SUPPLEMENTARY INFORMATION

Lipid II overproduction allows direct assay of transpeptidase inhibition by β-lactams

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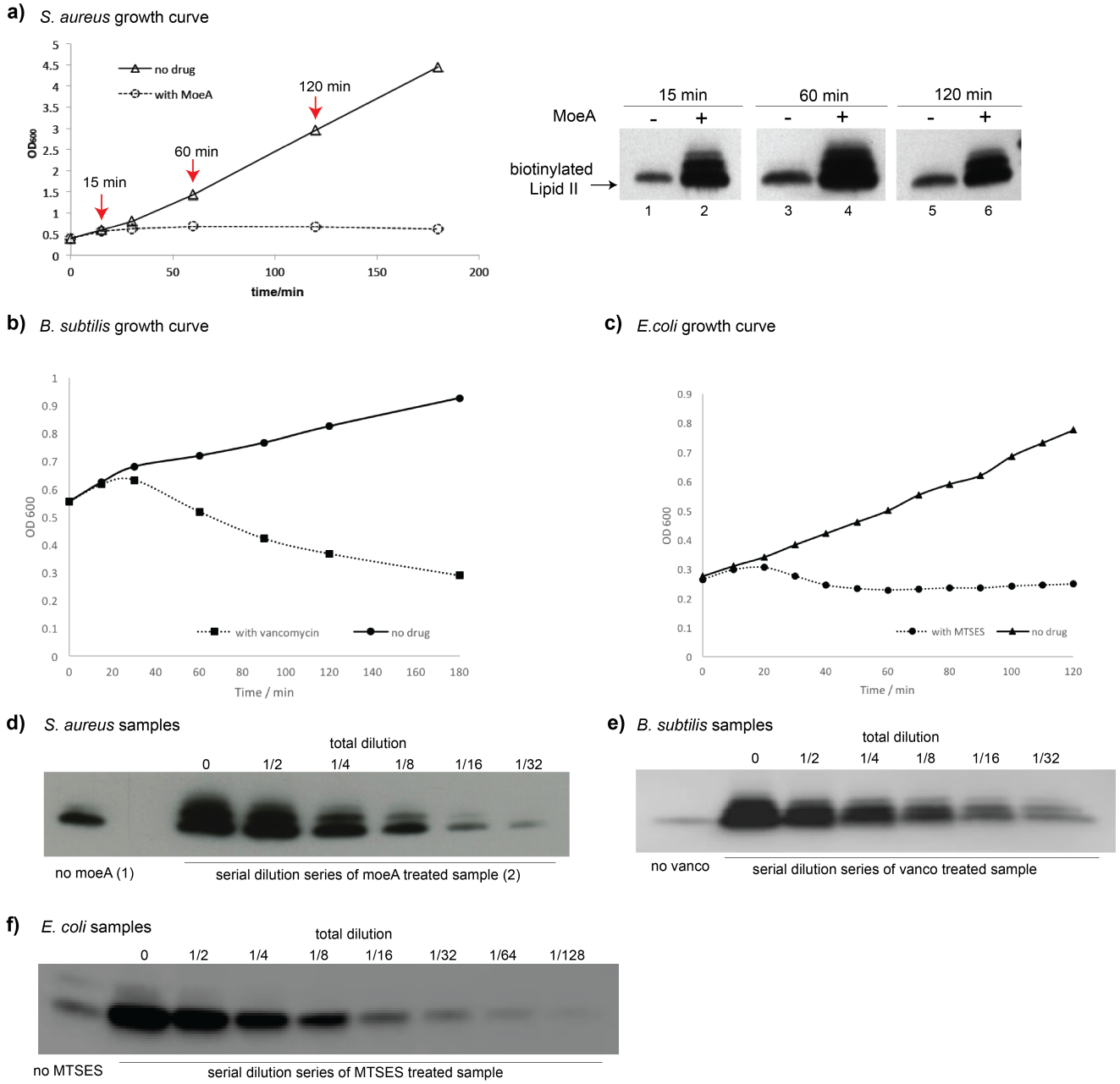
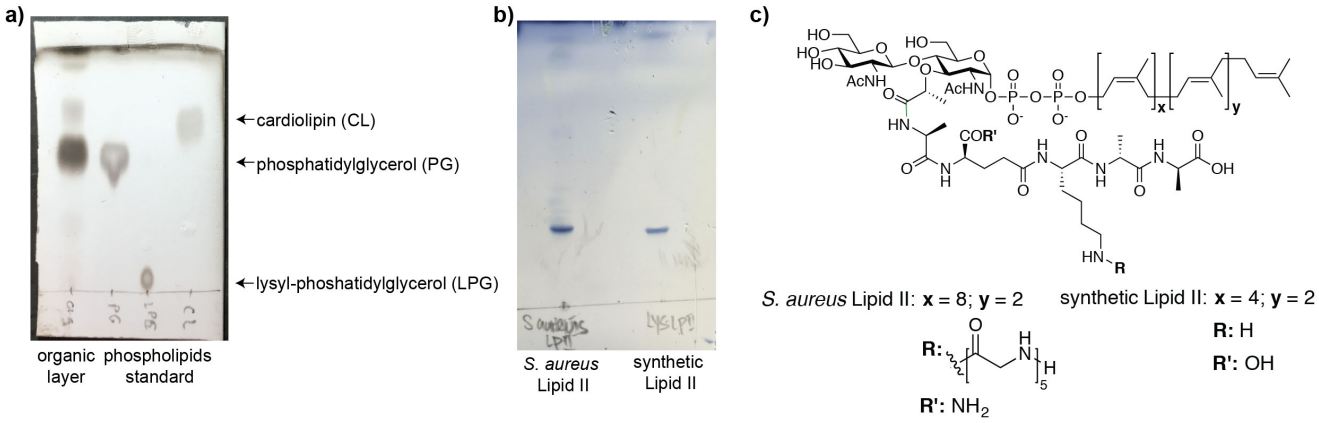
[†] These authors contributed to the work equally.

