SUPPORTING INFORMATION

Identification of a functionally unique family of penicillin-binding proteins

Michael A. Welsh,[1] Atsushi Taguchi,[1†] Kaitlin Schaefer,[1,2†] Daria Van Tyne,[3] François Lebreton,[3] Michael S. Gilmore,[1,3] Daniel Kahne,[2*] and Suzanne Walker[1*]

[1] Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts 02115

[2] Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138

[3] Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, Massachusetts 02114

[†] These authors contributed equally.

[*] Correspondence to: suzanne_walker@hms.harvard.edu, kahne@chemistry.harvard.edu

METHODS

Materials. All reagents and chemicals were purchased from Sigma-Aldrich, unless indicated otherwise. Lipid II from *Staphylococcus aureus* (**1**), *Escherichia coli* (**5**), and *Bacillus subtilis* (**6**) was extracted from cells as previously described.[1] Lipid II variant **4** was synthesized as reported.[2] Genomic DNA from *Streptococcus gordonii* was purchased from ATCC (ATCC 35105D-5). PBP2a from *Enterococcus faecalis* was expressed and purified as previously reported.[3] SgtB from *S. aureus* and its Y181D point mutant (abbreviated as SgtB* in the main text) were expressed and purified as previously described.[4] *E. coli* PBP5 was expressed and purified as reported previously.[5] Fmoc-biotin-D-lysine (BDL) was purchased from Bachem and de-protected as reported previously.[6] PCR primers were purchased from Integrated DNA Technologies. Restriction endonucleases were purchased from New England Biolabs.

Bacterial Culture. The bacterial strains and plasmids used in this study are listed in Table S1. Unless indicated otherwise, all strains were grown at 37 °C. *Enterococcus faecalis* strains were grown with shaking in brain-heart infusion broth (BHI, Hardy Diagnostics) or on agarized plates. *E. coli* strains were grown with shaking in LB broth (Beckton Dickinson) or on agarized plates. *Streptococcus gordonii* strains were grown in Todd Hewitt broth (THB, Beckton Dickinson) or on agarized BHI plates. *Streptococcus pneumoniae* strains were grown in THB containing 0.5% yeast extract (THY, Beckton Dickinson). *S. gordonii* and *S. pneumoniae* were grown without shaking at 37 °C in an atmosphere containing 5% CO_2. When growth on solid medium was required, *S. pneumoniae* strains were grown on pre-poured tryptic soy agar 5% sheep blood (TSAII 5% SB, Beckton Dickinson) plates with a 5 mL overlay of 1% nutrient broth (NB, Beckton Dickinson) agar containing any required antibiotics. Antibiotics were used at the following concentrations: carbenicillin, 100 μg/mL; erythromycin, 0.2 μg/mL (*S. pneumoniae*) or 10 μg/mL (*S. gordonii*); kanamycin, 50 μg/mL; spectinomycin, 500 μg/mL; tetracycline, 4 μg/mL.

Instrumentation. Mass spectrometry data was analyzed using Agilent MassHunter Workstation Qualitative Analysis software version B.06.00. LC-MS was conducted using an Agilent Technologies 1200 series HPLC in line with an Agilent 6520 Q-TOF mass spectrometer using electrospray ionization (ESI). Funding for the Agilent Q-TOF was supported, in part, by the Taplin Funds for Discovery program.

Generation of a sequence similarity network for the Peptidase_S11 Pfam family. Sequence alignment data was generated using the EFI Enzyme Similarity Tool[7] (http://efi.igb.illinois.edu/efi-est/index.php) using Option B with the Peptidase_S11 Pfam family (PF00768) as the input sequence set and with Pfam-defined domains enabled. A similarity network was then created using an alignment score corresponding to approximately 40% sequence identity and filtering for sequences between 190 and 260 residues in length. Data was plotted using Cytoscape version 3.2.0.

Cloning of *E. faecalis* PBPX. The *ef_3129 [T36-P429]* gene encoding truncated *E. faecalis* PBPX, lacking the N-terminal signal peptide and C-terminal transmembrane helices, was amplified from *E. faecalis* V583 genomic DNA

using the primers (5'-GTAGCTAGCACTCACGCAGAAGAAGATATTACCG-3') and (5'-ACTGGATCCTTAGGGAATAAAATGATTCGGTTCTGCAAC-3'). After gel purification and digestion with NheI and BamHI, the DNA fragment was ligated into pET28b(+) to produce plasmid pMW1010, which expresses *E. faecalis* PBPX [T36-P429] with an N-terminal His_6 tag. The *ef_3129 [T36-P429]* insert was confirmed by sequencing.

