Detection of Lipid-Linked Peptidoglycan Precursors by Exploiting an Unexpected Transpeptidase Reaction

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Supporting information

Materials

Lys-Lipid I, Lys-Lipid II, and mDAP-Lipid II were prepared as previously described.[1] N-terminus truncated *S. aureus* SgtB and *E. coli* PBP5 were purified as reported previously.[2] Fluorescein D-Lysine (FDL) was prepared as described.[3] PBP4 deletion *S. aureus strains (*Δ*pbp4*) and complementation (Δ*pbp4* complemented with *pbp4* gene), which were described previously, were obtained from the Cheung Lab.[4] Primers were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from New England Biolabs. Vectors and expression hosts were obtained from Novagen. Non-stick conical vials and pipet tips used for enzymatic reactions were from VWR. H-Gly-Gly-OH and H-Gly-Gly-Gly-OH were purchased from TCI. H-Gly-Gly-Gly-Gly-Gly-OH was purchased from Bachem. D-7-Azatryptophan was purchased from Santa Cruz Biotechnology Inc. Fmoc-D-Lys(biotinyl)-OH was purchased from VWR. D-Propargylglycine (DPG) was purchased from Peptech Corporation. D-Alanine-3,3,3-d_3 (D-Ala-d_3) was purchased from CDN Isotopes. Bocillin-FL penicillin, Na salt (Bo-FL) was purchased from Invitrogen. All other chemicals were purchased from Sigma Aldrich unless otherwise noted.

General Methods

LC/MS chromatograms were obtained on an Agilent Technologies 1100 series LC-MSD instrument using electrospray ionization (ESI). HRMS data was obtained on an Agilent 6520 LC-MS Q-TOF mass spectrometer instrument using ESI. Microscopic images were acquired with a Hamamatsu digital camera model ORCA-ER connected to a Nikon Eclipse TE2000-U microscope with X-cite 120 illumination system. A typhoon phosphorimager (GE Healthcare) was used to capture fluorescent SDS-PAGE gels. ImageJ was used to analyze microscopy images and western blots.

Cloning of *S. aureus* PBP4[Y21-Q383] and PBP4[Y21-Q383] S75A mutant

The *pbp4*[Y21-Q383] gene encoding truncated *S. aureus* PBP4[Y21-Q383], lacking the predicted transmembrane segments was PCR purified from *S. aureus* Newman strain using the following primer pair: 5'-CCTAGCTAGCTATGCACAAGCTACTAAC-3' and 5'-GCAGGGATCCTTACTGATGAACTTCTAC-3'. After digestion with NheI and BamHI, the PCR fragment was ligated into pET28a(+) to produce pPBP4[Y21-Q383]

as a N-terminal His_6 fusion. The inserted *pbp4*[Y21-Q383] gene was confirmed by sequencing (Genewiz or Beckman Sequencing Facility). *E. coli* novablue strain was used for cloning.

The catalytic serine residue (S75) of the transpeptidase domain of *S. aureus* PBP4 has been identified by sequence alignment with other PBPs.[5] The S75A mutation was made in the parent plasmid pPBP4[Y21-Q383] using techniques described in the QuickChange site-directed mutagenesis kit (Stratagene) to make the mutant plasmid pPBP4[Y21-Q383] S75A with the forward primer 5'-CTAAGTGGAATCCAGCGGCAATGACTAAATTAATGAC-3' and the reverse primer 5'-GTCATTAATTTAGTCATTGCCGCTGGATTCCACTTAG-3' (mutagenized codon is underlined).

Overexpression and purification of *S. aureus* PBP4[Y21-Q383] and PBP4[Y21-Q383] S75A mutant

Plasmid pPBP4[Y21-Q383] encoding for soluble *S. aurues* PBP4 and plasmid pPBP4[Y21-Q383] S75A encoding for *S. aureus* PBP4 TP* were collected from the cloning strains, and were transformed into *E. coli* BL21(DE3) cultures respectively for overexpression and purification. *E. coli* BL21 (DE3) cultures (LB medium supplemented with 50 µg/ mL kanamycin) were used to inoculate 1 L of LB medium (1:100) supplemented with 50 µg/mL kanamycin at 37 ℃ and grown to OD_{600} = 0.4-0.5 with shaking. Cells were cooled to 17 ℃ before induction with 0.5 mM IPTG for 17 hr with shaking. Cells were harvested by centrifugation (5250 x g, 20 min, 4 ℃) and pellets were resuspended on ice with 30 mL of Buffer A (20 mM Tris (pH = 7.5), 400 mM NaCl) with 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by three passages through a cell disrupter (3 x 10,000 psi, 4 ℃). The cell lysate was then pelleted by ultracentrifugation (90,000 x g, 30 min, 4 ℃). The resulting supernatant containing PBP4 protein was added to 1.5 mL washed Ni-NTA resin (Qiagen) and rocked at 4 ℃ for 40 min. After loading the column, the resin was washed with wash buffer (20 mL Tris (pH = 7.5), 40 mM NaCl, 0.1 % Triton-X-100, 40 mM imidazole). The protein was eluted with 200 mM imidazole in 10 mL of elution buffer (20 mM Tris (pH = 7.4), 500 mM NaCl, 0.1% reduced Triton-X-100, 200 mM imidazole). The eluent containing His_6-PBP4 was concentrated to < 1 mL using 30 kD MWCO Amicon Ultra Centrifuge Filter Devices (Millipore). The yield of purified protein was found to be ~ 8 mg/mL by DC protein assay (Biorad). Proteins were stored at -80 ℃. *S. aureus* PBP4 [Y21-Q383] is referred to PBP4 subsequently, and *S. aureus* PBP4 [Y21-Q383] S75A mutant is denoted as PBP4 TP*.

Bocillin-FL binding assay to ensure proper folding of TP domain of purified PBP4 protein

Purified *S. aureus* PBP4 protein (250 nM) was incubated with varying concentrations of penicillin G (penG) (1000, 100, 0 U/mL) in a buffer containing 20 mM potassium phosphate (pH = 7.5) and 140 mM NaCl (to reach a volume of 9 µL). The mixture was incubated at 37 °C for 1 hr with shaking. Bocillin-FL (1 µL of 100 µM stock) was added to the mixture (total reaction volume is 10 µL),[6] which was then incubated for 30 min at 37 °C with shaking. The reaction was quenched by adding 2x SDS loading dye, and heated for 20 min at 95 °C. The samples were loaded onto a SDS-PAGE gel, which was then scanned using Typhoon (fluorescence scan, excitation 488 nm, emission 526 nm). The scanned image was then analyzed using ImageJ (**Figure S1**).