SUPPLEMENTARY RESULTS

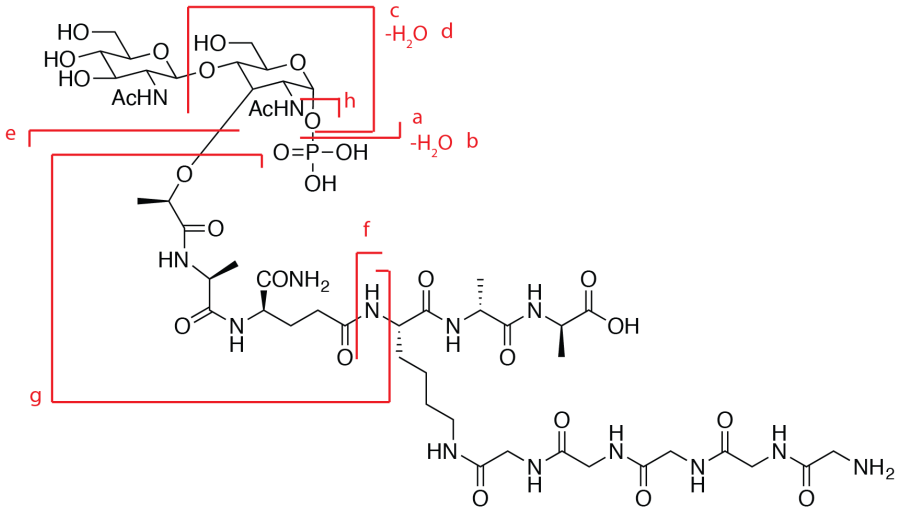


Supplementary Figure 1. Lipid II is substantially accumulated in bacteria treated with various chemical probes. (a) Treatment of moenomycin (0.6 μg/mL) to *S. aureus* stalls growth, while Lipid II accumulation in *S. aureus* is stable after prolonged moenomycin treatment. (b-c) Treatment of vancomycin (8 μg/mL) to *B. subtilis* and of MTSES to *E. coli* MurJ^{A29C} causes rapid cell lysis. (d-f) Serial dilutions of samples enable estimation that Lipid II is accumulated 10-fold, 30-fold, and 16-fold in *S. aureus*, *B. subtilis* and *E. coli* respectively when treated with the indicated chemical probes. Detection of cellular Lipid II follows a previously published protocol.[1]

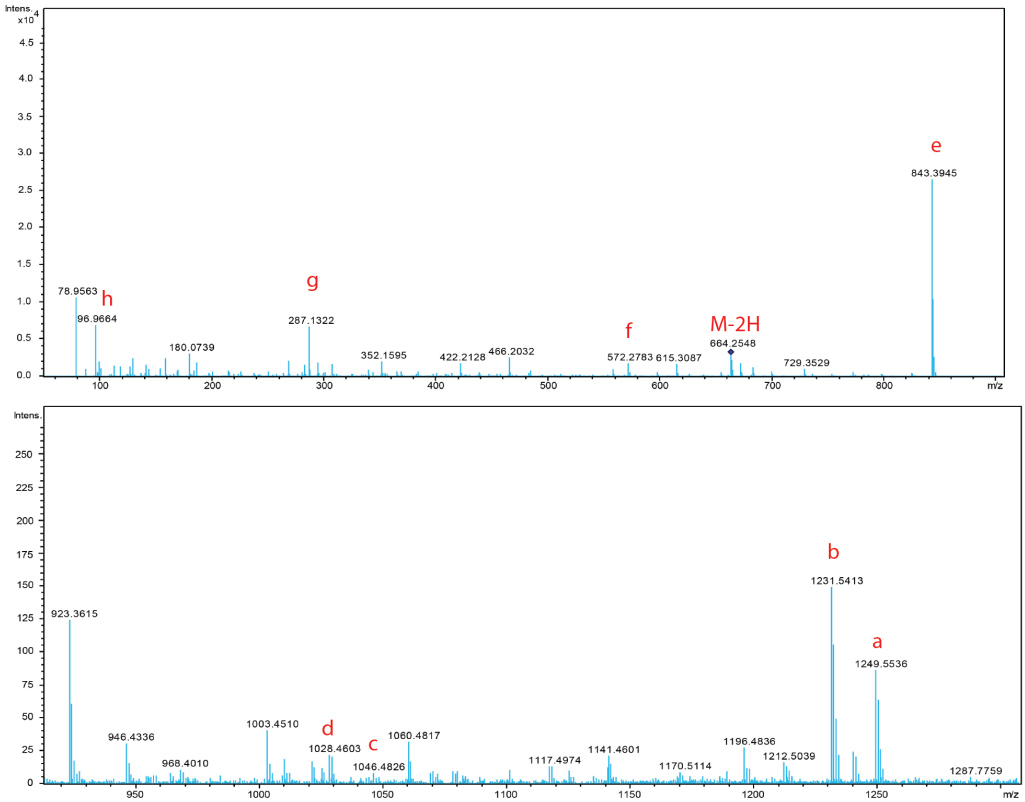


Supplementary Figure 2. Thin layer chromatography (TLC) analysis shows that the isolated Lipid II has good purity. Cellular phospholipids partition into the organic layer after the first ($MeOH/CHCl_3$) extraction (a); *S. aureus* Lipid II was obtained in the organic phase after the second (PyAc/BuOH) extraction (b). See materials and methods for TLC protocols.

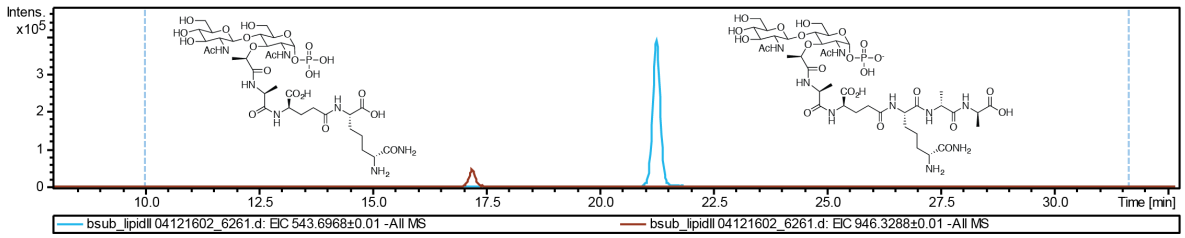
a) LC/MS/MS fragmentation pattern for *S. aureus* sample

