Supporting Information

MicroLESA: Integrating Autofluorescence Microscopy, *In Situ* Micro-Digestions, and Liquid Extraction Surface Analysis for High Spatial Resolution Targeted Proteomic Studies.

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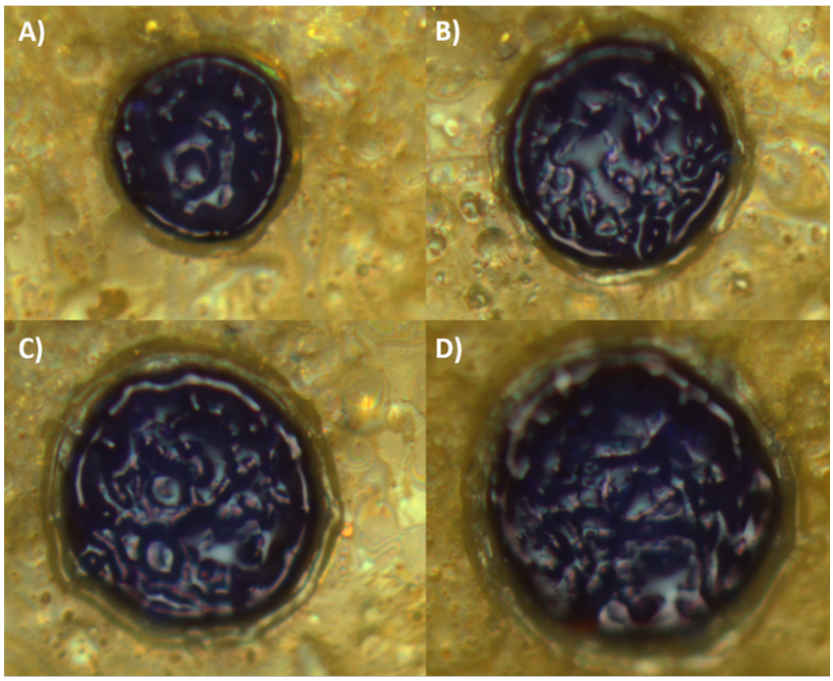
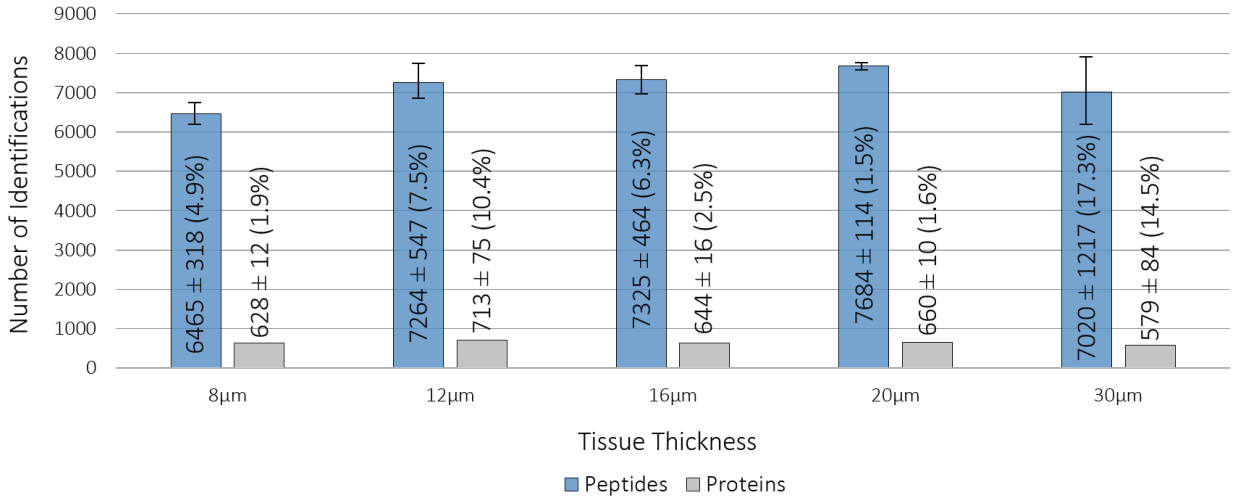


Run	Deposition Method	Average Diameter (µm)	Standard Deviation (µm)	Relative Standard Deviation
A	1 x 21	112.48	4.87	4.33
B	3 x 7	161.50	2.55	1.58
C	5 x 5	195.63	8.12	4.15
D	7 x 3	218.35	8.34	3.82

Supplemental Figure S1: The measured droplet diameters (volume ~220 pL) of trypsin micro-digests from water-sensitive paper. The total amount of droplets dispensed at a single time was varied from 1 to 7, while still varying the total number of dispension runs within a sample set to equate to the same total volume dispensed on a given spot (*i.e.* 1 spot dispensed 21 individual times yields the same total volume dispensed as 7 spots, dispensed 3 total times). Figure S1-A depicts the droplet diameter dispensing 1 single drop of trypsin at a time, 21 separate times, while S1-B through S1-D depict 3, 5, and 7 droplets being dispensed at a single time. Each run was completed a total of 5 times (N = 5) and bright field microscopy was used for measurement.