LC/MS assay to evaluate PBP4 TP activity *in vitro*

The procedure was modified from our recent report.[1b] Lys-Lipid II (40 µM) was incubated with SgtB (1 µM) and PBP4 (2 µM) in reaction buffer (12.5 mM HEPES (pH = 7.5), 2 mM $MnCl_2$, 20% DMSO, and 250 µM Tween-80) in a total of 10 µL reaction volume for 1 hr at 25 °C in the absence or presence of glycine oligopeptides or various D-amino acids (all solutions were used at 1 mM; except for Gly_3 and Gly_5 solutions that were acidified with 0.01 N HCl to facilitate solubility, and were used at a final concentration of 5 mM). The reaction was quenched at 95 °C for 5 min, and then treated with mutanolysin (from *Streptomyces globisporus*, Sigma, 1 U) for 1.5 hr at 37 °C followed by another 1 U aliquot for 1.5 hr. The resulting disaccharides were reduced with sodium borohydride (10 mg/mL, 30 min) to resolve the MurNAc anomers. Phosphoric acid (20%, 1.2 µL) was then added to adjust the pH to ~4. The reaction mixture was the lyophilized, redissolved in 12 µL H_2O and subjected to LC/MS analysis, conducted with ESI-MS operating in positive mode. The instrument was equipped with a Waters Symmetry Shield RP18 column (5 µM, 3.9 x 150 mm) with a matching column guard. The fragments were separated using the following method: 0.5 mL/min H_2O (0.1% formic acid) for 5 min followed by a gradient of 0% acetonitrile (ACN) (0.1% formic acid)/H_2O (0.1% formic acid) to 20% ACN (0.1% formic acid)/H_2O (0.1% formic acid) over 40 min. Molecular ions corresponding to expected disaccharide fragments were extracted from chromatograms (**Figure 1** and **S2**).

To analyze the ability of PBP4 to use polymeric PG and monomeric Lipid II as substrates, the above procedure is modified slightly. To analyze PBP4 activity on preformed PG polymer, Lys-Lipid II (40 µM) was incubated with SgtB (1 µM) for 1 hr at 25 °C, prior to inactivation of SgtB at 95 °C for 5 min. PBP4 (2 µM) and D-Tyr (1

mM) were added to the reaction mixture which was incubated for another hour at 25 °C before heat quenching. The digestion procedure is identical to above. To analyze PBP4 activity on Lipid II monomer, Lys-Lipid II (40 μM) was incubated with PBP4 (2 μM) and D-Tyr (1 mM) for 1 hr at 25 °C, prior to inactivation of PBP4 at 95 °C for 5 min. SgtB (1 μM) was added to the reaction mixture which was incubated for another hour at 25 °C before heat quenching. The digestion procedure is identical to above. Molecular ions corresponding to the expected disaccharide fragments were extracted from chromatograms (**Figure 4** and **S4a**). To analyze PBP4 activity to exchange unnatural D-amino acids into Lipid II monomers, the respective D-amino acid substrates were used instead of D-Tyr using the same protocol (**Figure S4b**).

Microscopy imaging analysis of FDL labeling in *S. aureus* strains

S. aureus strains (MW2 wildtype, Δ*pbp4*, and Δ*pbp4* complemented with *pbp4* gene)[4] were grown for 4 hr in TSB at 37 °C to stationary phase. The cultures were then diluted to reach OD_{600} = 0.1 and allowed to grow for an additional 30 min to reach mid-log phase. To 0.5 mL of *S. aureus* culture, 1 μL of FDL probe (2 mM in DMSO) was added (giving a final concentration of 4 μM). The cultures were incubated briefly for 10 min at 37 °C with shaking, and fixed with ice cold 70% ethanol immediately. The fixed cells were then incubated on ice for 15 min, before washing with PBS buffer for three times. The washed cell pellet was resuspended in 30 μL PBS, and 2 μL liquid was mounted on PBS pads containing 2% agarose. The samples were imaged using BODIPY-GFP channel. Images were adjusted to the same intensity scale to allow comparison using ImageJ.[7]

Deprotection of BDL probe

To Fmoc-D-Lys(biotinyl)-OH (0.016 mmol, 10 mg) in a glass vial, 2 mL of 20% piperidine/DMF and 0.3 mL toluene were added. The mixture was stirred to dissolve for 30 min at room temperature, and then concentrated to remove solvent *in vacuo*. The crude concentrated mixture was diluted in H_2O leaving a white byproduct precipitate, which was removed via filtration. The filtrate was concentrated, and the product was analyzed by MS direct injection in positive mode. Pure BDL probe was prepared as a stock solution of 10 mM in H_2O.

Preparation of Lipid I and Lipid II analogs with functionalized D-amino acids by PBP4

An aliquot of C20-Lys-Lipid I analog (50 nmol) in DMSO (20 μL)[1c,8] was briefly sonicated and then diluted with 20 μL of 10x reaction buffer (125 mM HEPES pH = 7.5, 20 mM $MnCl_2$, and 2.5 mM Tween-80). Biotinylated D-

Lysine (BDL) (5mM, final concentration) and PBP4 (20 μM, final concentration) were then added to the reaction mixture to reach a final volume of 200 μL. The reaction was incubated for 3 hr at 25 ℃, and then kept on ice before purification. The reaction mixture was loaded onto a pre-equilibrated Strata C18-E SPE column (Phenomenex), then eluted with ACN/25 mM NH_4HCO_3 using a step gradient of 0% to 100% ACN (500 μL each, 10% increment). The collected fractions were analyzed by MS direct injection in negative mode. The modified-Lipid I analog was eluted in 30%-40% ACN fractions. HRMS data was obtained for the fractions contain pure BDL-incorporated Lipid I analog (**Figure S5**). D-propargylglycine (DPG)-modified Lipid I analog was obtained using DPG in place of BDL following the same protocol (**Figure S5**).

The fractions that contained pure BDL-incorporated Lipid I analog were combined and concentrated by speed-vac. Lipid I to Lipid II conversion was carried out as previously described.[9] Briefly, the dried Lipid I analog (50 nmol) was re-dissolved in MeOH (24 μL), and then added 22.9 μL of 7x MurG buffer (350 mM HEPES pH 7.9, 35 mM $MgCl_2$), 5 μL UDP-GlcNAc (10 mM), and 96.1 μL H_2O. The mixture was vortexed before adding 8 μL alkaline phosphatase (20U/ μL, Roche) and 4 μL purified MurG (20 mg/mL) to reach a total final volume of 160 μL. After incubation at 25 ℃ for 1 hr, another aliquot of 4 μL MurG (20 mg/mL) was added to the reaction to incubate for another 1 hr. Cold H_2O (160 μL) was added to quench the reaction. The quenched mixture was loaded onto a pre-equilibrated BAKERBOND SPE C18-extraction column (J. T. Bakers), and washed with H_2O/ 0.1% NH_4OH (500 μL x 7), and then eluted with MeOH/ 0.1% NH_4OH (500 μL x 7). The fractions were analyzed by MS direct injection in negative mode. The modified-Lipid II analog was eluted in the initial MeOH/ 0.1% NH_4OH fraction (500 μL). HRMS data was also obtained for the pure BDL-modified Lipid II analog (**Figure 5a and S5b**). DPG-modified Lipid II analog was converted from DPG-Lipid I following the same protocol. (**Figure S5**).