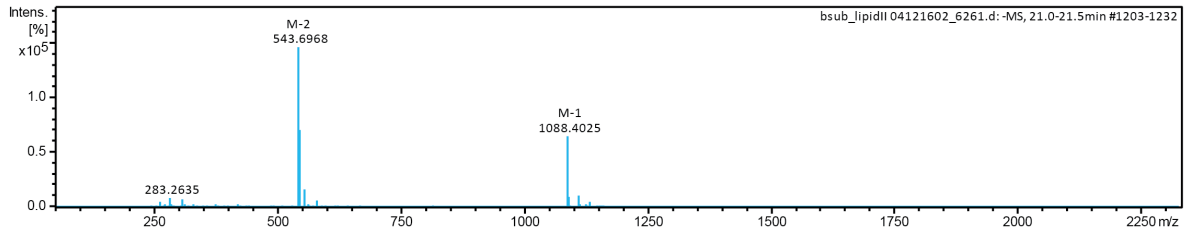


b) LC/MS/MS for *S. aureus* sample

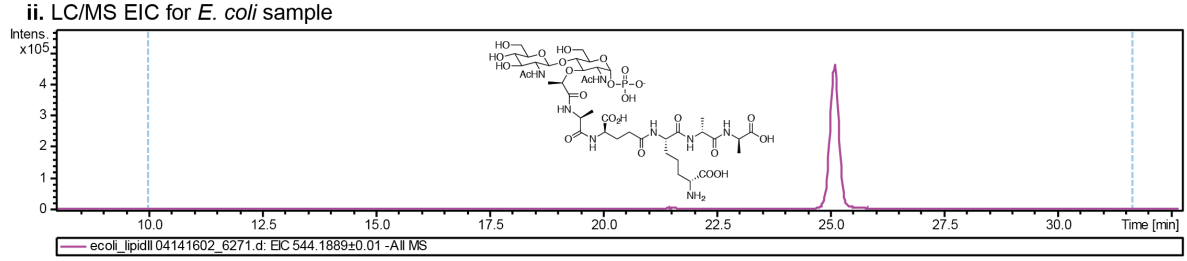


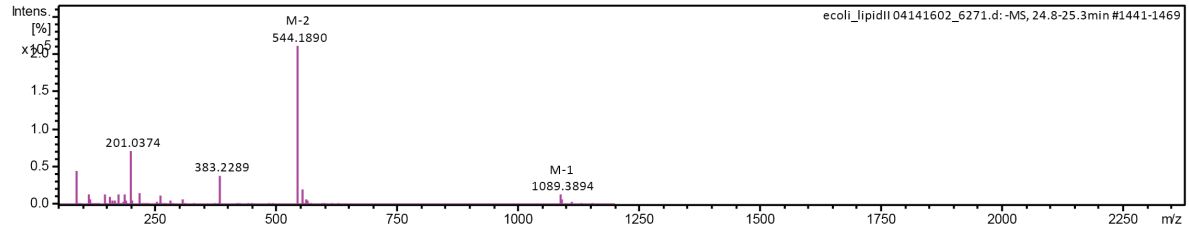
c) **i.** LC/MS EIC for *B. subtilis* sample



