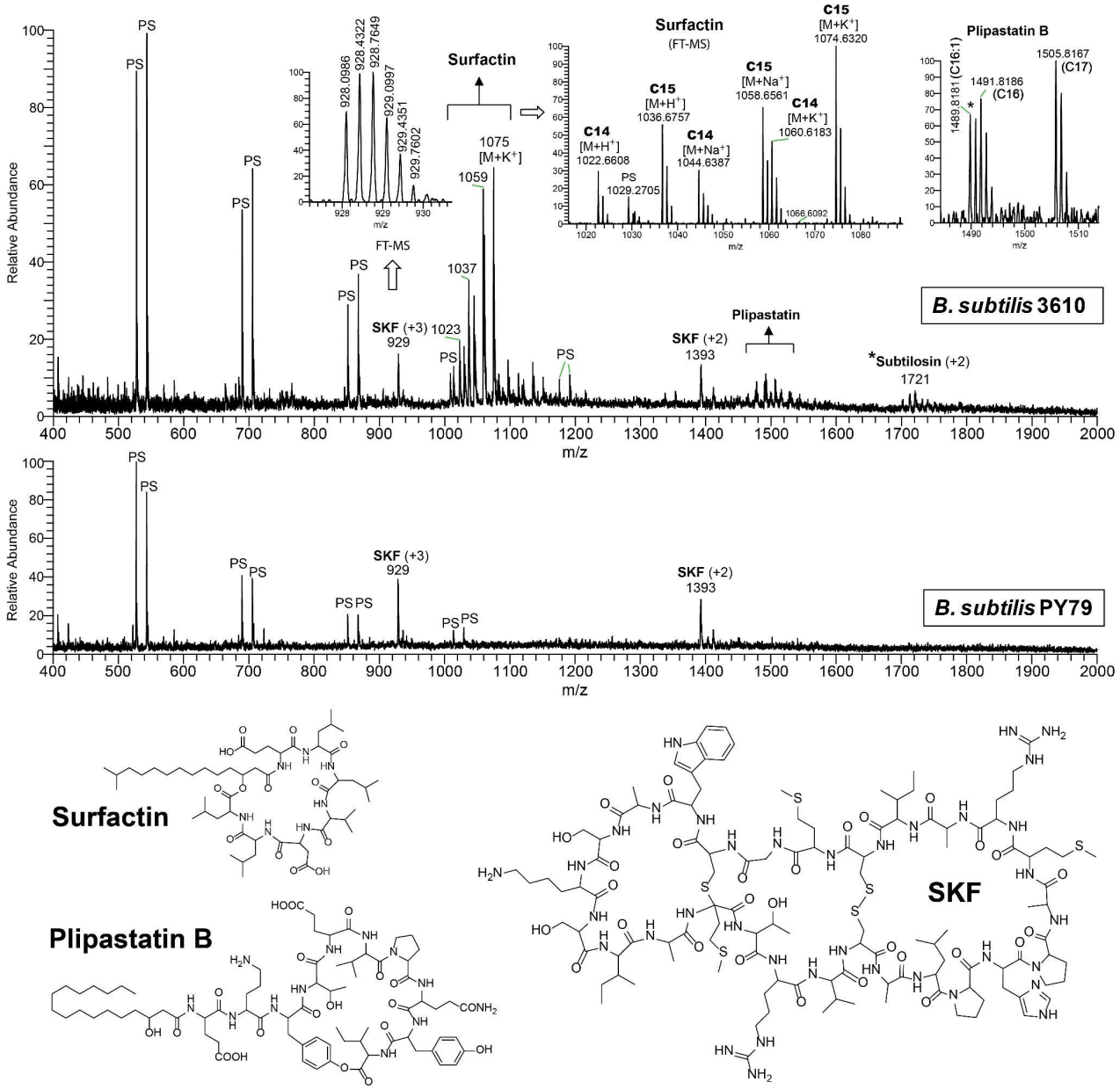


Figure S2. Analysis of *B. subtilis* wild-type and domesticated strains with flowprobe prototype. *Top*: Mass spectrum acquired on the surface of *B. subtilis* 3610 and PY79 biofilm. *Inset*: high resolution expanded FT-MS spectra of annotated metabolites secreted by *B. subtilis* 3610. The peaks for surfactin, plipastatin and SKF are confirmed by MS/MS as shown in the supporting information. Putative annotations are denoted with an asterisk. Ions of polysaccharides are labeled (PS). *Bottom*: structures of the found metabolites.

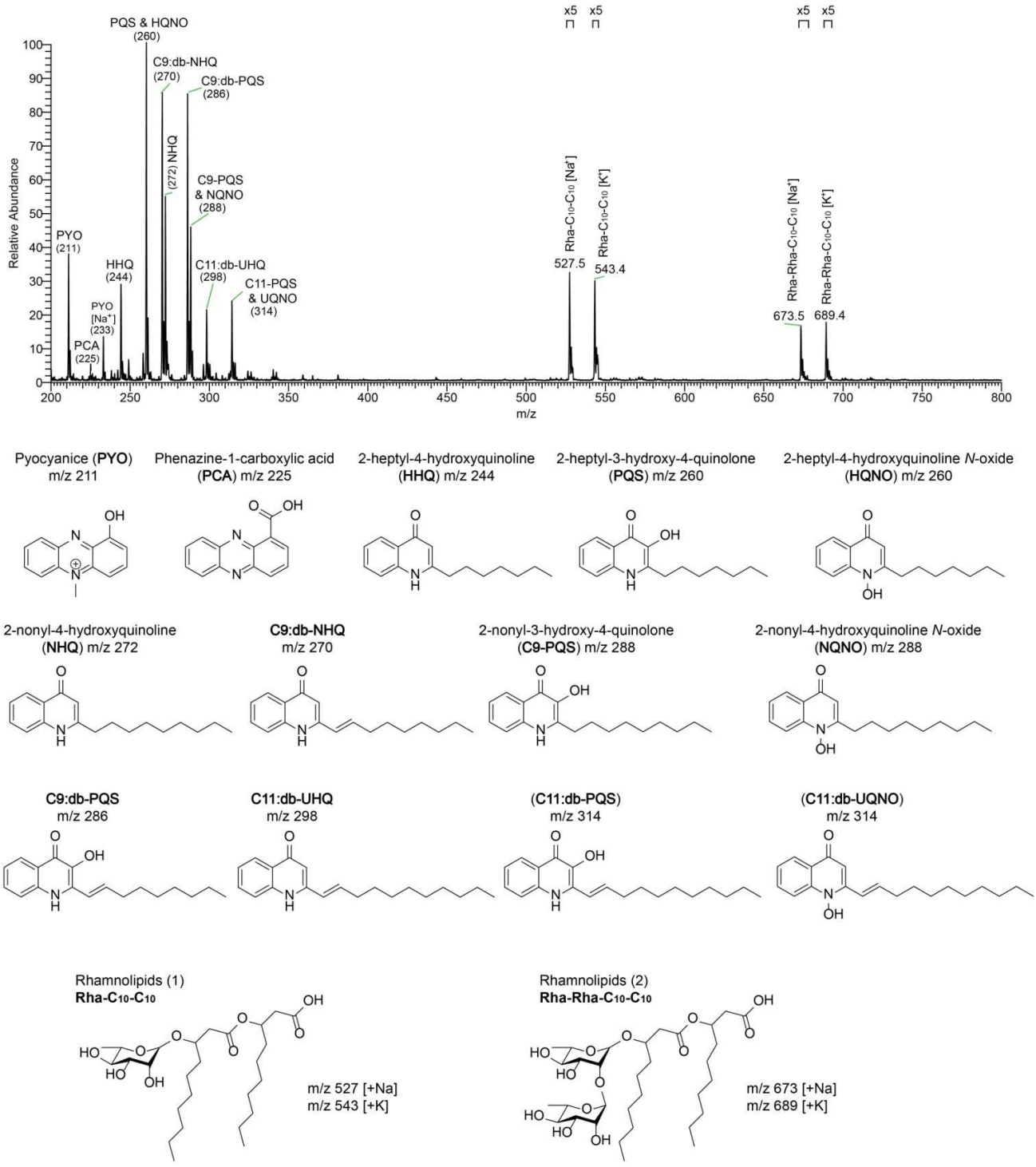


Figure S3. Analysis of pathogen *P. aeruginosa* PA14 biofilm using the flowprobe system. *Top*: Mass spectrum acquired during continuous extraction of surface of *P. aeruginosa* PA14 colony showing phenazines, quinolones and rhamnolipids that are secreted by *P. aeruginosa* PA14. *Bottom*: molecular structures of the metabolites identified.