Western blot detection of BDL-Lipid II *in vitro*

To a non-stick reaction vial, 3 μL H_2O and 1 μL of 10x reaction buffer (125 mM HEPES pH = 7.5, 20 mM $MnCl_2$, and 2.5 mM Tween-80) was added with 3 μL of BDL probe (10 mM in H_2O). The mixture was vortexed before adding 1 μL of Lys-Lipid II (200 μM in DMSO), 1 μL of DMSO, and 1 μL of PBP4 (40 μM) to reach a total volume of 10 μL. The reaction was incubated at 25 ℃ for 1 hr. To quench the reaction, 10 μL of 2x SDS loading buffer was added and the samples were heated at 95 ℃ for 5 min. 3 μL of the final mixture was loaded onto a 15% SDS polyacrylamide gel. The products were transferred to Immun-Blot PVDF membrane (BioRad).

BDL-Lipid II was detected by blotting with streptavidin-HRP (1:10000 dilution, Pierce), and visualized using ECL Prime Western Blotting Detection Reagent (GE Ambersham) and Biomax Light Film (Kodak). To confirm the presence of PBP4, the membrane was also blotted with anti-His_5-HRP (1:10000 dilution, Qiagen). Control reactions were performed with omitting one or two components (**Figure S6**).

Extraction of cellular lipid-linked PG precursors and western blot detection

The extraction of cellular lipids has been modified from a previously published protocol.[10] An overnight culture of *S. aureus* RN4220 was diluted to an OD_{600}= 0.1, and allowed to grow to mid-exponential phase at 37 ˚C. The culture was divided into 2 mL aliquots subsequently which were treated with antibiotics (concentrations of antibiotics were about 2x of their respective MICs against *S. aureus* RN4220, see Figure **S8**). Following 10 min of growth at 37 ˚C, cells were harvested by centrifugation for 10 min at 5,000 x g. The amount of cell pellet collected was normalized by OD_{600}, and resuspended in 500 µL PBS (pH 7.4). The resuspended pellet was transferred to a glass tube alongside 500 µL $CHCl_3$ and 1 mL MeOH. The mixture was vortexed repetitively for 10 min at 25 ˚C, following which any cell debris was removed with centrifugation for 10 min at 4,000 x g. The supernatant was collected and transferred to a new glass tube with 500 µL $CHCl_3$ and 500 µL PBS. The mixture was vortexed for 10 min, and centrifuged for 1 min at 4,000 x g to achieve phase separation. 1 mL of the organic layer was collected and concentrated *in vacuo*. The dried fractions were resuspended in 12 µL DMSO. 2 µL of each fraction was used (in place of Lipid II and DMSO) in a BDL labeling reaction as described earlier. Reactions were further analyzed using a western blot with the use of HRP-Conjugated Strepavidin as the antibody (**Figure 5b and S7**).

Measurement of minimum inhibitory concentration (MIC) of antibiotic against *S. aureus* RN4220

A serial dilution of antibiotic was prepared, and 1.5 µL of each was added to a clear 96-well plate (Corning). To each well containing antibiotic, 150 µL of a culture of *S. aureus* RN4220 at OD_{600}= 0.1 (diluted from a saturated overnight culture in TSB medium) was seeded. The plate was shaken at 37 ℃ and OD_{600} reading was taken after 10 hr. The concentration of antibiotic that showed no bacteria growth was designated the MIC.

References:

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Supplementary figures

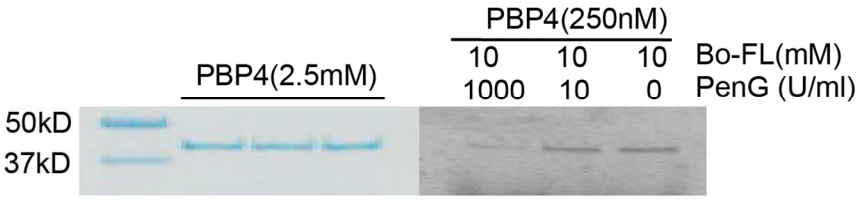
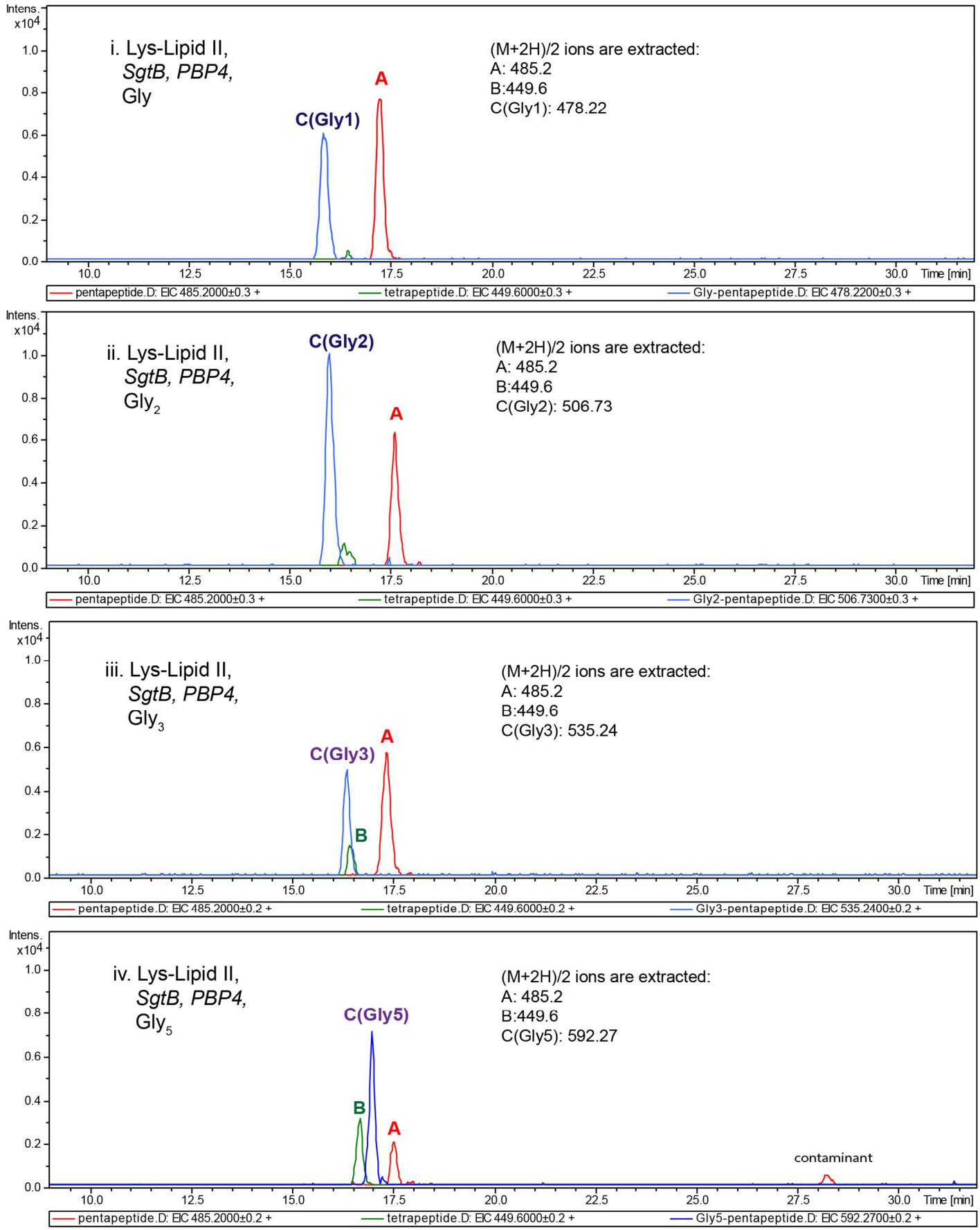