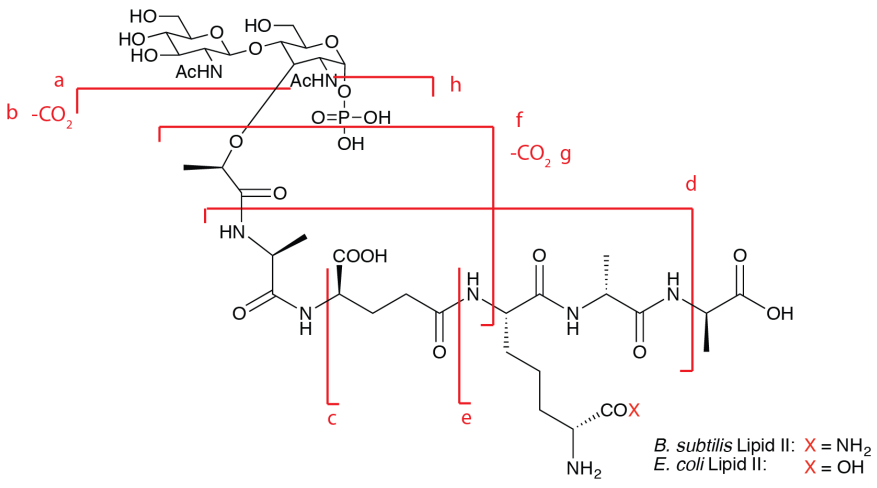


ii. LC/MS EIC for *E. coli* sample

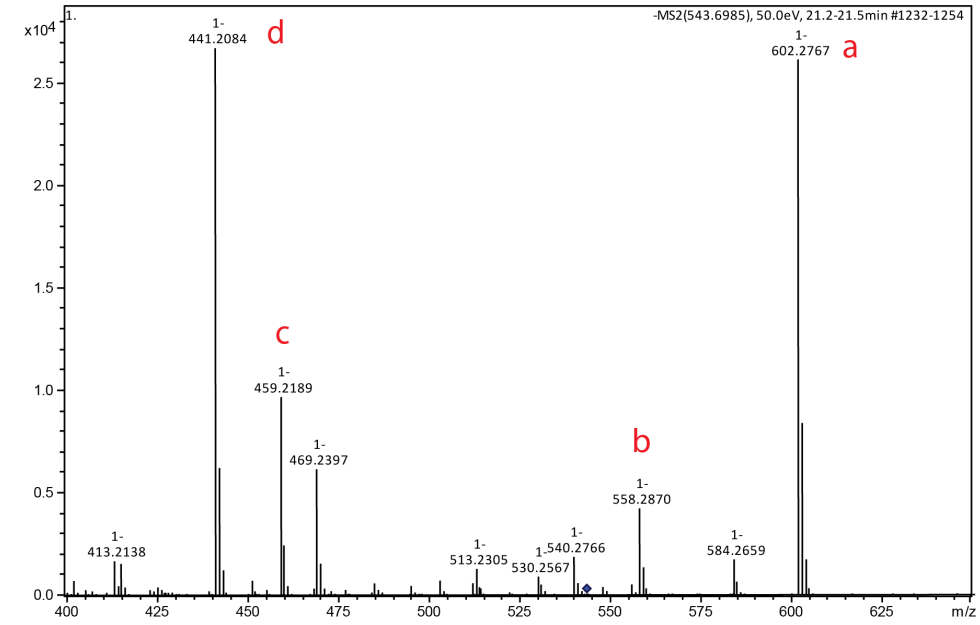




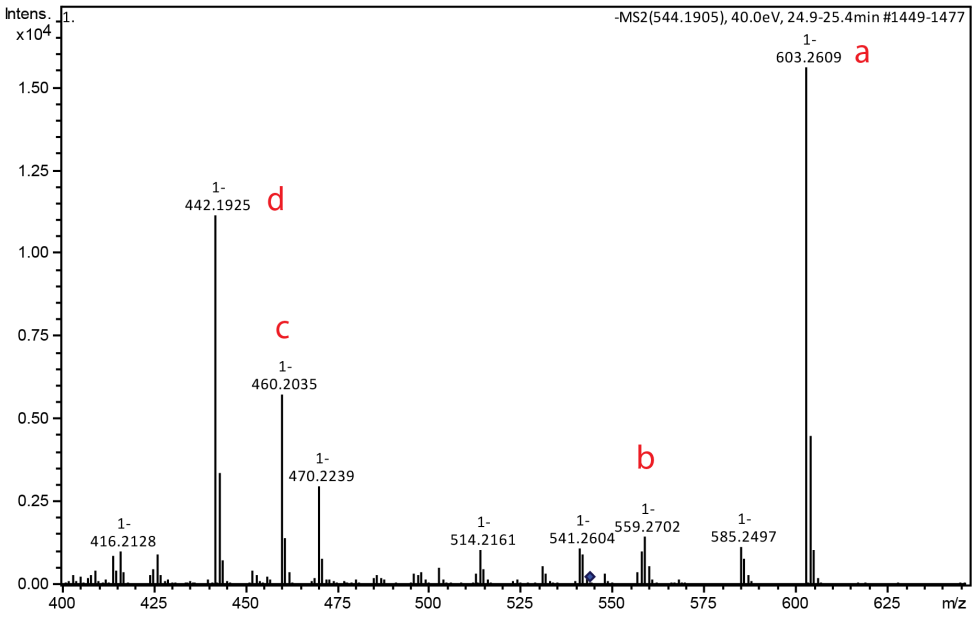
d) LC/MS/MS fragmentation pattern for *B. subtilis* and *E. coli* samples



e) Comparison of LC/MS/MS from *B. subtilis* and *E. coli* samples

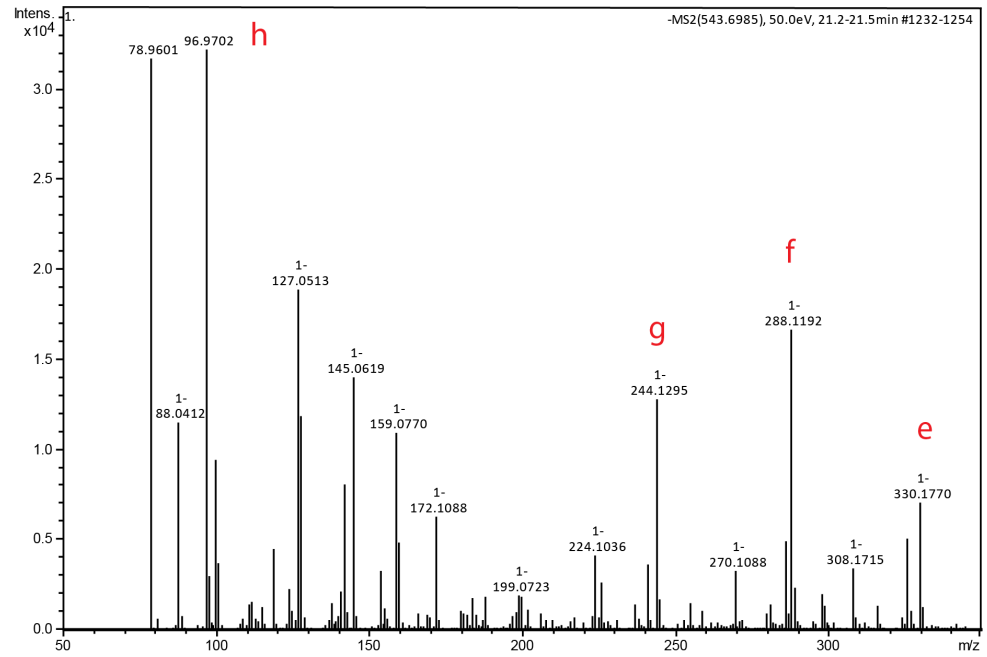


B. subtilis sample

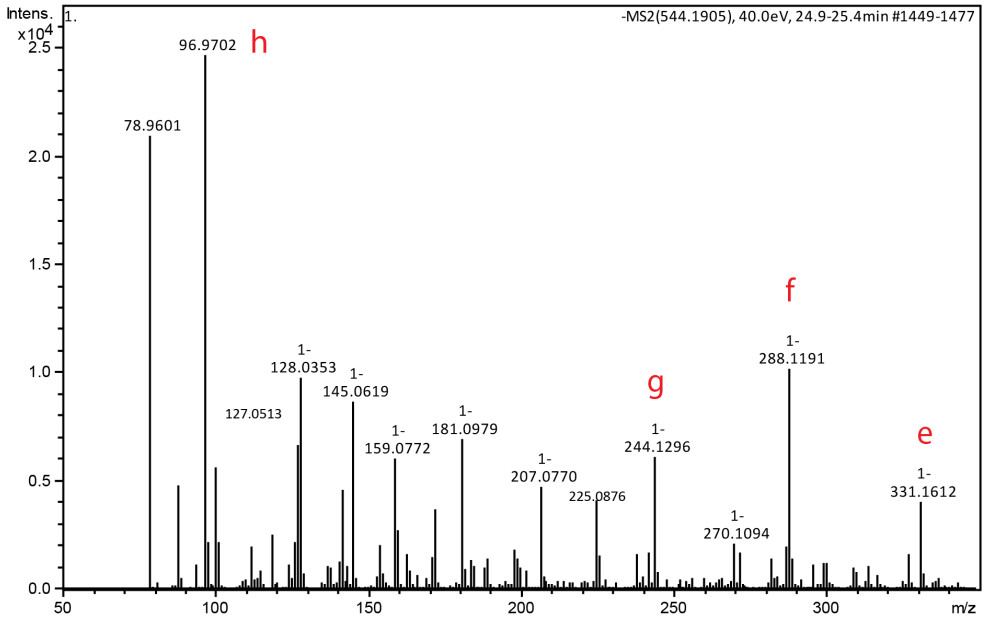


E. coli sample

e) Comparison of LC/MS/MS from *B. subtilis* and *E. coli* samples (continued)



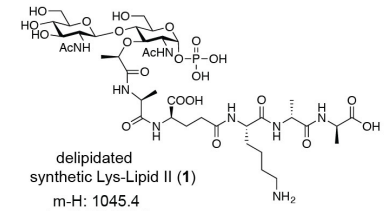
B. subtilis sample



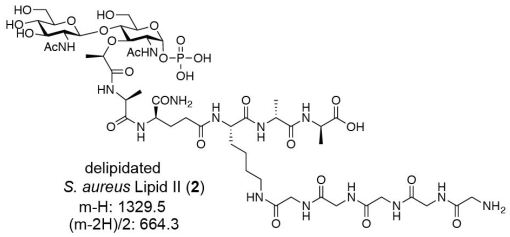
E. coli sample

Supplementary Figure 3. LC/MS and MS/MS analyses confirm the identity of isolated Lipid II from bacteria. *S. aureus* Lipid II contains an isoglutamine at the second position on the stem peptide and a pentaglycine branch (a-b); Fragmentation patterns reveal that *B. subtilis* Lipid II contains an amidated m-DAP residue at the third position of the stem peptide while *E. coli* Lipid II lacks the amidation on m-DAP (c-e).