Cloning of *S. gordonii* PBPX. The *sgo_1585 [E24-S392]* gene encoding truncated *S. gordonii* PBPX, lacking the N-terminal signal peptide and C-terminal transmembrane helix, was amplified from *S. gordonii* V288 (Challis) genomic DNA using the primers (5'-GTACATATGGAGCTGATGGATATTACTCGG-3') and (5'-ACTGGATCCTTAAGAATCCATAACTGGTGAGTC-3'). After digestion with NdeI and BamHI and gel purification of the digested product, the DNA fragment was ligated into pET28b(+) to produce plasmid pAT1, which expresses *S. gordonii* PBPX [E24-S392] with an N-terminal His_6 tag. The *sgo_1585 [E24-S329]* insert was confirmed by sequencing.

Knockout of *S. pneumoniae murMN*. The *murMN* genes, encoding the enzymes responsible for adding the branch peptide to Lipid II, were deleted via allelic replacement with an erythromycin resistance cassette (*erm*) according to a previously published method.[8] The two ~1 kb regions flanking *murMN* were amplified from *S. pneumoniae* R6 genomic DNA using the primers (5'- TGGCTAGAGCTCGTGTTCA -3') and (5'-TCCTGCCTTTCCTCCCTCTTTCCCAGTAGTACCACTCG -3') for the fragment upstream of *murM* and (5'-GAGAGCACAGATACGGCGGATGAAAAAGTCAGTATTTAGATTTC -3') and (5'-CCTCAATATTAGACTCCGTAACAA -3') for the fragment downstream of *murN*. The *erm* gene was amplified from *S. pneumoniae* D39 Δ*bga::erm* genomic DNA using the primers (5'- GAGGGAGGAAAGGCAGGA -3') and (5'- CGCCGTATCTGTGCTCTC -3'). The three DNA fragments were assembled and amplified by overlap extension PCR.

S. pneumoniae R6 was transformed using the following protocol.[8] Cells were grown to mid-log phase in THY broth and then back-diluted to an OD_{600} of 0.025. This culture was grown to OD_{600} ~0.2 and back-diluted again to an OD_{600} of 0.03 into fresh THY containing 1 mM $CaCl_2$ and 0.2% bovine serum albumin. Competence was stimulated by adding 500 pg/mL competence stimulating peptide (CSP-1, AnaSpec) and the cells incubated for 15 min. To 1 mL of cell suspension, 200 ng of PCR product was added and the resulting culture grown for 1 h. Aliquots of this culture (100 μL) were combined with 5 mL molten 1% NB agar, supplemented with erythromycin, and the mixture poured over fresh TSAII 5% SB plates. Transformants were selected after overnight incubation at 37 °C.

Knockout of *S. gordonii* PBPX. The *sgo*_1585 gene was deleted via allelic replacement with *erm*. The two ~1kb regions flanking *sgo_1585* were amplified from *S. gordonii* genomic DNA using the primers (5'-TTAAAATATGGAAATTTCTATCTTGAC-3') and (5'-TCCTGCCTTTCCTCCCTCAGCCTCCTAATTTTTCATCGT-3') for the fragment upstream of *sgo_1585* and (5'-GAGAGCACAGATACGGCGCCAGCCCTTTTGTAATGTTAGA-3') and (5'-

CATGTTTAGAAGCCAAGGTCAA-3') for the fragment downstream of *sgo_1585*. The two fragments, together with the *erm* gene amplified from *S. pneumoniae* D39 Δ*bga::erm* genomic DNA, were assembled and amplified by overlap extension PCR.

The linear PCR product was inserted into *S. gordonii* V288 (Challis) according to a previously published protocol.[9] An overnight 1 mL culture was back-diluted to 20 mL THB containing 5% heat-inactivated horse serum (THB-HS). This culture was grown for 2 h and the resulting 1 mL culture was diluted again in 20 mL THB-HS and incubated for another 2 h. For transformation, 50 μL of this culture was combined with 450 μL THB-HS and 1 μg PCR product and incubated for 2 h. Transformants were selected by plating the cells on BHI plates, supplemented with erythromycin, and incubating overnight at 37 ºC.