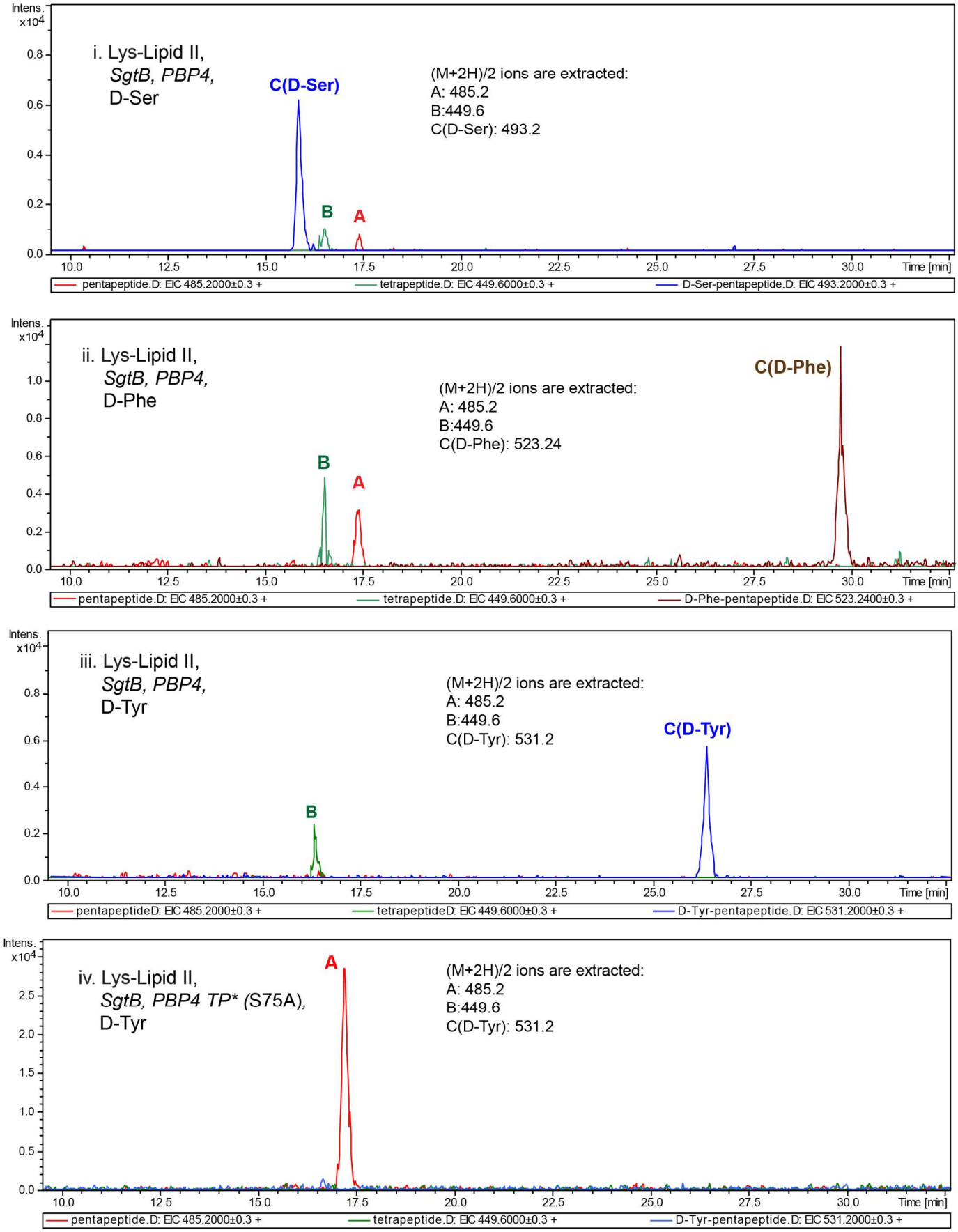
Figure S1. The TP domain of purified *S. aureus* PBP4 is properly folded. Coomassie blue stained SDS-PAGE gel shows purified *S. aureus* PBP4 protein (2.5 μM) with expected molecular weight of 42 kD (left panel). Fluorescent image shows binding of Bocillin-FL (Bo-FL, 10 μM) to PBP4 (250 nM) is inhibited by pencillin G (penG, 1000-0 U/mL) in a dose-dependent manner (right panel).

Figure S2a. *S. aureus* PBP4 shows TP activity *in vitro* as it incoprorates glycine oligopeptides.



Detailed experimental description is on Page S12

Figure S2b. *S. aureus* PBP4 incoprorates D-amino acids into PG *in vitro*.



Detailed experimental description is on Page S12

Figure S2c. LMW PBP (*E. coli* PBP5) only demonstrates CP activity.