Tandem Mass Analysis

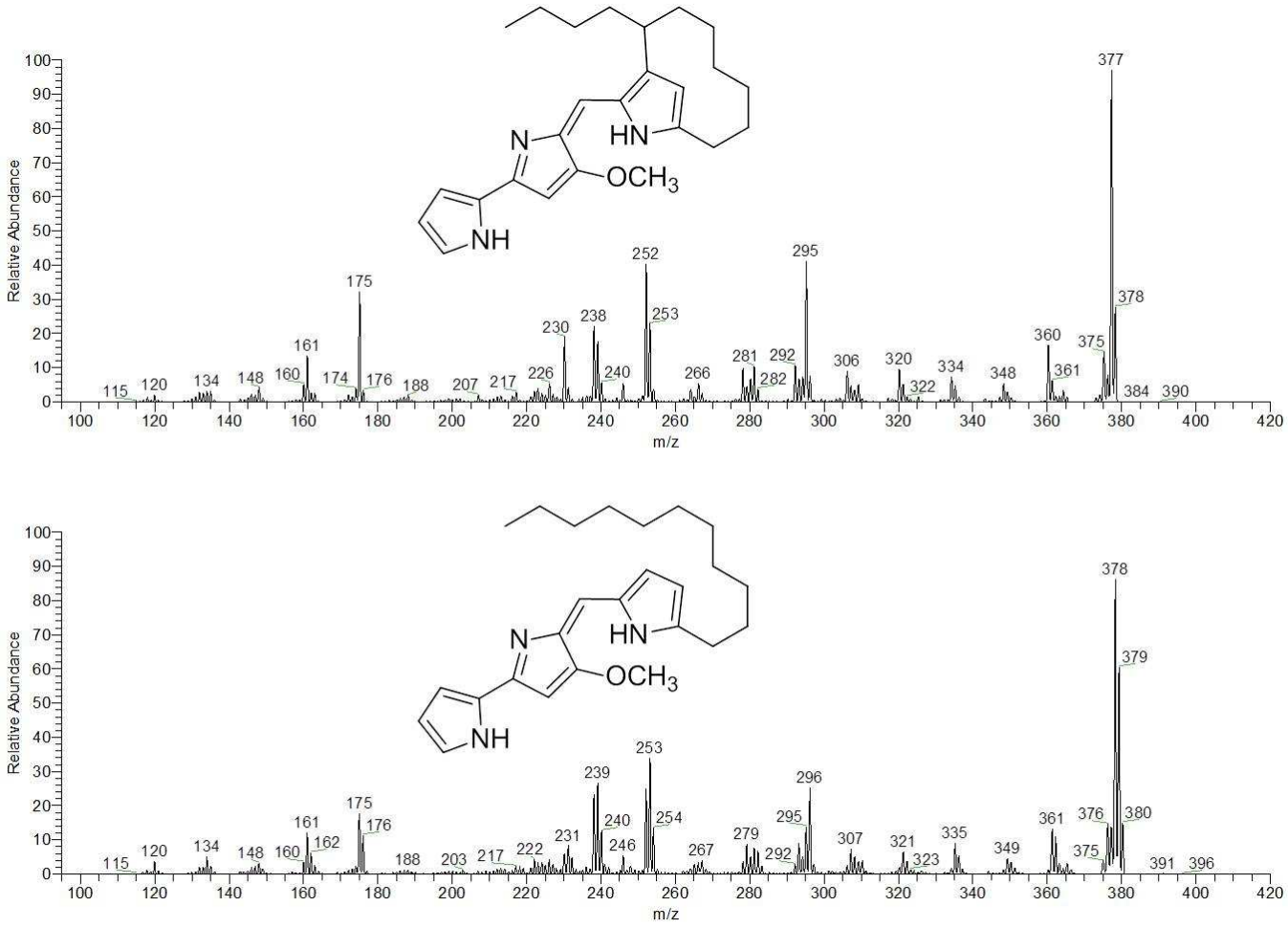


Figure S4. MS/MS validation of prodiginines (*m/z* 392 and 394) produced by *S. coelicolor* A3(2) using LMJ-SSP. The fragmentation products are consistent with the previous report published by Watrous et al.[5]