Supplemental Figure S2: The tissue thickness as a function of unique proteins and peptides identified was studied using micro-digests combined with a LESA extract. Three digestion spots per extract were analyzed using LC-MS/MS. the results show there is no statistical change in the number of peptide and protein identifications between the sample sets (ANOVA: p-value 0.289 for peptides identified and p-value 0.0919 for unique proteins identified). These results suggest that the digestion may only be occurring on the surface of the tissue. All experiments were completed at 12 µm for both imaging and microLESA.

METHODS

3) Micro-Digestions and Autofluorescence Microscopy: Spotting specific ROI's with Trypsin

In order to spot any liquid to specific ROI's on a tissue section, a custom-designed fiducial slide was used to register the spotting system. Briefly, the spotting system uses a camera to recognize a point of origin on the sample for which to spot from. An arbitrarily chosen fiducial was utilized prior to any ROI generation and used as the origin for which all spots would deposited from. For all spotting runs, the Piezo was initially tuned while dispensing pure water, then while dispensing a solution of trypsin, and in between all trypsin depositions to ensure a stable droplet. The PDC 50 was tuned to these approximate settings: 70-90 V, 40-60 µs, a frequency of 500 Hz,

and an LED delay of 200 µs. These settings generated stable spot volumes of approximately 250 pL using a PDC 50 nozzle with relative standard deviations under 10%.

4) Bottom-Up LC-MS/MS and Data Analysis

For the microLESA optimization experiments, an analytical column was packed with 22 cm of C18 reverse phase material (Jupiter, 3 µm beads, 300Å, Phenomenox) directly into a laser-pulled emitter tip (Sutter Instrument Company, Novato, CA, USA). Peptides were loaded on the capillary reverse phase analytical column (360 µm O.D. x 100 µm I.D.) using a Dionex Ultimate 3000 nanoLC and autosampler. The mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). Peptides were eluted with a gradient at a flow rate of 400 nL/min. A 60-minute gradient was performed as follows: 1-2 min, 2% B (sample loading from autosampler); 2-44 min, 2-35% B; 44-49 min, 35-95% B; 49-49.5 min, 95% B; 49.5-50 min, 95-2% B; 50-60 min (column re-equilibration), 2% B. A Thermo Q Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), equipped with a nanoelectrospray ionization source, was used to analyze the eluting peptides. The instrument method consisted of MS1 using an MS AGC target value of 3x106, followed by up to 15 MS/MS scans of the most abundant ions detected in the preceding MS scan. A maximum MS/MS ion accumulation time of 60 ms was used with a MS2 AGC target of 1x105 and an intensity threshold of 5x104. Dynamic exclusion was set to 10s, HCD collision energy was set to 27 normalized collisional energy, and peptide match and isotope exclusion were enabled.

For the microLESA experiments targeting the abscess in mouse infected with *S. aureus,* tryptic peptides from the tissue extracts were injected and gradient eluted on a pulled tip emitter column (360 µm O.D. x 100 µm I.D. x 35 cm) packed in-house with C18 material (Waters BEH C18, 1.7 µm, 130 Å). The column was heated to 60 °C with a flow rate of 400 nL/min during operation using an Easy-nLC 1000 UHPLC (Thermo Scientific, San Jose, CA, USA), where the mobile phase A consisted of 0.1% formic acid, 99.9% water, and mobile phase B consisted of 0.1% formic acid, 99.9% acetonitrile. Peptides were eluted on the reverse phase column using a linear gradient of 2-20% B for 100 minutes, followed by 20-32% B for 20 minutes, and lastly 32-95% B for 1 minute. An Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA, USA) was used to mass analyze the eluting peptides. MS1 scans were acquired using the Orbitrap at 120k resolution, a mass range of 400-1600 *m/z*, an automatic gain control (AGC) target

of $1.0x10^6$, and a maximum injection time of 100 ms. The top 17 most abundant ions measured in the MS1 scans were then mass isolated using a quadrupole mass filter at 2 *m/z* window width to undergo fragmentation in the HCD cell using 35% normalized collision energy. The fragmented ions are subsequently mass analyzed in the linear ion trap using an AGC target of $1x10^4$, a maximum injection time of 35 ms, and the normal scanning rate setting. Dynamic exclusion of 30 s was used for all MS2 scans.