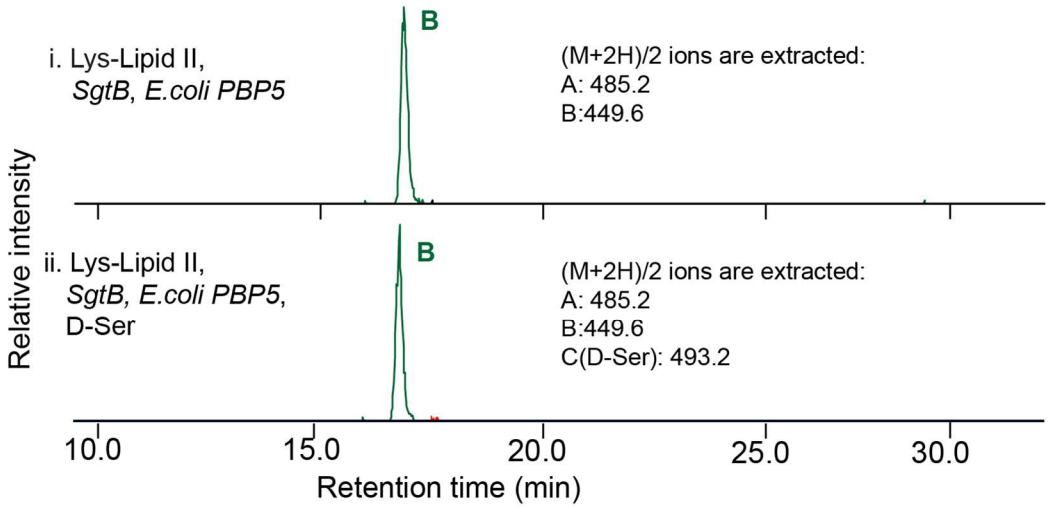


Figure S2. *S. aureus* PBP4 demonstrates *in vitro* TP activity, unlike LMW PBP (*E. coli* PBP5) that shows only carboxypeptidase (CP) activity. (a) Reactions of PBP4 (2 μM) and SgtB (1 μM) with Lys-Lipid II (40 μM) in the presence of glycine oligoglycine (Gly_X: Gly_{1-3} and Gly_5; Gly_1 and Gly_2: 1mM, Gly_3 and Gly_5: 5mM) incubated for 1 hr at 25 ℃. The reaction mixtures were then digested as described above. Extracted chromatograms (EICs) of all reactions (**S2a**, trace **i-iv**) give peak **Cx,** the muropeptide products with glycine oligopeptides incorporated. Comparing peak **Cx** and peak **B** in each chromatogram shows that PBP4 preferentially incorporates glycine oligopeptides over water, the model nucleophiles for crosslinking. (M+2)/2 ions were extracted: A: 485.2, B: 449.6, C_1: 478.2, C_2: 506.73, C_3: 535.24, C_5: 592.27. (b) Reactions of PBP4 (2 μM) and SgtB (1 μM) with Lys-Lipid II (40 μM) in the presence of various D-amino acids (1 mM) incubated for 1 hr at 25 ℃. The reaction mixtures were then digested as described above. EICs give peak **C,** illustrating D-Ser, D-Phe, D-Tyr amino acids were all incorporated by PBP4 (**S2b**, trace **i-iii**), whereas PBP4 TP* (S75A) does not exchange D-Tyr (**S2b**, trace **iv**). (M+2)/2 ions were extracted: A: 485.2, B: 449.6, C(D-Ser):493.2, C(D-Phe): 523.24, C(D-Tyr): 531.20. (c) *E. coli* PBP5 is a previously characterized LMW PBP that demonstrates only hydrolysis activity. Reactions of *E. coli* PBP5 (2 μM) and SgtB (1 μM) with Lys-Lipid II (40 μM) in the absence (**S2c**, trace **i**) and in the presence (**S2c**, trace **ii**) of D-Ser (1mM) both give peak **B**, the hydrolysis product only. (M+2)/2 ions were extracted: A: 485.2, B: 449.6, C(D-Ser): 493.2 (for **S2c**, trace **ii**).

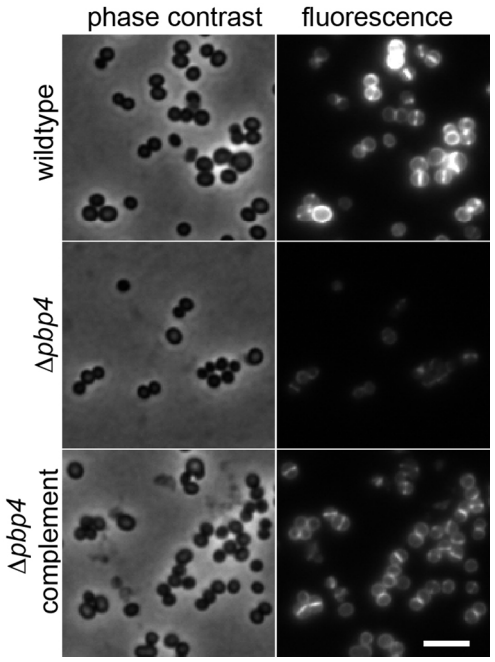


Figure S3. *S. aureus* PBP4 shows TP activity *in vivo*, and is a major pathway for D-amino acid incorporation in *S. aureus* PG. *S. aureus* wildtype strain incorporates a fluorescent D-lysine probe (FDL) efficiently, but Δ*pbp4* strain shows dramatic decrease in FDL incorporation efficiency. Complementation with *pbp4* gene restores the incorporation efficiency (Scale bar: 2 μM). Sample preparation is described above.

Figure S4a. PBP4 efficiently incorporates D-Tyr into Lys-Lipid II.

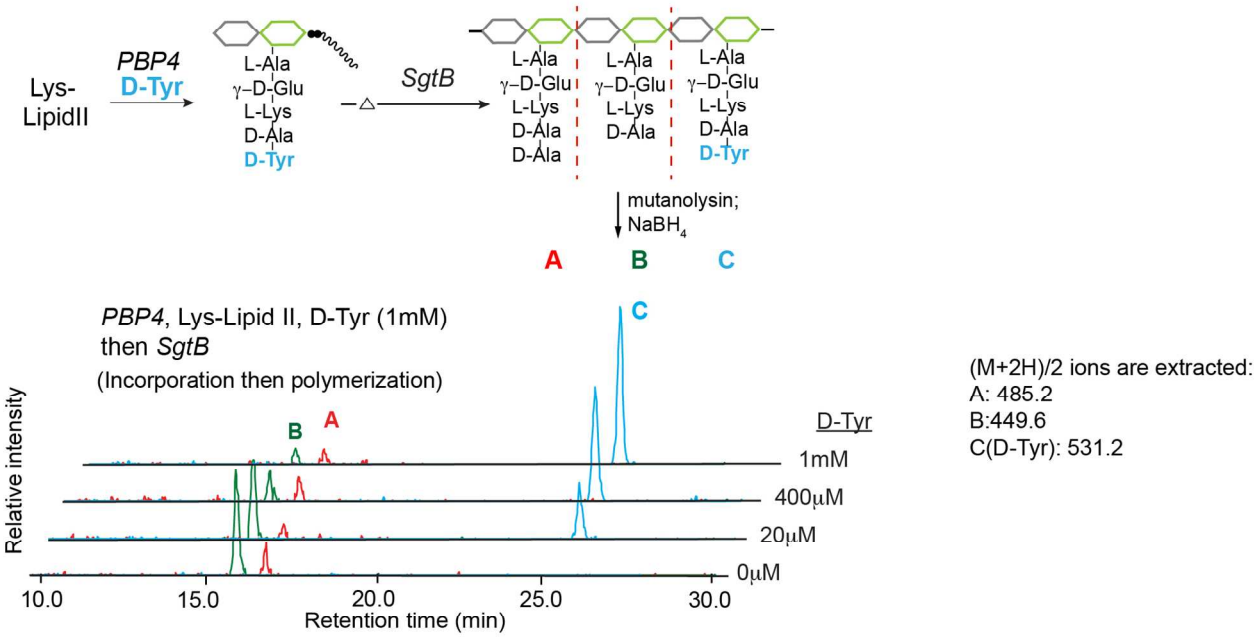


Figure S4b. PBP4 efficiently incorporates modified D-amino acids into Lys-Lipid II and mDAP-Lipid II.