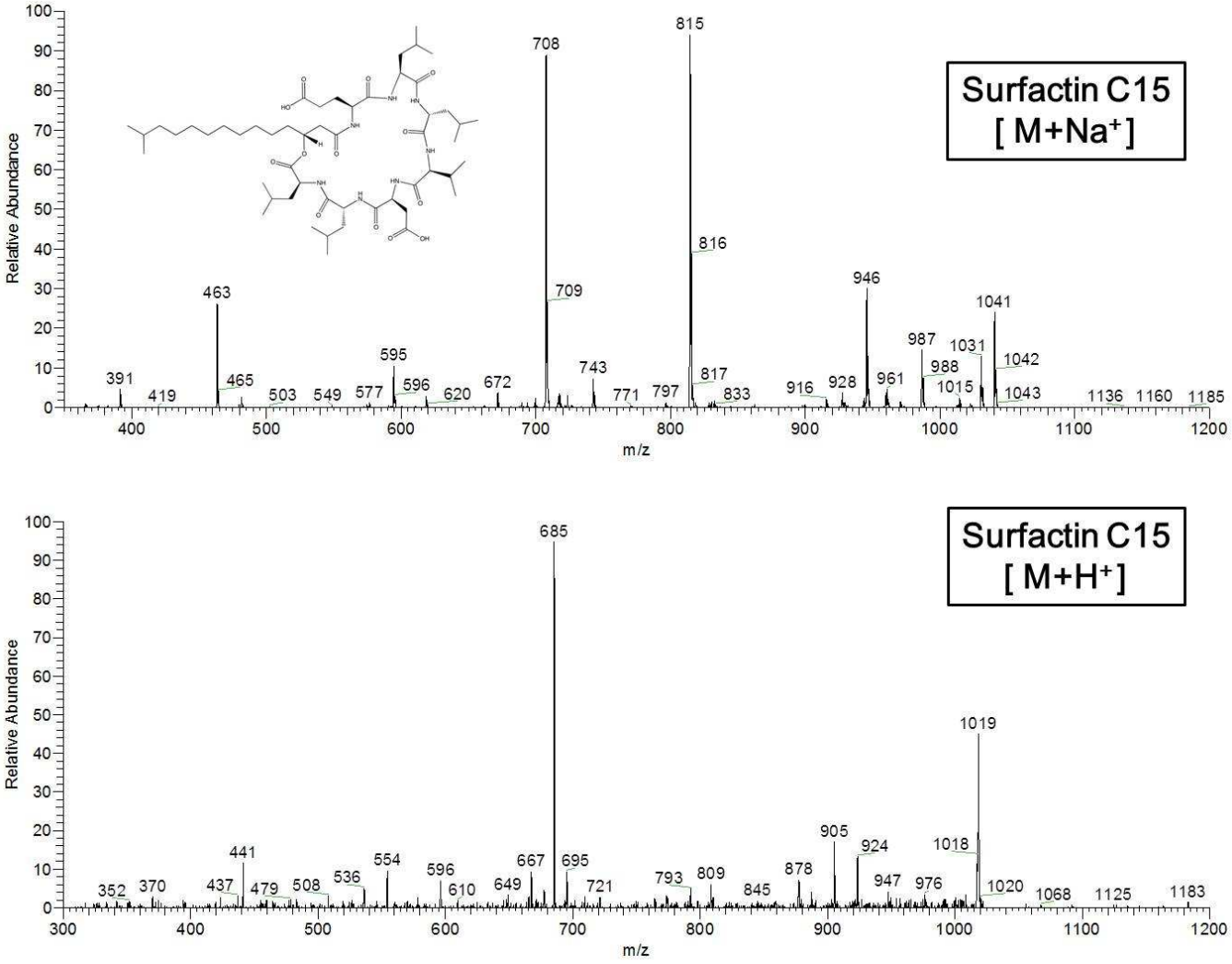


Figure S5. MS/MS validation of surfactin (*m/z* 1037 and 1059, H^+ and Na^+ adducts respectively) secreted by *B. subtilis* 3610 using LMJ-SSP. The fragmentation products are consistent with the previous report published by Watrous et al. (H^+) and Song et al. (Na^+).[5,6]

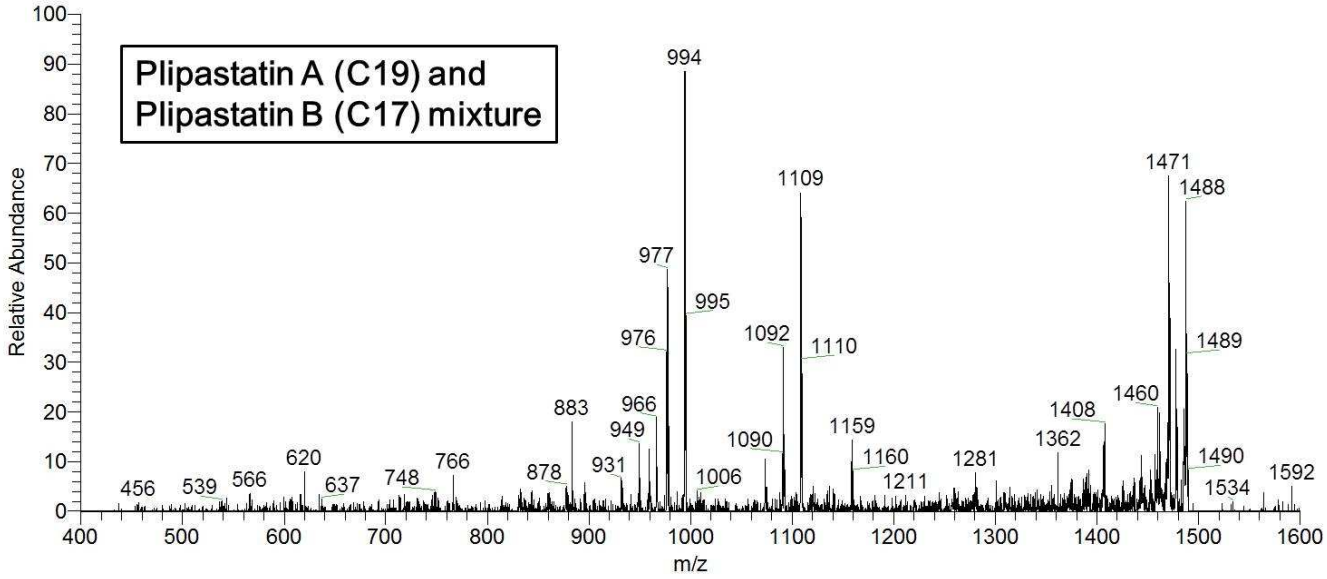


Figure S6. MS/MS validation of plipastatin mixture (*m/z* 1506) produced by *B. subtilis* 3610 using LMJ-SSP. The fragmentation products are consistent with the previous report published by Watrous et al.[5]

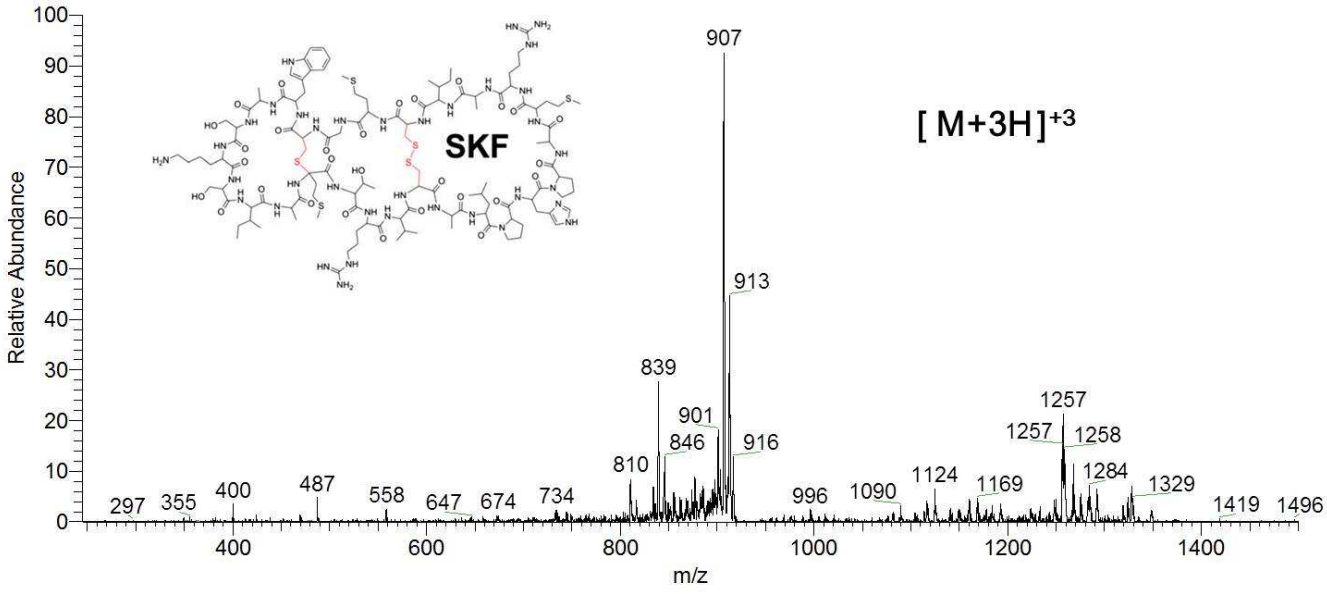


Figure S7. MS/MS validation of SKF (*m/z* 928) produced by *B. subtilis* PY79 using LMJ-SSP. The fragmentation products are consistent with the previous report published by Watrous et al. and Liu et al. using purified SKF.[5,7]