Expression and Purification of LMW PBPs: General Method. *Sa*PBP4, *Ef*PBPX, and *Sg*PBPX were expressed and purified via a previously reported protocol.[6] The appropriate PBP expression plasmid was transferred to *E. coli* BL21(DE3). Overnight cultures were diluted 1:100 into 1 L fresh LB medium supplemented with appropriate antibiotics and grown at 37 °C with shaking until the OD_{600} was 0.4-0.5. The cultures were cooled to 16 °C and protein expression was induced with 500 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 18 h with shaking. Cells were harvested by centrifugation (5,300 × g, 20 min, 4 °C) and the pellet suspended in 30 mL binding buffer (20 mM Tris pH 7.5, 400 mM NaCl) supplemented with 1 mM phenylmethylsulfonylfluoride (PMSF) and 500 μg/mL DNase. Cells were lysed by three passages through a cell disrupter at 10,000 psi and the cell lysate pelleted by ultracentrifugation (90,000 × g, 30 min, 4 °C). The resulting supernatant was supplemented with 40 mM imidazole, added to 1.5 mL washed Ni-NTA resin (Qiagen), and rocked at 4 °C for 40 min. After loading the gravity column, the resin was washed with 20 mL wash buffer (20 mM Tris pH 7.5, 40 mM NaCl, 40 mM imidazole, 0.1% Triton X-100). The protein was eluted in 10 mL of elution buffer (20 mM Tris pH 7.5, 500 mM NaCl, 200 mM imidazole, 0.1% reduced Triton X-100). The eluate was concentrated to ~1 mL using a 30 kD MWCO Amicon Ultra Centrifuge Filter and the final protein concentration was measured via BCA assay. The protein was then diluted to 200 μM in binding buffer (above), aliquoted, and stored at -80 °C.

Bocillin-binding Assay for PBP Folding. Purified PBPs (2.5 μM) were incubated at 37 °C for 1 h with varying concentrations of penicillin-G (PenG) in assay buffer (20 mM potassium phosphate, 140 mM NaCl, pH 7.5) in a total volume of 9 μL. Bocillin-FL (1 μL of a 100 μM stock, ThermoFisher) was added and the proteins incubated for an additional 30 min at 37 °C. The reactions were quenched with 2x loading dye and the proteins separated via SDS-PAGE. The gels were then imaged using a Typhoon scanner at excitation 488 nm and emission 526 nm.

Large Scale Extraction of Lipid II from *E. faecalis*. Lipid II was extracted from 6 L (4 × 1.5 L) of *E. faecalis* MMH594 culture according to a previously published protocol, with modifications.[1a] Overnight cultures of *E. faecalis* MMH594 (10 mL) were grown in brain-heart infusion (BHI) broth. A 10 mL overnight was used to inoculate each 1.5 L of fresh BHI broth and the cultures grown at 37 °C with shaking until the OD_{600} was 0.5-0.6. To accumulate Lipid II, vancomycin and moenomycin A were added at final concentrations of 10 μg/mL and 5

μg/mL, respectively, to each culture and the flasks incubated at room temperature with periodic swirling for 15 min. The cultures were then poured into pre-chilled centrifuge bottles and the cells immediately harvested by centrifugation (3,500 × g, 20 min, 4 °C). The pellets from 6 L of culture were suspended in phosphate-buffered saline (PBS, pH 7.4) at a combined final volume of 30 mL. The cell suspension was then divided equally (15 mL) into two 125 mL Erlenmeyer flasks, each of which contained 52.5 mL chloroform:methanol (1:2). The mixture was stirred vigorously at room temperature for 1 h to lyse the cells. The cell lysate from each Erlenmeyer flask was divided equally between two 50 mL Teflon centrifuge tubes and the insoluble material pelleted at 4,000 × g for 10 min at 4 °C. The supernatants from two Teflon tubes were combined into a 125 mL Erlenmeyer flask containing 30 mL chloroform and 22.5 mL PBS (pH 7.4). The mixture was stirred vigorously (to ensure thorough mixing of layers) at room temperature for 1 h. The pH of the final homogenized suspension was neutralized by dropwise addition of 4 M NaOH and the mixture divided between three 50 mL Teflon tubes. The tubes were centrifuged at 4,000 × g for 10 min at 4 °C to separate the layers and then stored at 4 °C overnight. Upon warming to room temperature, a white layer at the aqueous-organic interface, enriched in Lipid II and Park's nucleotide, was observed in each tube.[1a] The interface layers were carefully isolated from the aqueous and organic layers using a Pasteur pipette, combined, and dried *in vacuo*.