For identification of peptides from both instruments, tandem mass spectra were searched using Protalizer software (Vulcan Analytic, Birmingham, Alabama, USA) against a rat, mouse, or a *Staphylococcus aureus* (strain USA300) database created from the UniprotKB protein database (www.uniprot.org). Variable modifications such as glycosylation, phosphorylation, methionine oxidation, and deamidation were included in the database search. Proteins were identified with at least 2 peptides per protein, with a false discovery rate of 1%.

5) MALDI Protein IMS

In all experiments except the tissue thickness optimization, tissue was sectioned (12 µm thickness) at -15 °C using a CryoStar^{TM} NX70 Cryostat (Thermo Fisher Scientific, San Jose, CA, USA), thaw mounted onto conductive indium-tin-oxide coated slides with custom printed fiducials in black ink (Delta Technologies, Loveland, CO, USA)), and dried in a vacuum desiccator for at least 20 minutes prior to preparation for analysis. Tissue sections underwent a washing prior to any imaging, micro-digestion, or surface extraction to remove interfering lipids and salts. The wash steps were as follows: 70% ethanol (30s), 100% ethanol (30s), Carnoy's Wash (6:3:1 ethanol:chloroform:acetic acid), 100% ethanol (30s), water (30s), and 100% ethanol (30s) as described previously.[52]

For intact protein IMS, tissue was covered homogenously with DHA using a robotic sprayer (TM Sprayer, HTX Technologies, Carrboro, NC, US) at a concentration of 15 mg/mL in 9:1, ACN:H_2O (0.1% formic acid). The sprayer nozzle was set to spray at 85 °C using a carrier solvent of 9:1 ACN:H_2O at a flow rate of 0.1 mL/min and a drying sheath gas of dry nitrogen set to 10 psi. Four passes of matrix were applied using alternating offsets (1 mm) and directional rotations (90 degrees) with a 2 mm track spacing. The spray velocity was set to 700 mm/min with a 2 s dry time between passes and 40 mm nozzle height. The matrix layer on the sample was recrystallized prior to MALDI analysis as previously described using 1.0 mL of 1:1, TFA:H_2O at

37 °C for 3 minutes.[53] The image was acquired in positive ion mode at 60 μm spatial resolution on a Bruker SolariX 15T FTICR MS (Bruker Daltonics, Billerica, MA, USA). The instrument employs a Smartbeam II 2 kHz frequency tripled Nd:YAG (355 nm) laser, as well as an Apollo II dual MALDI/ESI ion source. Each pixel was the sum of 2000 laser shots, using the smallest laser focus (~50 μm), while random-walking the target within the 125 μm pixel. The mass spectrometer was externally calibrated prior to analysis using a protein mixture (insulin, cytochrome C, trypsinogen, and apomyoglobin). Data were collected from *m/z* 1,385 - 20,000 with a time-domain file size of 512K (FID length: 1.6078 s), yielding a resolving power of ~42,000 at *m/z* 5000. In order to generate an image with a higher mass range, the ion optics were tuned as follows: accumulation hexapole (1.4 MHz, 1700 Vpp), time-of-flight delay (2.1 ms), funnel RF amplitude (200 Vpp), transfer optics (2 MHz, 380 Vpp), and ICR cell (sweep excitation power: 40%). The source pressure was lowered to 950 mTorr in order to maximize the transmission of higher *m/z* species.