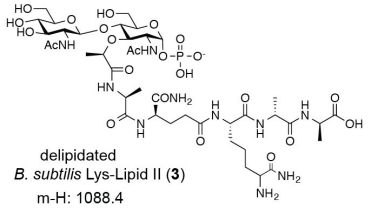
a)



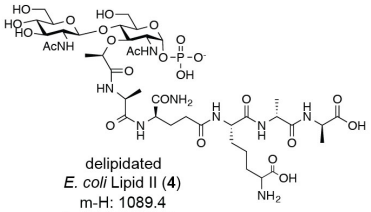
delipidated synthetic Lys-Lipid II (**1**)
m-H: 1045.4
(m-2H)/2:522.2



delipidated *S. aureus* Lipid II (**2**)
m-H: 1329.5
(m-2H)/2: 664.3

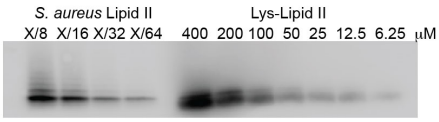


delipidated *B. subtilis* Lys-Lipid II (**3**)
m-H: 1088.4
(m-2H)/2:543.7



delipidated *E. coli* Lipid II (**4**)
m-H: 1089.4
(m-2H)/2: 544.2

b)



BDL-*S.aureus* Lipid II	
conc./μM	intensity
X/4	15407.7
X/8	9845.0
X/16	4413.4
X/32	2582.7

estimated X: 1210 μM

BDL-Lys-Lipid II	
conc./μM	intensity
400	28760.6
200	19705.6
100	10625.4
50	5365.6
25	3897.7
12.5	2574.5
6.5	1311.7

Estimation of total *S. aureus* Lipid II:
1210 μM x 2000 μL x 2000 g/mol =
4.8 mg per 6 L culture
(~800 μg *S. aureus* Lipid II per 1 L culture)

c)

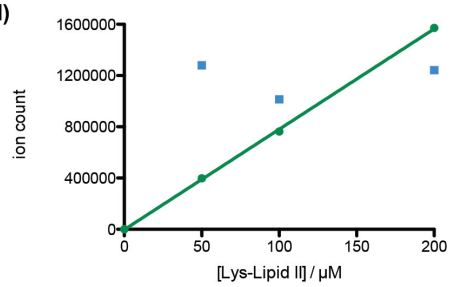
	integrated areas of (m-H) & (m-2H)/2	
	1	**2**
A	374458	519397.7
B	185928.1	534487.4
C	107485.8	525003.7
D	-	494418.8

estimated X: 540 μM

Estimated concentration = 540 μM

Estimation of total *S. aureus* Lipid II:
540 μM x 2000 μL x 2000 g/mol =
2 mg per 6 L culture
(~333 μg *S. aureus* Lipid II / 1 L culture)

d)



$y = 7835x + 2362$
$R^2 = 0.999$

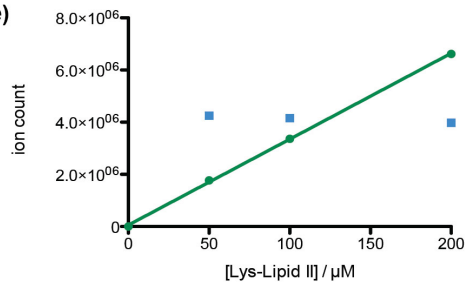
Lys-Lipid II
B. subtilis Lipid II

	integrated areas of (m-H) & (m-2H)/2	
	1	**4**
A	6.6190×10^6	3.9769×10^6
B	3.3630×10^5	4.1599×10^6
C	1.7727×10^6	4.2477×10^6

Estimated concentration = 150 μM

Sample dilution = 1x
Estimation of total B. subtilis Lipid II:
150 μM x 100 μL x 1918 g/mol =
29 μg per 1.5 L culture
(20 μg B. subtilis lipid II / 1 L culture)

e)



$y = 16462 x + 57870$
$R^2 = 0.999$

Lys-Lipid II
E. coli Lipid II

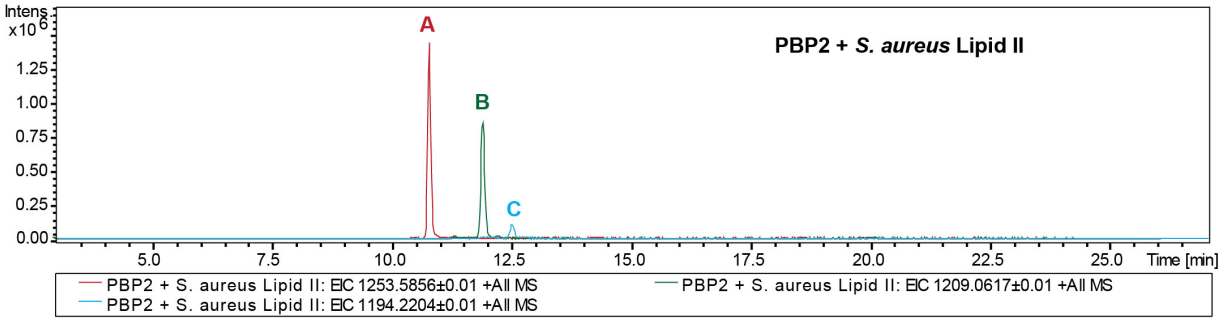
	integrated areas of (m-H) & (m-2H)/2	
	1	**3**
A	1.5713×10^6	1.2421×10^6
B	7.6324×10^5	1.0137×10^6
C	3.9813×10^6	1.2798×10^6

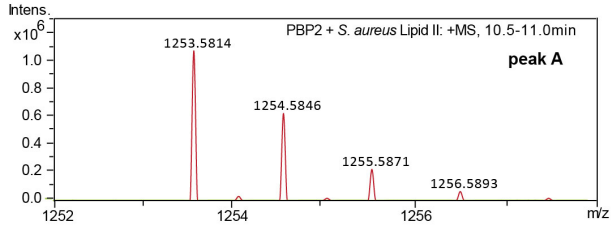
Estimated concentration = 247 μM

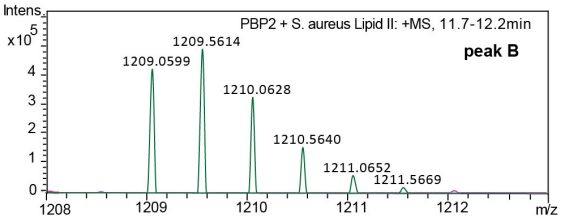
Sample dilution = 3.1x
Initial concentration = 760 μM
Estimation of total E. coli Lipid II:
760μM x 100 μL x 1919 g/mol =
145 μg per 1.5 L culture
(97 μg E.coli lipid II / 1 L culture)