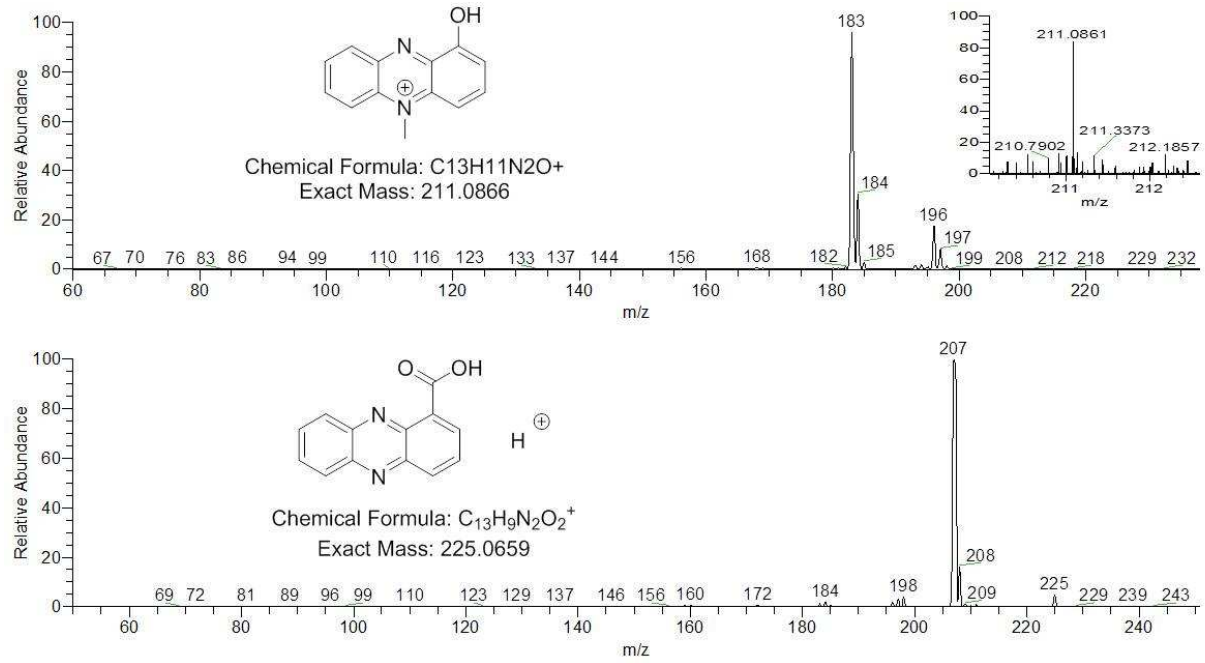


Figure S8. MS/MS validation of two phenazines, PYO (*m/z* 211) and PCA (*m/z* 225) produced by *P. aeruginosa* PA14 using LMJ-SSP. The fragmentation products are consistent with the previous report published by Moree et al. using purified compounds.[8] *Inset*: high-resolution FT-MS spectrum of PYO parent ion. Mass error: -2 ppm.

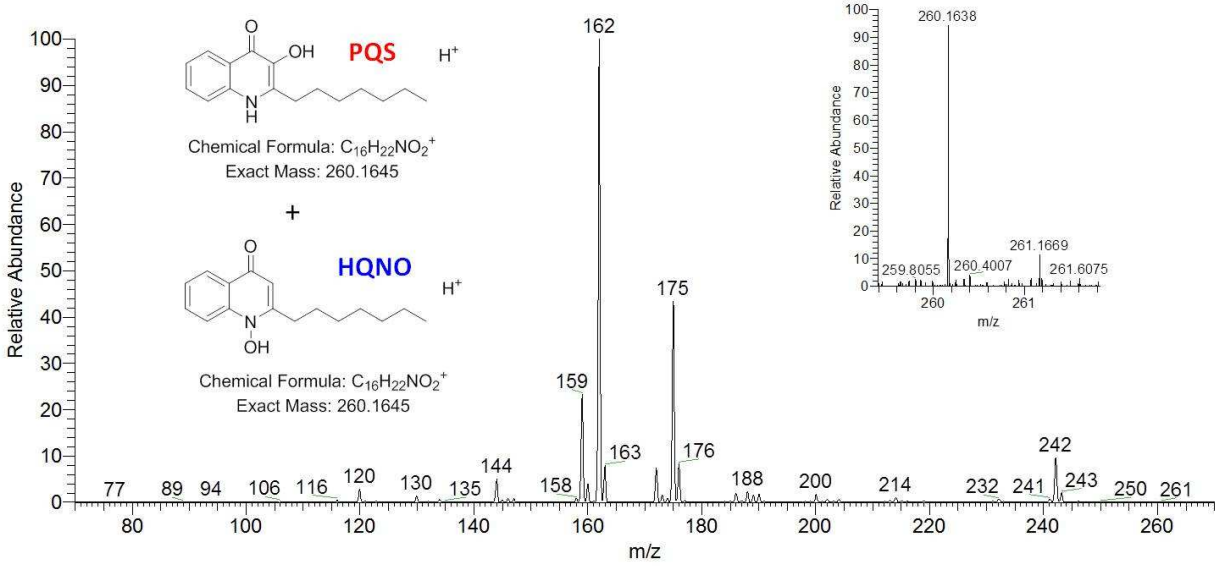


Figure S9. MS/MS validation of PQS and HQNO mixture (*m/z* 260) produced by *P. aeruginosa* PA14 using LMJ-SSP. The fragmentation products are consistent with the previous report published by Moree et al.[8] *Inset*: high-resolution FT-MS spectrum of parent ion. Mass error: -2 ppm.

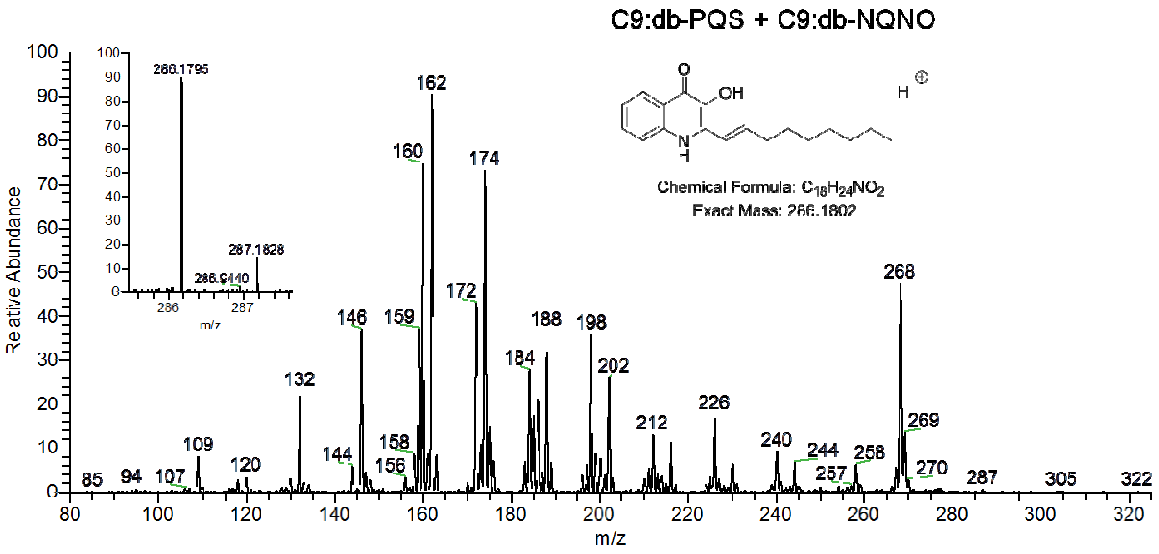


Figure S10. MS/MS validation of C9:db-PQS and C9:db-HQNO (structure not shown) mixture (*m/z* 286) produced by *P. aeruginosa* PA14 using LMJ-SSP. The fragmentation products are consistent with the previous report published by Moree et al.[8] *Inset*: high-resolution FT-MS spectrum of parent ion. Mass error: -2 ppm.