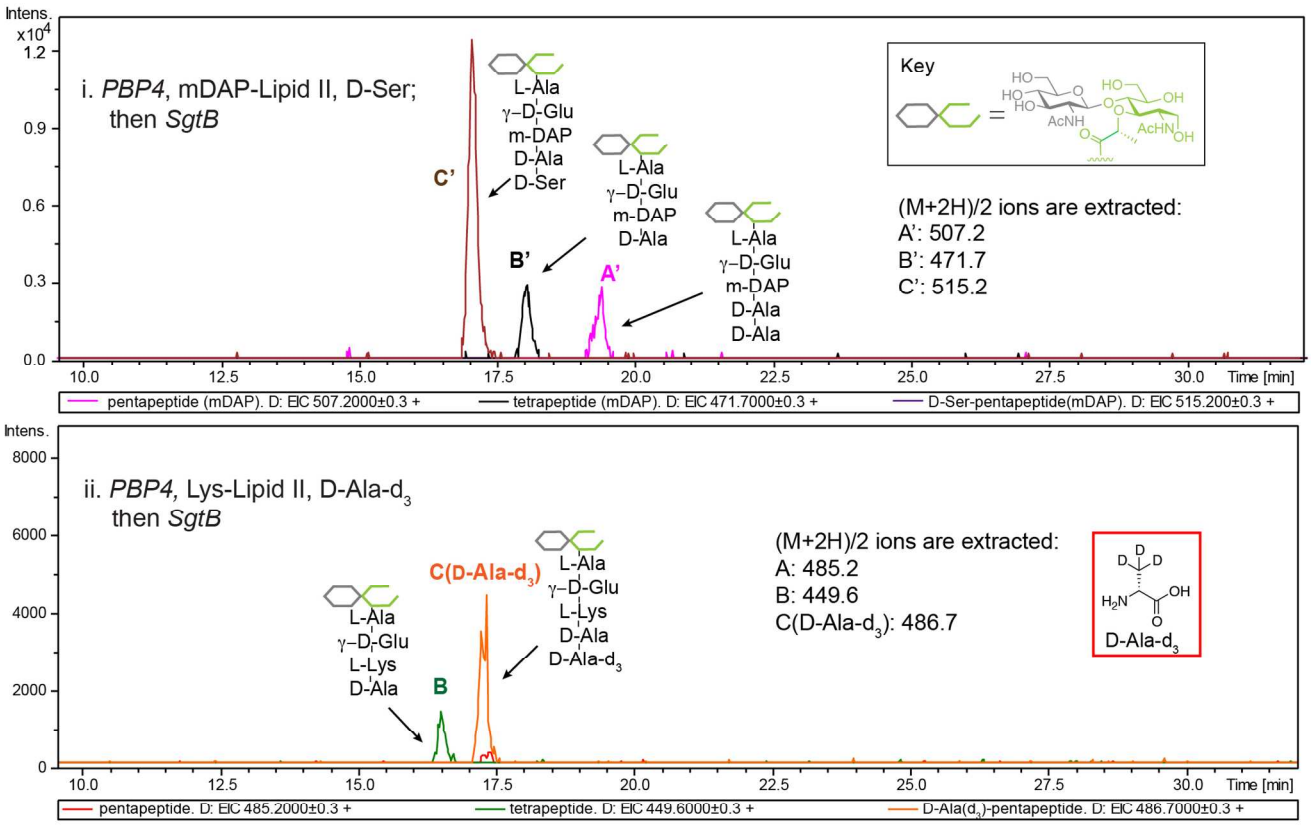


Figure S4b trace i-ii

Figure S4b. PBP4 efficiently incorporates modified D-amino acids into Lys-Lipid II and mDAP-Lipid II.