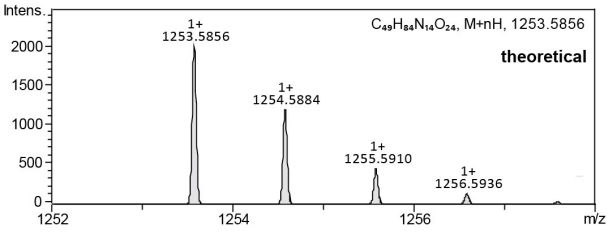
Supplementary Figure 4. Sufficient quantities of Lipid II can be isolated from bacteria. *S. aureus* Lipid II was quantified by two orthogonal methods: western blot analysis of biotinylated Lipid II (b) and LC/MS analysis of delipidated Lipid II (c). The estimated yields by both methods agree. LC/MS analysis of delipidated Lipid II was used to quantify *B. subtilis* and *E. coli* Lipid II (d-e). Structures of delipidated Lipid II species are shown (a).

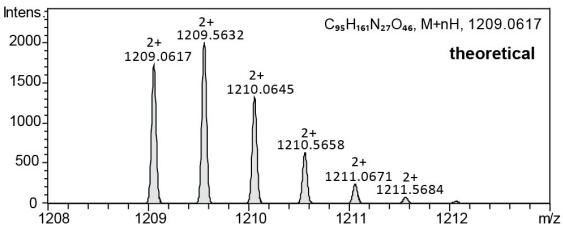
a)

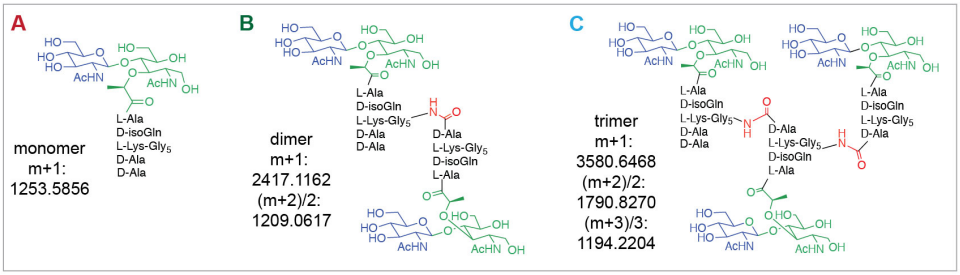

Intens.
x10^6
A
B
C
PBP2 + S. aureus Lipid II
Time [min]
PBP2 + S. aureus Lipid II: EIC 1253.5856±0.01 +All MS
PBP2 + S. aureus Lipid II: EIC 1194.2204±0.01 +All MS
PBP2 + S. aureus Lipid II: EIC 1209.0617±0.01 +All MS


PBP2 + S. aureus Lipid II: +MS, 10.5-11.0min
peak A
1253.5814
1254.5846
1255.5871
1256.5893
m/z

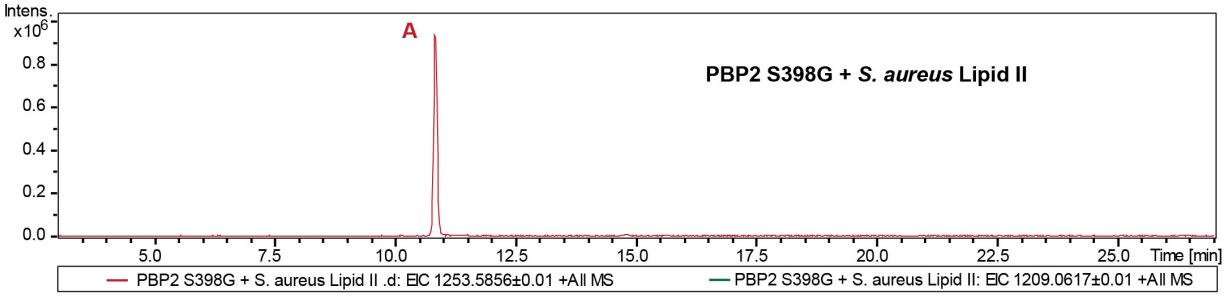

PBP2 + S. aureus Lipid II: +MS, 11.7-12.2min
peak B
1209.0599
1209.5614
1210.0628
1210.5640
1211.0652
1211.5669
m/z


C49H84N14O24, M+nH, 1253.5856
theoretical
1+ 1253.5856
1+ 1254.5884
1+ 1255.5910
1+ 1256.5936
m/z

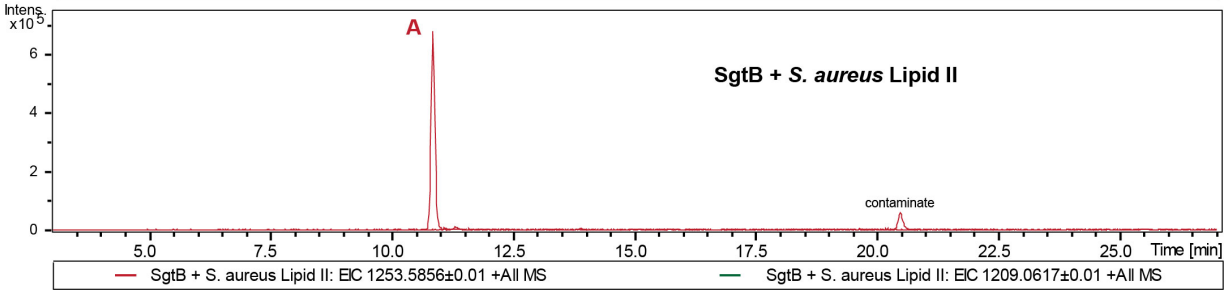

C95H161N27O46, M+nH, 1209.0617
theoretical
2+ 1209.0617
2+ 1209.5632
2+ 1210.0645
2+ 1210.5658
2+ 1211.0671
2+ 1211.5684
m/z


A
monomer
m+1:
1253.5856
B
dimer
m+1:
2417.1162
(m+2)/2:
1209.0617
C
trimer
m+1:
3580.6468
(m+2)/2:
1790.8270
(m+3)/3:
1194.2204