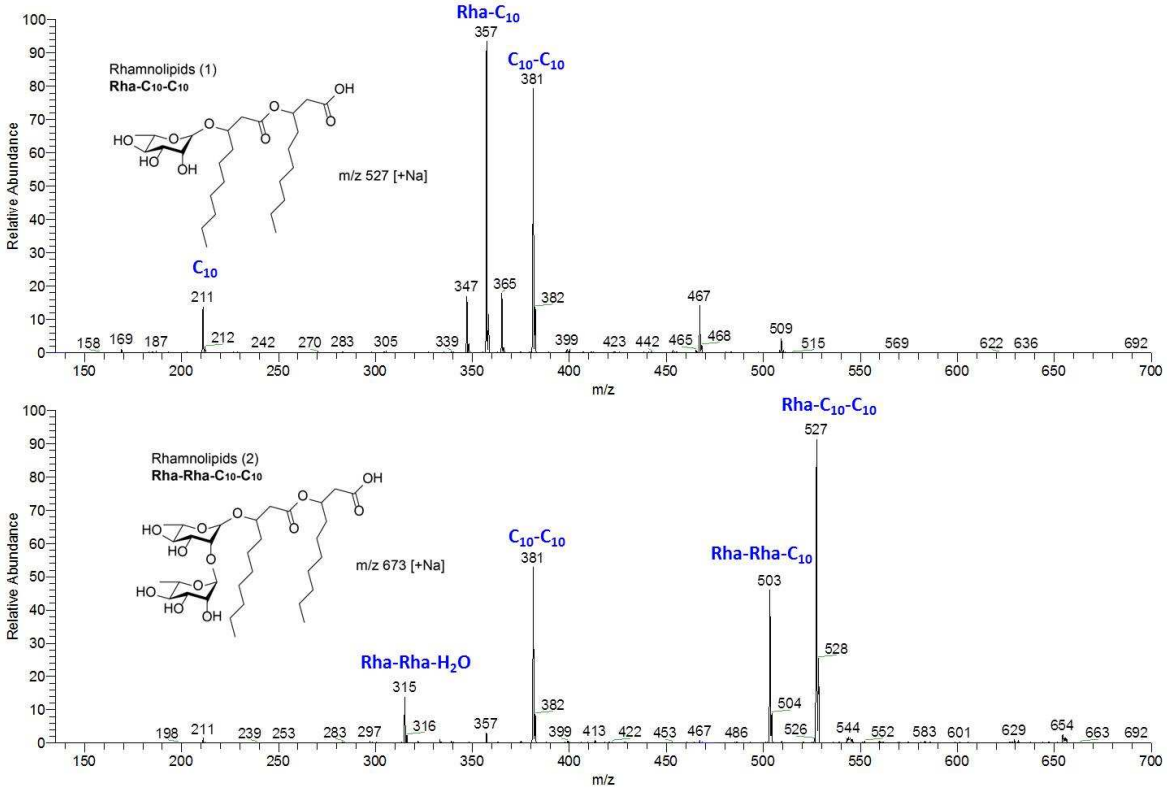
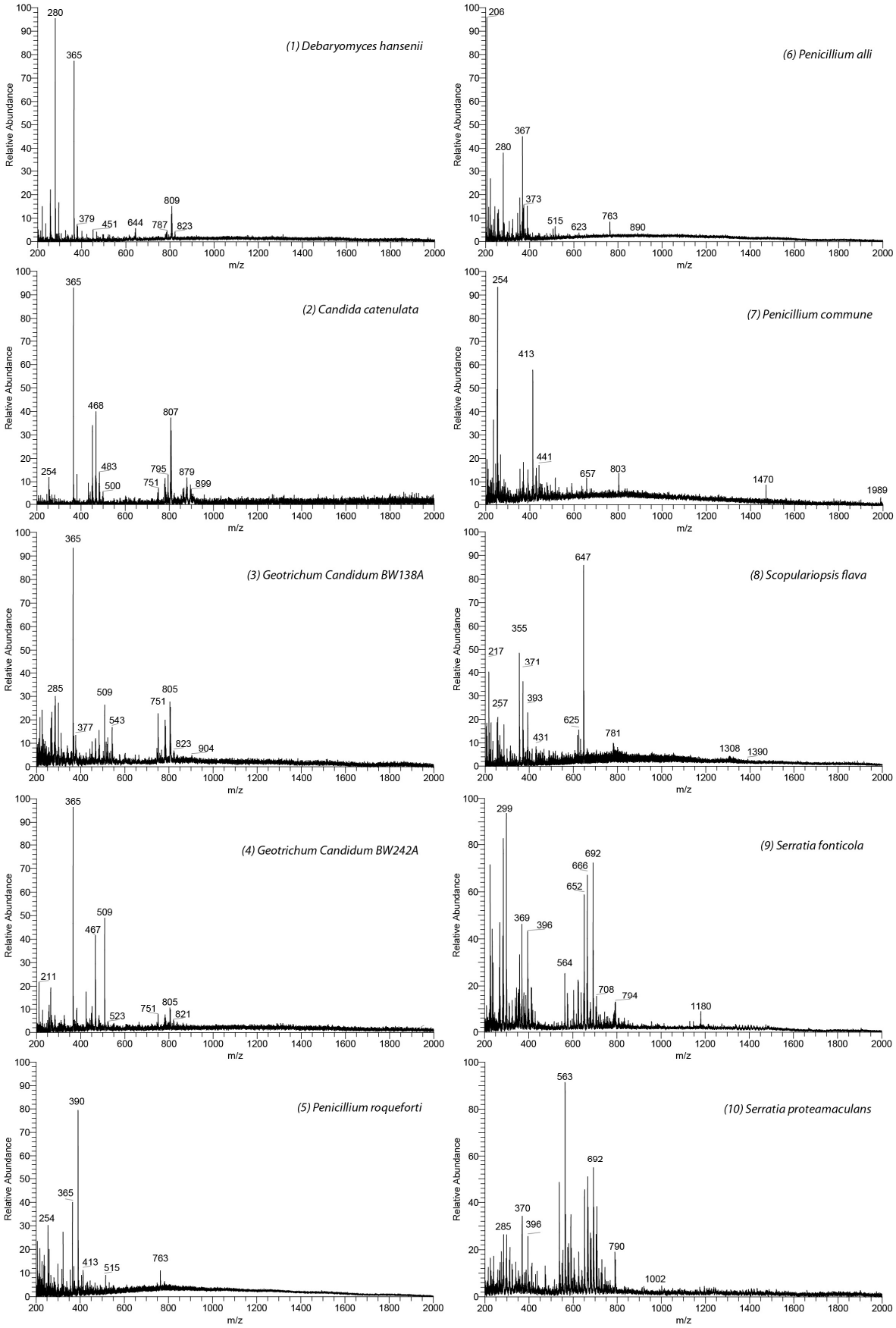
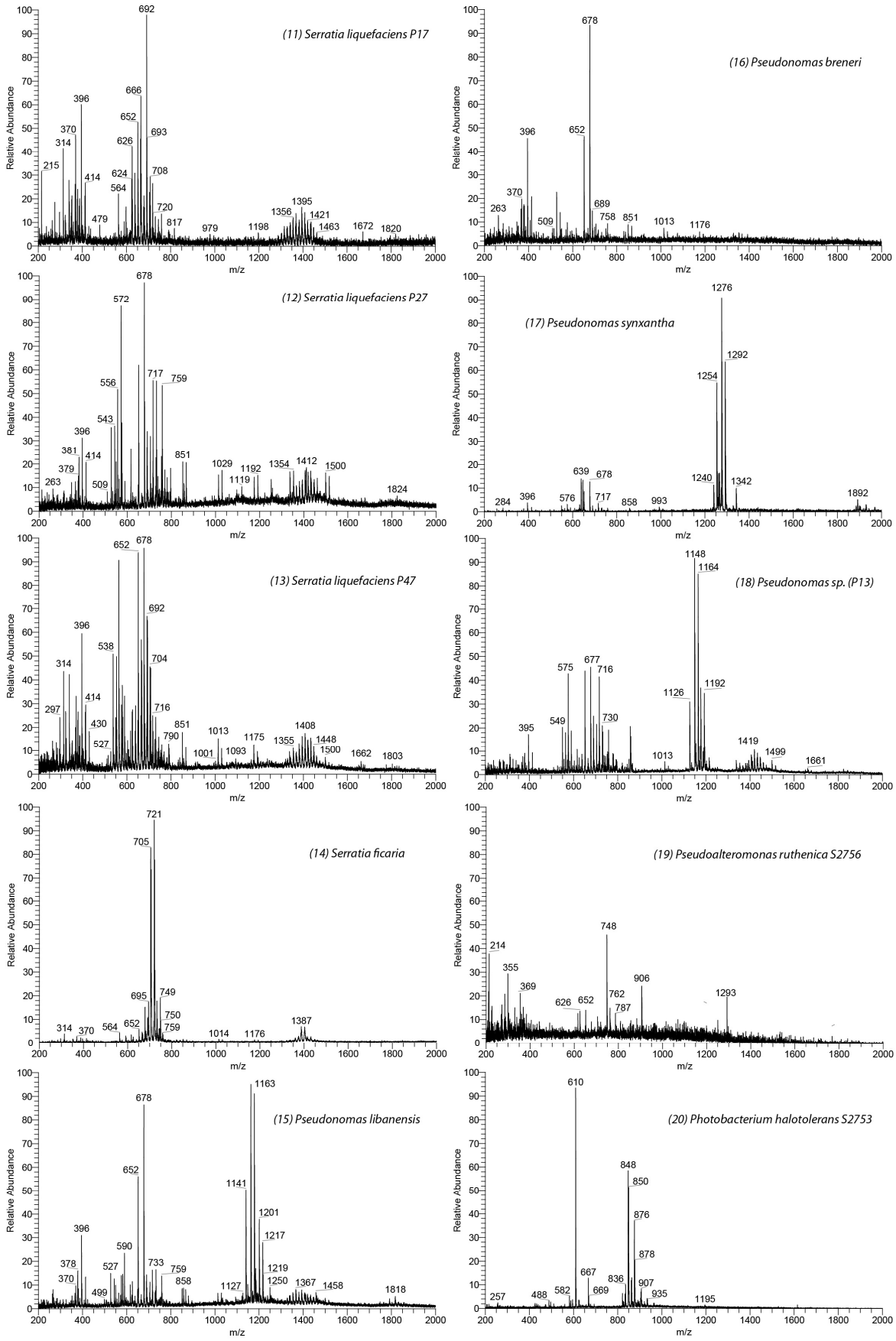
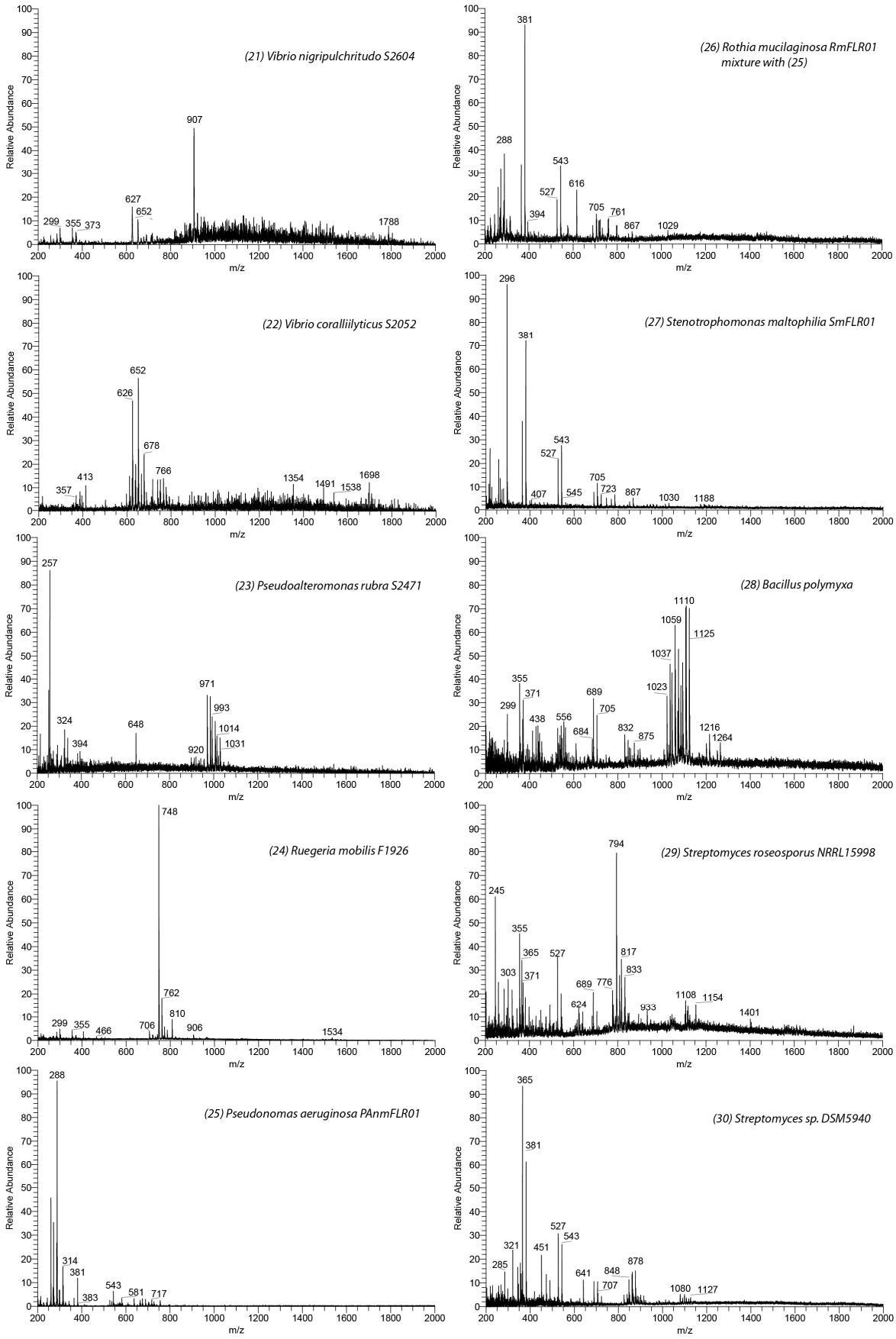