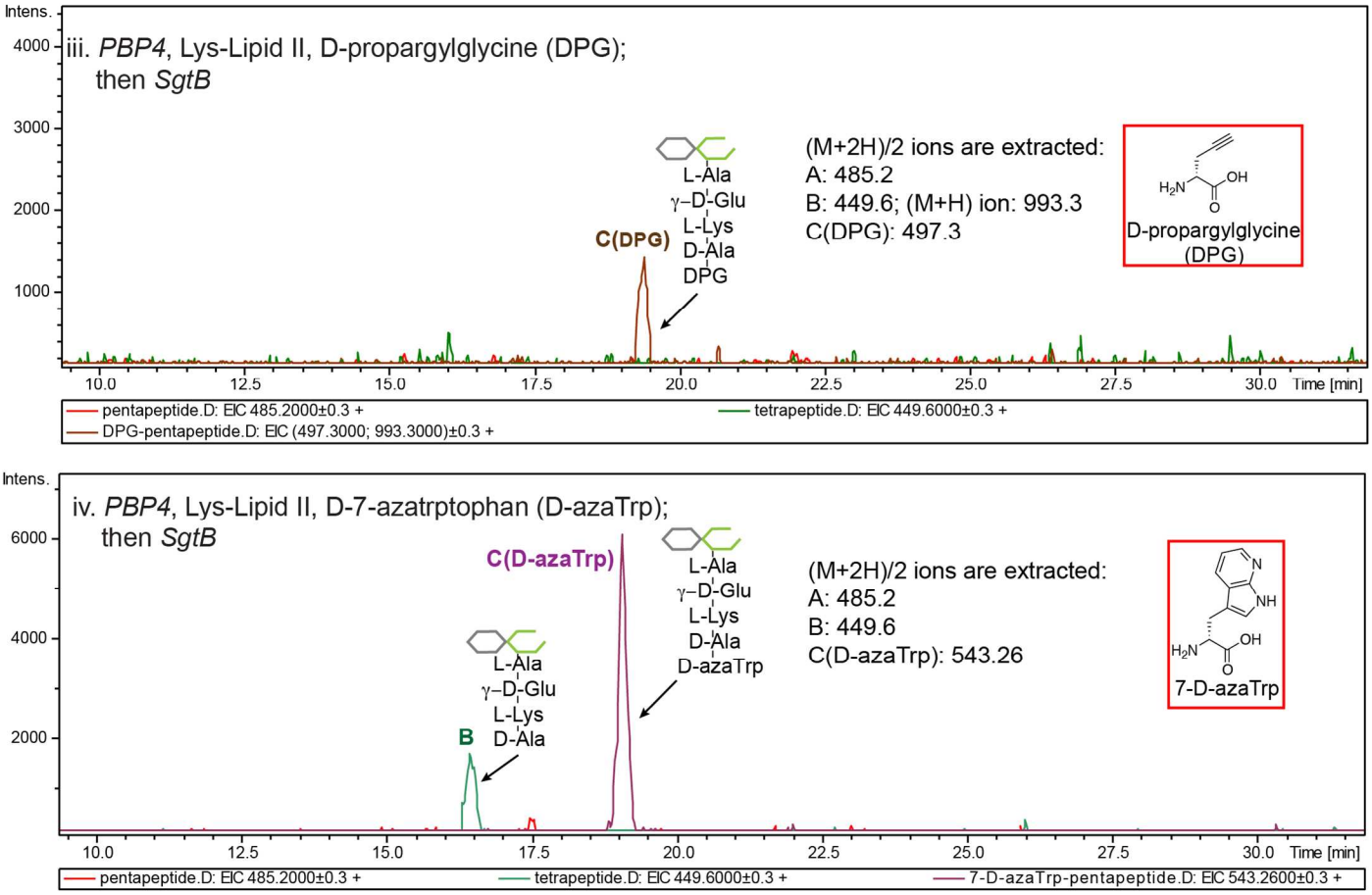


Figure S4b trace iii-iv

Figure S4. PBP4 exchanges D-amino acids in monomeric Lipid II efficiently. (a) Schematic for analysis of PBP4 activity with Lys-Lipid II monomers, which is also described in **Figure 4a**. Reactions of PBP4 (2 μM) with Lys-Lipid II (40 μM) and D-Tyr (0 μM, 20 μM, 400 μM and 1 mM) were first incubated at room temperature for 30 min before quenching at 95 ℃ for 5 min to inactivate PBP4. SgtB (1 μM) was then added to the mixture for incubation at room temperature for 1 hr. Reactions were then digested according to procedures described above. EICs show that the magnitude of peak **C** increases as concentration of D-Tyr increases. Approximately, 10-fold excess of D-Tyr (400 μM) to Lys-Lipid II (40 μM) led to complete exchange of the terminal D-Ala. (M+2)/2 ions were extracted: A: 485.2, B: 449.6, C(D-Tyr): 531.2. (b) EICs show *S. aureus* PBP4 can exchange various functionalized D-amino acids (D-Ala-d_3, D-propargylglycine (DPG) and D-7-azatrptophan (D-azaTrp) into Lys-Lipid II as well as mDAP-Lipid II. Structures of the D-amino acids used are shown. (M+2)/2 ions were extracted: A': 507.2, B': 471.7, C': 515.2 (**S4b**, trace **i**); A: 485.2, B: 449.6 (**S4b**, trace **ii–iv**), and C(D-Ala-d_3): 486.7 (**S4b**, trace **ii**); C(DPG): 497.3 and 993.3 (M+1) (**S4b**, trace **iii**); C(D-azaTrp): 543.26 (**S4b**, trace **iv**).

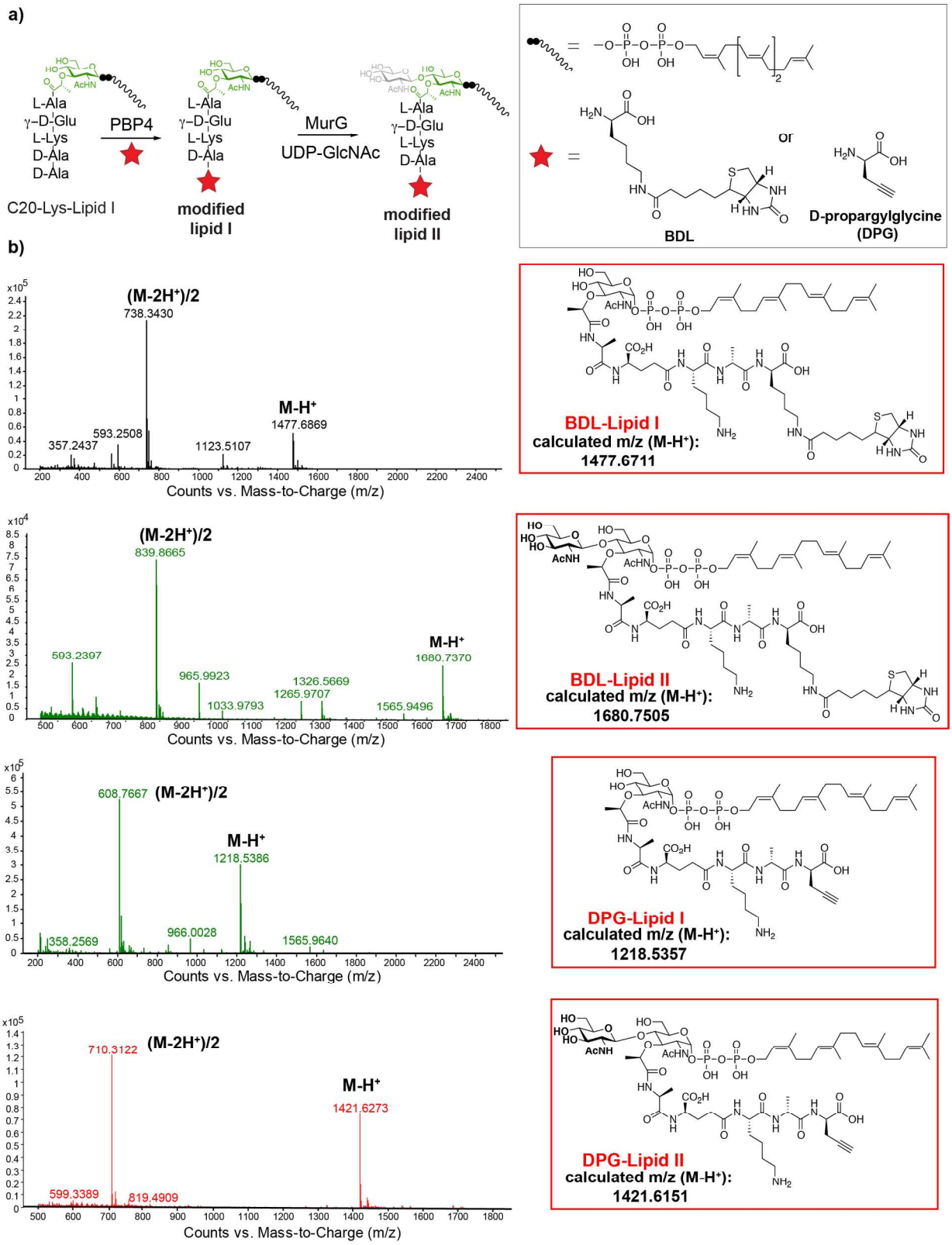


Figure S5. *S. aureus* PBP4 can be used to prepare Lipid I and Lipid II analogs with functionalized D-amino acids. (a) The chemoenzymatic route of generating modified Lipid I and Lipid II. BDL and DPG are two examples. (b) High resolution QTOF mass spectra of BDL-Lipid I, BDL-Lipid II, DPG-Lipid I and DPG-Lipid II. Structures are shown in red boxes. The protocol is described above.