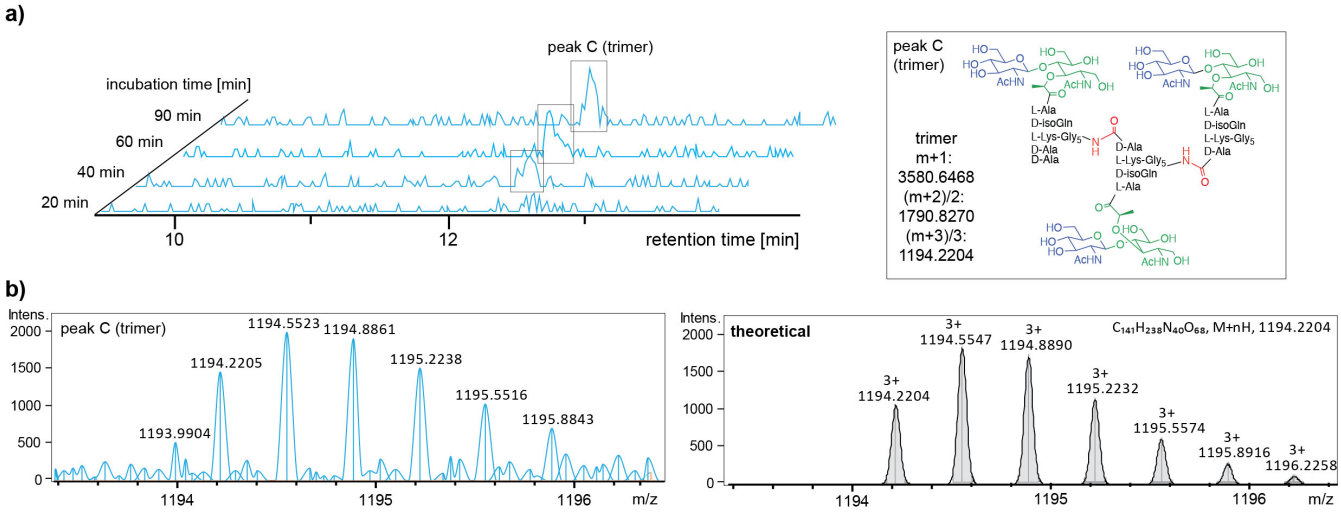
b)


Intens.
x10^6
A
PBP2 S398G + S. aureus Lipid II
Time [min]
PBP2 S398G + S. aureus Lipid II .d: EIC 1253.5856±0.01 +All MS
PBP2 S398G + S. aureus Lipid II: EIC 1209.0617±0.01 +All MS

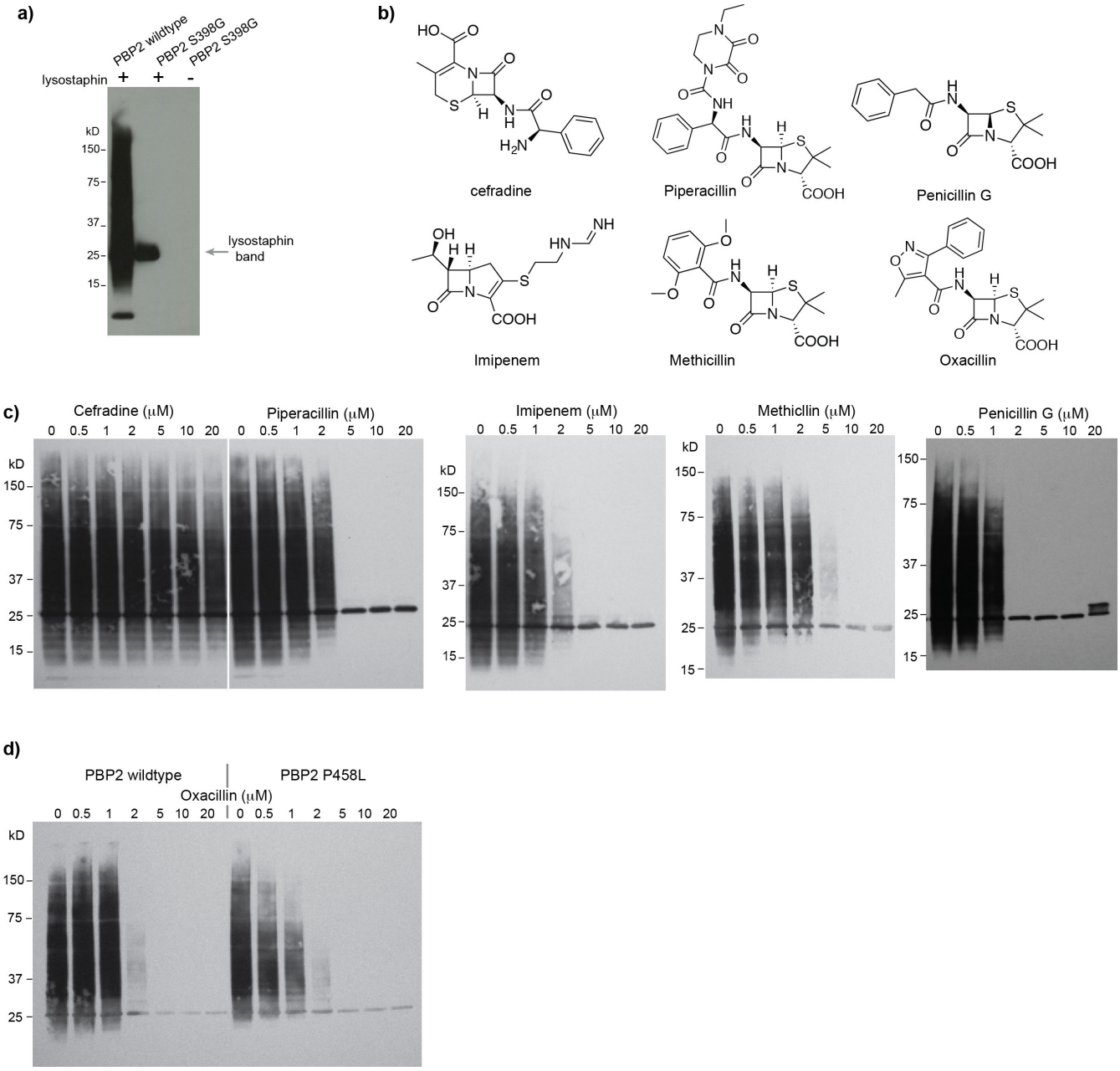
c)


Intens.
x10^5
A
SgtB + S. aureus Lipid II
contaminate
Time [min]
SgtB + S. aureus Lipid II: EIC 1253.5856±0.01 +All MS
SgtB + S. aureus Lipid II: EIC 1209.0617±0.01 +All MS