6) *Staphylococcus aureus* Protein Identifications

Protein Name	Protein ID
sp|Q2FDQ7|LDH2_STAA3 L-lactate dehydrogenase 2 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=ldh2 PE=3 SV=1	Q2FDQ7
sp|Q2FDV3|ROCA_STAA3 1-pyrroline-5-carboxylate dehydrogenase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rocA PE=3 SV=1	Q2FDV3
sp|Q2FDV8|CLPL_STAA3 ATP-dependent Clp protease ATP-binding subunit ClpL OS=Staphylococcus aureus (strain USA300) OX=367830 GN=clpL PE=3 SV=1	Q2FDV8
sp|Q2FE81|GPMA_STAA3 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=gpmA PE=3 SV=1	Q2FE81
sp|Q2FEC8|Y2315_STAA3 Uncharacterized lipoprotein SAUSA300_2315 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=SAUSA300_2315 PE=3 SV=1	Q2FEC8
sp|Q2FEN8|RS10_STAA3 30S ribosomal protein S10 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsJ PE=3 SV=1	Q2FEN8
sp|Q2FEP2|RL2_STAA3 50S ribosomal protein L2 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rplB PE=3 SV=1	Q2FEP2
sp|Q2FEP5|RS3_STAA3 30S ribosomal protein S3 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsC PE=3 SV=1	Q2FEP5
sp|Q2FEQ0|RL24_STAA3 50S ribosomal protein L24 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rplX PE=3 SV=1	Q2FEQ0
sp|Q2FEQ3|RS8_STAA3 30S ribosomal protein S8 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsH PE=3 SV=1	Q2FEQ3
sp|Q2FEQ5|RL18_STAA3 50S ribosomal protein L18 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rplR PE=3 SV=1	Q2FEQ5
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sp|Q2FF08|RL31B_STAA3 50S ribosomal protein L31 type B OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpmE2 PE=3 SV=1	Q2FF08
sp|Q2FF22|ATPA_STAA3 ATP synthase subunit alpha OS=Staphylococcus aureus (strain USA300) OX=367830 GN=atpA PE=3 SV=1	Q2FF22
sp|Q2FF24|ATPB_STAA3 ATP synthase subunit beta OS=Staphylococcus aureus (strain USA300) OX=367830 GN=atpD PE=3 SV=1	Q2FF24
sp|Q2FF95|CH60_STAA3 60 kDa chaperonin OS=Staphylococcus aureus (strain USA300) OX=367830 GN=groL PE=3 SV=1	Q2FF95
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sp|Q2FFF7|CHIPS_STAA3 Chemotaxis inhibitory protein OS=Staphylococcus aureus (strain USA300) OX=367830 GN=chp PE=3 SV=1	Q2FFF7
sp|Q2FFF8|SCIN_STAA3 Staphylococcal complement inhibitor OS=Staphylococcus aureus (strain USA300) OX=367830 GN=scn PE=3 SV=1	Q2FFF8
sp|Q2FFJ6|GATB_STAA3 Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B OS=Staphylococcus aureus (strain USA300) OX=367830 GN=gatB PE=3 SV=1	Q2FFJ6
sp|Q2FFQ0|Y1795_STAA3 UPF0342 protein SAUSA300_1795 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=SAUSA300_1795 PE=3 SV=1	Q2FFQ0
sp|Q2FFQ5|PRSA_STAA3 Foldase protein PrsA OS=Staphylococcus aureus (strain USA300) OX=367830 GN=prsA PE=3 SV=1	Q2FFQ5
sp|Q2FFV5|PCKA_STAA3 Phosphoenolpyruvate carboxykinase (ATP) OS=Staphylococcus aureus (strain USA300) OX=367830 GN=pckA PE=3 SV=1	Q2FFV5
sp|Q2FFV6|METK_STAA3 S-adenosylmethionine synthase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=metK PE=3 SV=1	Q2FFV6
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sp|Q2FG27|ACKA_STAA3 Acetate kinase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=ackA PE=3 SV=1	Q2FG27