Figure S11. MS/MS validation of rhamnolipids produced by *P. aeruginosa* PA14 using LMJ-SSP. The fragmentation products are consistent with the previous reports for rhamnolipids.[8,9]


(1) Debaryomyces hansenii
280
365
809
379
451
644
787
823
(2) Candida catenulata
365
468
807
254
483
795
879
500
751
899
(3) Geotrichum Candidum BW138A
365
285
509
805
751
543
377
823
904
(4) Geotrichum Candidum BW242A
365
509
467
211
805
751
523
821
(5) Penicillium roqueforti
390
365
254
413
515
763
(6) Penicillium alli
206
367
280
373
515
623
763
890
(7) Penicillium commune
254
413
441
657
803
1470
1989
(8) Scopulariopsis flava
647
355
217
371
257
393
625
781
431
1308
1390
(9) Serratia fonticola
299
692
666
652
369
396
564
708
794
1180
(10) Serratia proteamaculans
563
692
370
285
396
790
1002
Relative Abundance
m/z


(11) Serratia liquefaciens P17
(16) Pseudonomas breneri
(12) Serratia liquefaciens P27
(17) Pseudonomas synxantha
(13) Serratia liquefaciens P47
(18) Pseudonomas sp. (P13)
(14) Serratia ficaria
(19) Pseudoalteromonas ruthenica S2756
(15) Pseudonomas libanensis
(20) Photobacterium halotolerans S2753
Relative Abundance
m/z


(21) Vibrio nigripulchritudo S2604
(26) Rothia mucilaginosa RmFLR01
mixture with (25)
(22) Vibrio coralliilyticus S2052
(27) Stenotrophomonas maltophilia SmFLR01
(23) Pseudoalteromonas rubra S2471
(28) Bacillus polymyxa
(24) Ruegeria mobilis F1926
(29) Streptomyces roseosporus NRRL15998
(25) Pseudonomas aeruginosa PAnmFLR01
(30) Streptomyces sp. DSM5940
Relative Abundance
m/z