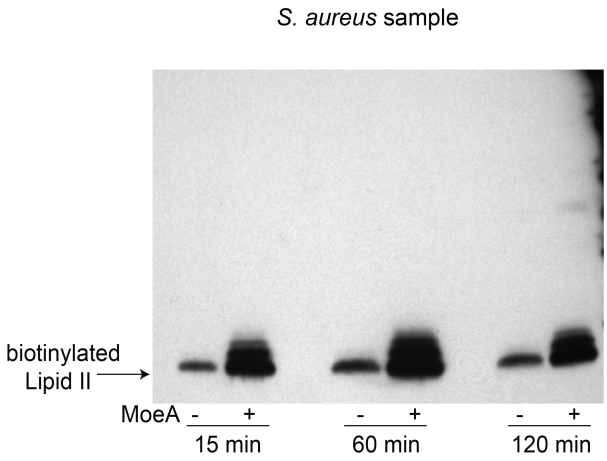
Supplementary Figure 5. Reconstitution of crosslinked peptidoglycan by *S. aureus* PBP2 using native Lipid II. (a) Extracted ion chromatogram (EIC) of muropeptide products. Peak A is the monomeric muropeptide, peak B is the crosslinked dimer, and peak C is the crosslinked trimer. High-resolution mass spectra of peak A and B are close to the theoretical mass spectra. The following ions were extracted: A: 1253.5856 (M+1), B: 1209.0617 ((M+2)/2), C: 1194.2204 ((M+3)/3). (b-c) Reaction with *S. aureus* $PBP2^{S398G}$ (TP inactive mutant) or SgtB, a monofunctional PGT does not yield crosslinked muropeptides.



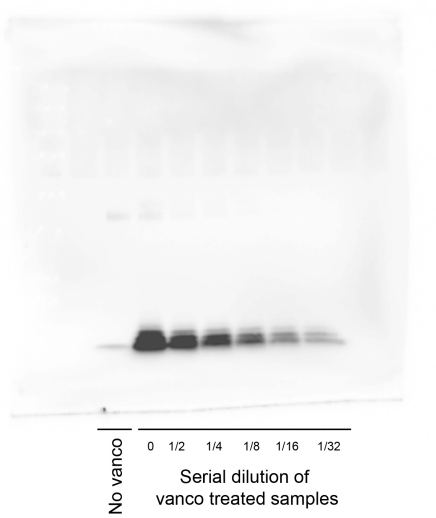
Supplementary Figure 6. The amount of the crosslinked trimer muropeptide increases as PBP2 reaction proceeds. (a) The intensity of peak C (trimer muropeptide) increases as PBP2 reaction time increases. The following ion was extracted: C: 1194.2204 ((M+3)/3). (b) The high-resolution mass spectrum of peak C is close to the theoretical mass spectrum.



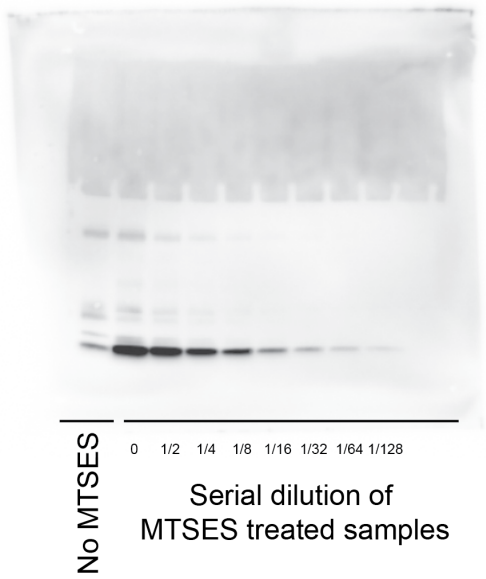
Supplementary Figure 7. A direct transpeptidase activity assay enables characterization of inhibitory potencies of different beta-lactams. (a) Western blot of crosslinked peptidoglycan produced by PBP2 with (left) requires active transpeptidase activity (middle-right). Product detection was enabled by BDL incorporation during PBP2 reaction. (b) Structures of beta-lactams examined in c-d. (c) Cefradine does not inhibit PBP2 activity up to the highest concentration tested in the experiment; whereas the other beta-lactams show potent inhibition. (d) The cefitzoxime-resistant mutant protein, $PBP2^{P458L}$, shows no notable resistance to oxacillin compared to wild-type PBP2. For all experiments, 1 μM of enzyme was used.



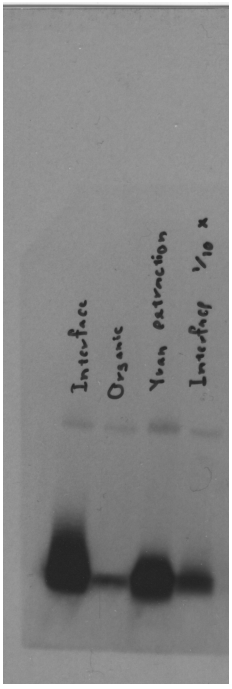
Supplementary Figure 8. A full gel of *S. aureus* Lipid II accumulation. Western blot of Lipid II isolated from *S. aureus* treated with and without moenomycin at various time points. The 15-min time points are cropped to use in Figure 2a.



Supplementary Figure 9. A full gel of *B. subtilis* Lipid II accumulation. Western blot of Lipid II isolated from *B. subtilis* treated with and without vancomycin at various time points. The first two lanes were cropped to use in Figure 2a.



Supplementary Figure 10. A full gel of *E. coli* Lipid II accumulation. Western blot of Lipid II isolated from *E. coli* ($MurJ^{A29C}$) treated with and without MTSES at various time points. The first two lanes are cropped to use in Figure 2b.



Supplementary Figure 11. A full gel of Lipid II comparison between the interface and the organic layer. The first two lanes are cropped to use in Figure 3b.

Primer Name	Sequence (5'-3')
F'pET42a_PBP2	AGGAGATATACATATGAAAGCACCTGCTTTTACCGAAGC
R'pET42a_PBP2	GTGGTGCTCGAGAGATTGTTGAGATCTAGTATTGTTATTTGATTGTGCAGT
F'pET42a	ATCTCAACAATCTCTCGAGCACCACCACC
R'pET42a	AGGTGCTTTCATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTC
F'PBP2_S398G	CAACAGATCCTCACCCTACTGGTGGATCTTTAAAACCTTTCTTAGCGTAT
R'PBP2_S398G	ATACGCTAAGAAAGGTTTTAAAGATCCACCAGTAGGGTGAGGATCTGTTG
F'PBP2_P458L	GACAAAGTTTCAATATCCTAGCTTTAAAAG
R'PBP2_P458L	CTTTTAAAGCTAGGATATTGAAACTTTGTC

Supplementary Table 1. Primers used in this study.

Reference:

1 Qiao, Y. *et al.* Detection of lipid-linked peptidoglycan precursors by exploiting an unexpected transpeptidase reaction. *J. Am. Chem. Soc.* **136**, 14678-14681, (2014).