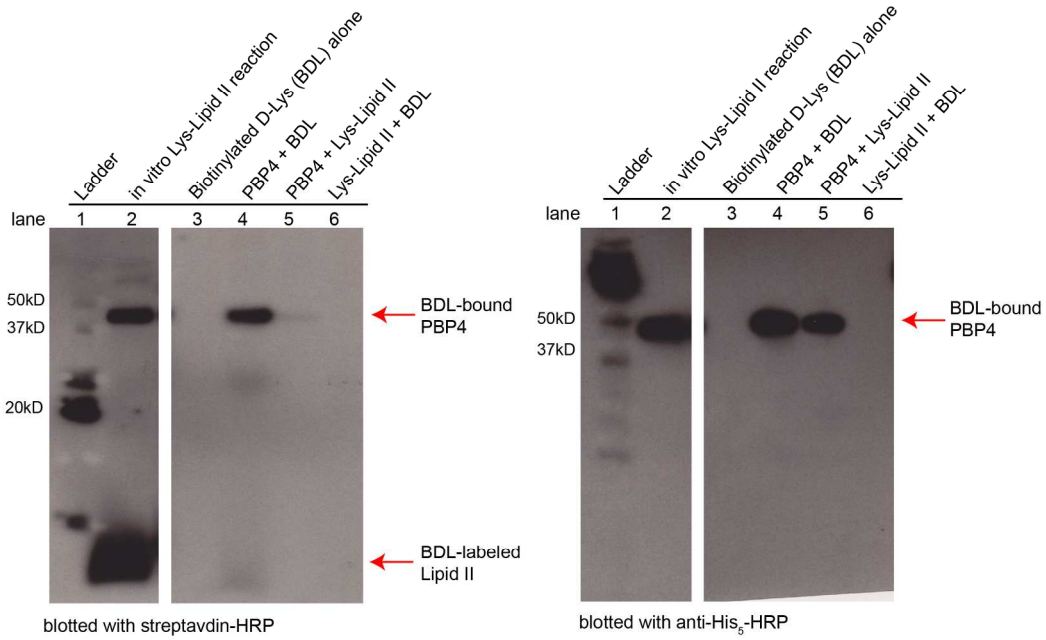


Figure S6. Western blot allows detection of i*n vitro* synthesized BDL-Lipid II. (a) Left gel: *In vitro* reaction of synthetic Lys-Lipid II, PBP4 and BDL probe (lane 2) shows two bands when blotted with streptavdin-HRP. The upper band corresponds to BDL-bound PBP4 (Mw~42kD); the lower band is the BDL-Lipid II. Lane 3-6 are control reactions with BDL alone (lane 3, BDL migrates off gel), PBP4 plus BDL (lane 4), PBP4 plus Lys-Lipid II (lane 5) and Lys-Lipid II plus BDL (lane 6) do not show the BDL-labeled Lipid II band; lane 4 gives the BDL-bound PBP4 band due to interactions between BDL and PBP4. Right gel: the presence of PBP4 (lane 2, 4 and 5) is confirmed by blotting with anti-His_5-HRP antibody.

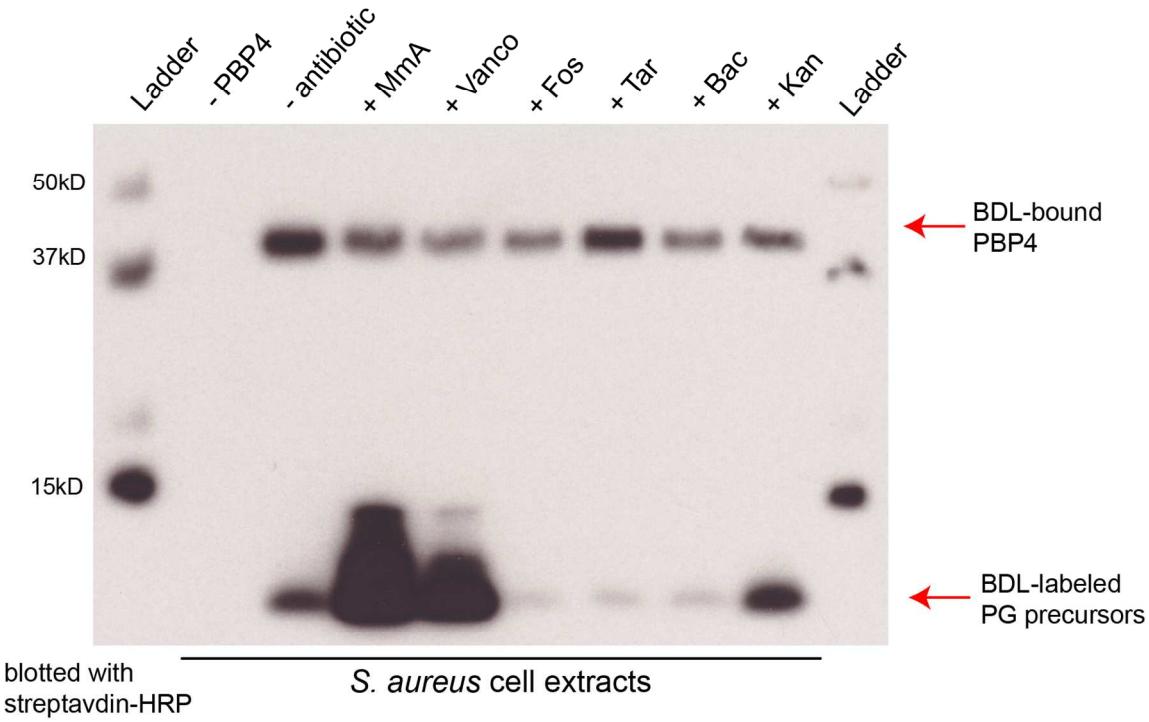


Figure S7. *S. aureus* PBP4 allows detection of cellular lipid-linked PG precursors. 2 mL culture of *S. aureus* was treated with different antibiotics for 10 min, lipid-linked PG precursors were extracted and subjected to BDL labeling by PBP4. The precursors extraction method and BDL labeling method are described above. Higher molecular weight intermediates were observed in MmA and Vanco treated samples, which could be higher molecular weight oligomers of Lipid II due to either glycosylation or transpeptidation and could contain other modifications. Studies to characterize these species are currently underway.

	MIC	Amount used
Moenomycin (MmA)	0.125 μg/ml	0.3 μg/ml
Vancomycin (Vanco)	4 μg/ml	10 μg/ml
Fosfomycin (Fos)	12.5 μg/ml	28 μg/ml
Targocil (Tar)	1 μg/ml	2 μg/ml
Bacitracin (Bac)	250 μg/ml	500 μg/ml
Kanamycin (Kan)	4 μg/ml	10 μg/ml

Figure S8. Minimum inhibitory concentrations (MICs) of various antibiotics against *S. aureus* (grown in TSB medium), and concentrations of antibiotics selected for experiment in **Figure 5b** and **S7**.