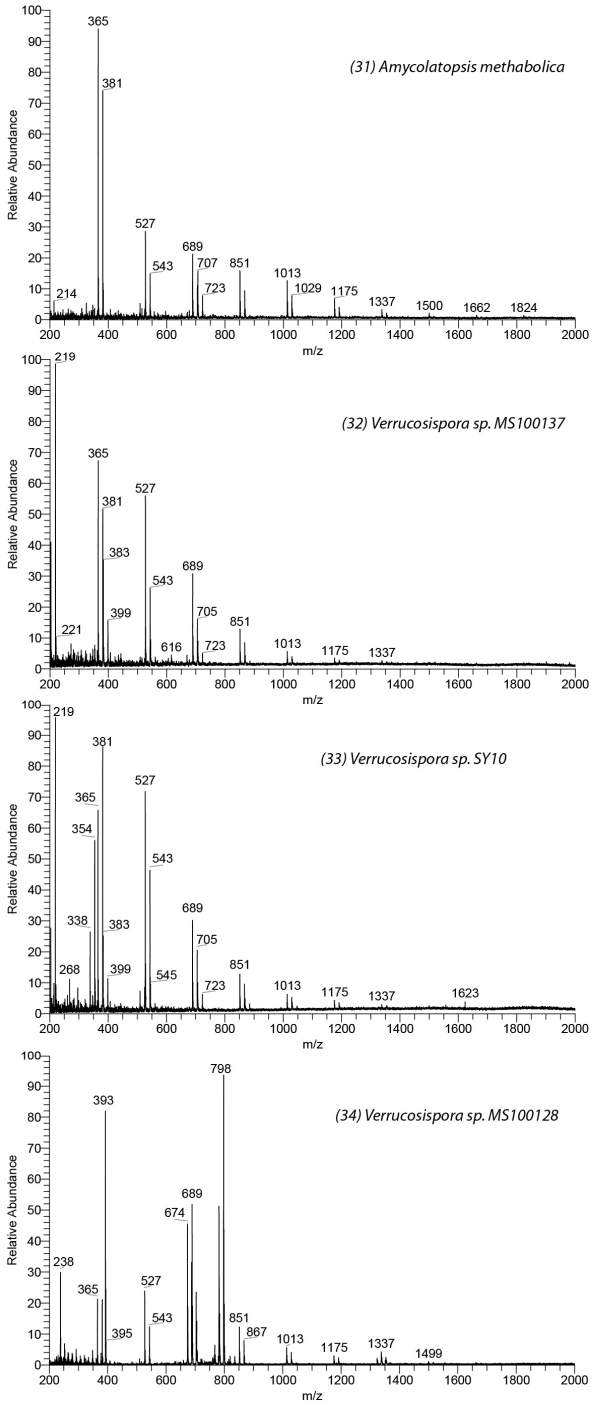


Figure S12. Real-time mass spectra acquired on the surfaces of diverse microbial colonies **1-34**.

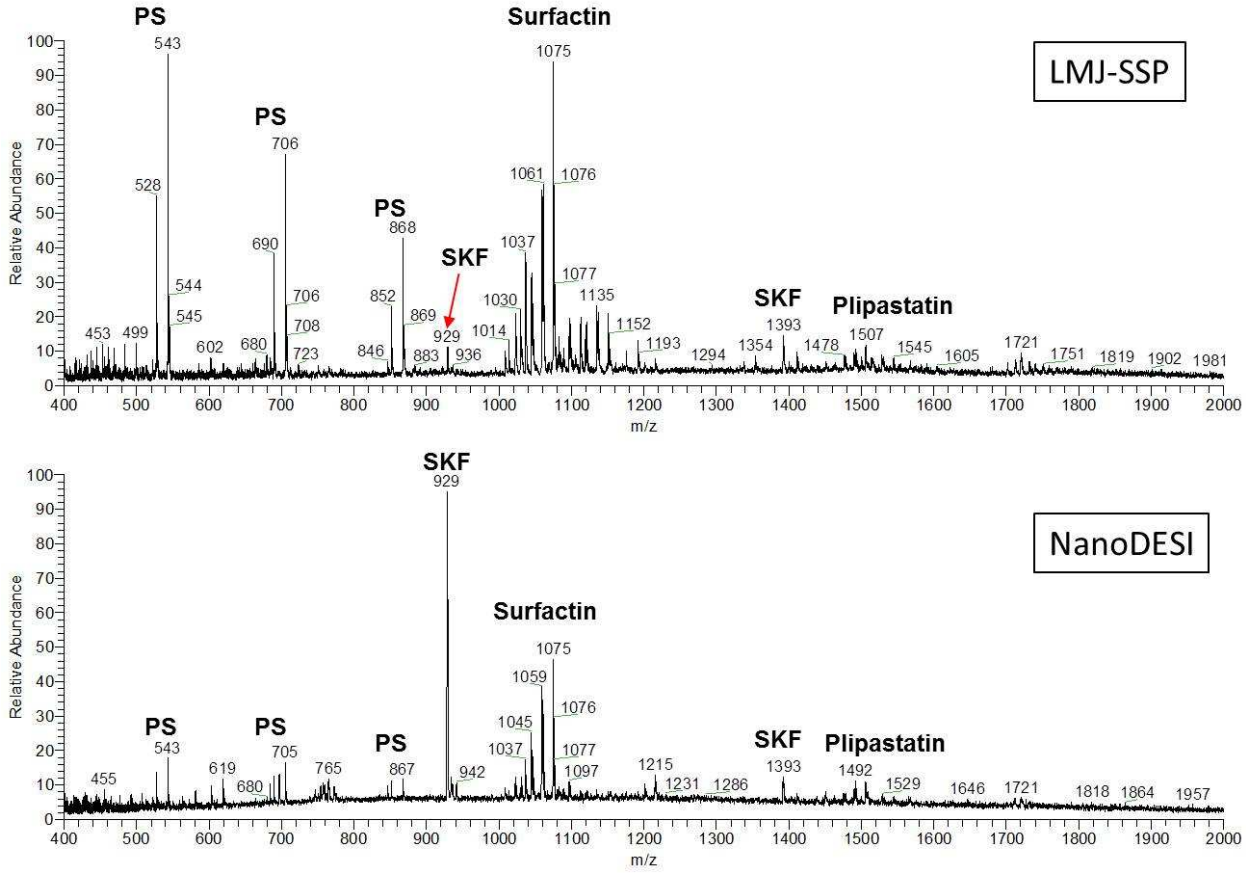


Figure S13. Mass spectra acquired on the surface of *B. subtilis* 3610 biofilm using LMJ-SSP and nanoDESI. The result shows that all of the cyclic peptides produced by *B. subtilus* 3610 measured via LMJ-SSP are also found via nanoDESI. PS = polysaccharide from the agar. MS spectra of *B. subtilus* 3610 measure by MALDI have published elsewhere.[7,10]

Experimental Section

Instrumental

The experimental parameters of LMJ-SSP have been described in the manuscript. The nanoDESI setup was described previously.[5] The solvent system for both setups were both 65/35 acetonitrile/0.1% formic acid aqueous solution, solvent choices were based on extractive procedures already used with other extractive based techniques to microbial metabolomics studies.[5,11] Both ionization sources were coupled to a 6.4 T Finnigan LTQ-FT-ICR MS (Thermo-Electron Corporation, San Jose, CA) that is capable of collision-induced dissociation. All analyses were performed in positive ion mode in the mass range of

m/z from 200 to 2,000. The instrument scan cycle consisted of two segments. The first segment had two profile mode MS scans: one full scan in the IT mode with 200 ms max fill time as the dependent scan; one full scan in the FT cell (50,000 resolution) with 2 sec max inject time. The second segment followed by a data-dependent tandem mass acquisition consisted of 8 scans in a cycle with a $\Delta m/z = 3$ isolation window. These data-dependent scans (IT mode, profile spectra) consisted of a maximum 1000 ms fill time, 35% normalized collision energy, 0.25 activation Q, and 0.05 s activation time. Data were acquired for 15 minutes for each microbial plates.