sp|Q2FG28|Y1656_STAA3 Putative universal stress protein SAUSA300_1656 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=SAUSA300_1656 PE=3 SV=1 Q2FG28
sp|Q2FG40|KPYK_STAA3 Pyruvate kinase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=pyk PE=3 SV=1 Q2FG40
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sp|Q2FG61|TIG_STAA3 Trigger factor OS=Staphylococcus aureus (strain USA300) OX=367830 GN=tig PE=3 SV=1 Q2FG61
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sp|Q2FGZ4|GPSB_STAA3 Cell cycle protein GpsB OS=Staphylococcus aureus (strain USA300) OX=367830 GN=gpsB PE=3 SV=1 Q2FGZ4
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sp|Q2FHI2|RS2_STAA3 30S ribosomal protein S2 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsB PE=3 SV=1 Q2FHI2
sp|Q2FHI3|CODY_STAA3 GTP-sensing transcriptional pleiotropic repressor CodY OS=Staphylococcus aureus (strain USA300) OX=367830 GN=codY PE=3 SV=1 Q2FHI3
sp|Q2FHJ3|SUCC_STAA3 Succinate--CoA ligase [ADP-forming] subunit beta OS=Staphylococcus aureus (strain USA300) OX=367830 GN=sucC PE=3 SV=1 Q2FHJ3
sp|Q2FHK0|RS16_STAA3 30S ribosomal protein S16 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsP PE=3 SV=1 Q2FHK0
sp|Q2FHK6|ACP_STAA3 Acyl carrier protein OS=Staphylococcus aureus (strain USA300) OX=367830 GN=acpP PE=3 SV=1 Q2FHK6
sp|Q2FHR7|ARCC1_STAA3 Carbamate kinase 1 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=arcC1 PE=3 SV=1 Q2FHR7
sp|Q2FHS7|FLIPR_STAA3 FPRL1 inhibitory protein OS=Staphylococcus aureus (strain USA300) OX=367830 GN=flr PE=3 SV=1 Q2FHS7
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sp|Q2FIK4|EMP_STAA3 Extracellular matrix protein-binding protein emp OS=Staphylococcus aureus (strain USA300) OX=367830 GN=emp PE=3 SV=1	Q2FIK4
sp|Q2FIL7|ENO_STAA3 Enolase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=eno PE=3 SV=1	Q2FIL7
sp|Q2FIS2|LTAS_STAA3 Lipoteichoic acid synthase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=ltaS PE=3 SV=1	Q2FIS2
sp|Q2FJ29|SYR_STAA3 Arginine--tRNA ligase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=argS PE=3 SV=1	Q2FJ29
sp|Q2FJ31|ADH_STAA3 Alcohol dehydrogenase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=adh PE=3 SV=1	Q2FJ31
sp|Q2FJ56|Y569_STAA3 Putative heme-dependent peroxidase SAUSA300_0569 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=SAUSA300_0569 PE=3 SV=1	Q2FJ56
sp|Q2FJ87|Y538_STAA3 Uncharacterized epimerase/dehydratase SAUSA300_0538 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=SAUSA300_0538 PE=3 SV=1	Q2FJ87
sp|Q2FJ92|EFTU_STAA3 Elongation factor Tu OS=Staphylococcus aureus (strain USA300) OX=367830 GN=tuf PE=3 SV=1	Q2FJ92
sp|Q2FJ93|EFG_STAA3 Elongation factor G OS=Staphylococcus aureus (strain USA300) OX=367830 GN=fusA PE=3 SV=3	Q2FJ93
sp|Q2FJ94|RS7_STAA3 30S ribosomal protein S7 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsG PE=3 SV=2	Q2FJ94
sp|Q2FJ95|RS12_STAA3 30S ribosomal protein S12 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsL PE=3 SV=1	Q2FJ95
sp|Q2FJA0|RL7_STAA3 50S ribosomal protein L7/L12 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rplL PE=3 SV=1	Q2FJA0
sp|Q2FJA1|RL10_STAA3 50S ribosomal protein L10 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rplJ PE=3 SV=1	Q2FJA1
sp|Q2FJA3|RL11_STAA3 50S ribosomal protein L11 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rplK PE=3 SV=3	Q2FJA3
sp|Q2FJB0|SYC_STAA3 Cysteine--tRNA ligase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=cysS PE=3 SV=1	Q2FJB0
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sp|Q2FJG3|Y453_STAA3 Nucleoid-associated protein SAUSA300_0453 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=SAUSA300_0453 PE=3 SV=1	Q2FJG3
sp|Q2FJN4|AHPC_STAA3 Alkyl hydroperoxide reductase C OS=Staphylococcus aureus (strain USA300) OX=367830 GN=ahpC PE=3 SV=1	Q2FJN4
sp|Q2FJP8|RS6_STAA3 30S ribosomal protein S6 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=rpsF PE=3 SV=1	Q2FJP8
sp|Q2FK15|TARI1_STAA3 Ribitol-5-phosphate cytidylyltransferase 1 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=tarI1 PE=3 SV=1	Q2FK15

sp|Q2FK29|LDH1_STAA3 L-lactate dehydrogenase 1 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=ldh1 PE=3 SV=2	Q2FK29
sp|Q2FK44|PFLB_STAA3 Formate acetyltransferase OS=Staphylococcus aureus (strain USA300) OX=367830 GN=pflB PE=3 SV=1	Q2FK44
sp|Q2FK96|HDOX2_STAA3 Heme oxygenase (staphylobilin-producing) 2 OS=Staphylococcus aureus (strain USA300) OX=367830 GN=isdI PE=3 SV=1	Q2FK96