Preparation of Microbial Colonies

Bacillus subtilis and *Streptomyces coelicolor*

B. subtilis 3610/PY79 and *Streptomyces coelicolor* A3(2) colonies were prepared by inoculating 1 μL of liquid cultures (*B. subtilis*) and harvested spores (*S. coelicolor*) onto ISP2 nutrient agar (7.5 g agar, 5 g malt extract, 2 g yeast extract, and 2 g dextrose in 500 mL milli-Q water) and allowed to grow for 54 hours at the 30 °C incubator. *B. subtilis* 3610/PY79 liquid cultures were first prepared by inoculating 1 μL of cell stock into 4 mL of LB broth and incubated at 30 °C until an OD_{600} of 0.4 was reached. *B. subtilis* 3610 was originally acquired from Bacillus Genetic Stock Center (BGSC) (Ohio State Univ., Columbus), collected as Marburg strain by Robert S. Breed, chief bacteriologist at the Geneva station.[1] *B. subtilis* PY79 was first isolated when the auxotrophic markers of strain CU1769 (*glnA100* and *metB5*) were removed by two cycles of PBS1-mediated transduction, using lysates grown on strain 168.[1,2] *Streptomyces coelicolor* A3(2) is the model representative of a group of soil-dwelling organism with a complex lifecycle involving mycelial growth and spore formation.

Pseudomonas aeruginosa PA14

Pseudomonas aeruginosa PA14 came from the D. Hung lab (Harvard Medical School, USA) and originated from F.M. Ausubel's lab (Massachusetts General Hospital, USA). *P. aeruginosa* PA14 was inoculated on ISP2 agar (10 mL) in Petri dish. The sample was incubated for 48 hours at 30 ^{o}C.

Yeasts and Filamentous Fungi

Yeasts (**1-4**) and filamentous fungi (**5-8**) strains were isolated from variant cheese sources that were made by Jasper Hill Farm in Greensboro, Vermont. The strains were identified using the ribosomal

internal transcribed spacer (ITS) region. Purified fungal colonies were grown and maintained on PCAMS agar plates (5 g tryptone, 2.5 g yeast, 1 g dextrose, 1 g whole milk powder, 10 g NaCl, 15 g agar) at 25 ^{o}C for 3 weeks before mass spectrometry analysis.

Bat-associated strains (Serratia and Pseudomonas)

Serratia (**9**-**14**) and *Pseudomonas* (**15**-**18**) strains were isolated from several bat species collected from caves in Czech Republic by swabbing and were identified by 16S rRNA gene sequence analysis. Strains were prepared by inoculation onto ISP2 nutrient agar and allowed to grow for 28 days at 10 °C.

Marine bacterial strains

Marine strains **19**-**24** were acquired from the Galathea 3 expedition strain collection.[3] *Pseudoalteromonas ruthenica* S2756 (**19**) and *Photobacterium halotolerans* S2753 (**20**) were isolated from swabs of mussel collected near the Solomon Islands. *Vibrio nigripulchritudo* S2604 (**21**) was isolated from the Solomon Sea. *Vibrio coralliilyticus* S2052 (**22**) was isolated from the sediment in the Indian Ocean northwest to Australia. *Pseudoalteromonas rubra* S2471 (**23**) was isolated near Coral sea. *Ruegeria mobilis* F1926 (**24**) was isolated from the Indian Sea. Strains were grown on Marine Agar 2216 (MA, BD DifcoTM) for 48 hours at room temperature. Then cultured in agitated Marine Broth 2216 (MB, BD DifcoTM) before spotting 20 μL on an MA plate, which was inoculated for 24 hours at room temperature.

Marine strains **31**-**34** were isolated using oatmeal agar from a sediment sample collected in April 2010 from the South China Sea (20° 9.795□ N, 118° 18.124□ E) at 2733 m below sea level and were identified as *Amycolatopsis methanolica* (**31**) and *Verrucosispora* spp. (**32**-**34**), respectively, using 16S rRNA gene sequence analysis. These strains were cultivated on ISP2 agar medium at 28°C for 7 days before mass spectrometry analysis.

Cystic Fibrosis related strains

Bacterial isolates PAnmFLR01 (**25**) and SmFLR01 (**27**) were obtained by culturing on various selective media at the UCSD adult CF clinic clinical lab, La Jolla, CA. The isolates were identified as *Psuedomonas aeruginosa* and *Stenotrophomonas maltophilia* by phenotypic methods and then sent to the Rohwer lab at San Diego State University for culturing. *Rothia mucilaginosa* strain RmFLR01 (**26**) was obtained by initial culturing of a patient's sputum on the gram-positive selective media CN agar.

Single colonies were verified as *R. mucilaginosa* by 16S rRNA gene sequence analysis. Isolates were maintained on Todd Hewitt agar at 37°C after initial isolation. Prior to the mass spectrometry analysis the bacteria were grown on ISP2 media for 48 hours.

Streptomyces roseosporus and Streptomyces sp. DSM5940

Streptomyces roseosporus NRRL 15998 (**29**) was acquired from the Broad institute, MIT/Harvard, MA, USA, whose parent strain *S. roseosporus* NRRL 11379 was isolated from soil from Mount Ararat in Turkey. *Streptomyces sp.* DSM5940 (**30**) was originally isolated from a soil sample collected from the Andaman Islands, India.[4] Both strains were maintained and grown on ISP2 nutrient agar medium. The cultures were incubated for 4 days at 30 °C before mass spectrometry analysis.

Bacillus polymyxa

Bacillus polymyxa (**28**) obtained from Davies lab (The University of British Columbia). The strain were grown and maintained on ISP2 agar medium at room temperature for 25 days before mass spectrometry analysis.

REFERENCES

(1) Zeigler, D. R.; Prágai, Z.; Rodriguez, S.; Chevreux, B.; Muffler, A.; Albert, T.; Bai, R.; Wyss, M.; Perkins, J. B.; *J. Bacteriol.* **2008**, *190*, 6983–6995.

(2) Youngman, P.; Perkins, J.; Losick, R. *Plasmid* **1984**, *12*, 1–9.

(3) Gram, L.; Melchiorsen, J.; Bruhn, J. B. *Mar. Biotechnol*. **2010**, *12*, 439-451.

(4) Chatterjee, S.; Nadkarni, S. R.; Vijayakumar, E. K. S.; Patel, M. V.; Ganguli; B. N. *J. Antibiot*. **1994**, *47*, 595–598.

(5) Watrous, J.; Roach, P.; Alexandrov, T.; Heath, B. S.; Yang, J. Y.; Kersten, R. D.; van der Voort, M.; Pogliano, K.; Gross, H.; Raaijmakers, J. M.; Moore, B. S.; Laskin, J.; Bandeira, N.; Dorrestein, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, E1743–E752.

(6) Song, Y.; Talaty, N.; Datsenko, K.; Wanner, B. L.; Cooks, R. G. *Analyst*, **2009**, *134*, 838–841.

(7) 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. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 16286–16290.

(8) Moree, W.J.; Phelan, V.V.; Wu, C. H.; Bandeira, N.; Cornett, D. S.; Duggan, B. M.; Dorrestein, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 13811–13816.

(9) Watrous, J. D.; Phelan, V. V.; Hsu, C.-C.; Moree, W. J.; Duggan, B. M.; Alexandrov, T.; Dorrestein, P. C. *ISME J*. **2013**, *7*, 770–780.

(10) Yang, Y.-L.; Xu, Y.; Straight, P.; Dorrestein, P. C. *Nat. Chem. Bio.* **2009**, *5*, 885–887.

(11) Rath, C. M. et. al., *Anal. Chem.* **2012**, *84*, 9259–9267.