To remove Park's nucleotide, the dried interface layer was dissolved in 15 mL of 6 M pyridinium acetate:*n*-butanol (1:2) and washed with 15 mL *n*-butanol-saturated water. The aqueous layer was removed and re-extracted with 10 mL of 6 M pyridinium acetate:*n*-butanol (1:2). Combined organic layers were washed three times with 10 mL *n*-butanol-saturated water and the final, clean organic layer dried *in vacuo*. The residue was dissolved in a small volume of methanol, transferred to a sample vial (residual pyridinium acetate precipitate was removed by filtration, if present), and the volatiles removed under blowing N_2 gas. The final Lipid II residue was dissolved in a small amount of DMSO and stored at -20 °C. The Lipid II was quantified by amino acid analysis. The estimated yield from this procedure was 200 μg Lipid II per L of culture.

Extraction of Lipid II from *S. pneumoniae* Δ*murMN*. A culture of *S. pneumoniae* Δ*murMN* was grown to mid-log phase in THY at 37 °C in an atmosphere of 5% CO_2. The culture was back-diluted 1:100 into 6 x 0.5 L fresh THY medium and the cultures grown to an OD_{600} of 0.6. To accumulate Lipid II, vancomycin was added at a final concentration of 8 μg/mL and the cultures grown for an additional 20 min. The cells were then pelleted and the Lipid II isolated via the same procedure as for *E. faecalis*, above.

Biotinylation of Lipid II/peptidoglycan and detection via western blot. The protocol for Lipid II biotinylation and detection was adapted from a previously published method.[6] Briefly, 10× reaction buffer (500 mM HEPES, 100 mM $CaCl_2$, pH 7.5), BDL (3 mM), Lipid II (3 μM), and PBP (10 μM) were combined in a final volume of 10 μL (10% DMSO) in an Eppendorf tube. For peptidoglycan biotinylation, Lipid II was first polymerized, under the same conditions, with $SgtB^{Y181D}$ (5 μM) for 10 min before addition of the PBP. Reactions were incubated at room temperature for 1 h and quenched by addition of 10 μL 2× SDS loading dye. The samples were then loaded into a 4-20% gradient polyacrylamide gel and run at 200 V. The products were then transferred onto a PVDF membrane

(BioRad). The membrane was blocked with SuperBlock TBS blocking buffer (ThermoFisher) for 1 h at room temperature and the biotinylated products detected by incubation with a streptavidin-horseradish peroxidase conjugate (Pierce, 1:10,000 in SuperBlock) for an additional hour. Membranes were washed 3 x 10 min with TBST (0.01% Tween 20). Blots were then visualized by incubating the membranes in ECL reagent (ThermoFisher) and exposing to CL-Xposure film (ThermoFisher) or imaging using an Amersham Imager 600 CCD camera.

Lipid II labeling reactions and LC-MS analysis. This procedure was adapted from prior reports.[6, 10] Unless indicated otherwise, Lipid II labeling reaction conditions (optimized) were as follows. For *S. aureus* Lipid II (**1**): 8 μM Lipid II, 20 μM PBP, 25 mM D-amino acid, 1x reaction buffer (12.5 mM HEPES, 2 mM $MnCl_2$, 0.25 mM Tween-80, pH 7.5), total volume 30 μL, 10% DMSO. For *E. faecalis* Lipid II (**2**): 10 μM Lipid II, 10 μM PBP, 10 mM D-amino acid, 1× reaction buffer (50 mM HEPES, 10 mM $CaCl_2$, pH 7.5), total volume 30 μL, 5% DMSO. Reactions with **2** and *Sa*PBP4 were conducted under the same conditions but with 30% DMSO.

Reactions were incubated at room temperature for 1 h. For reactions with *Ef*PBPX and *Sg*PBPX, an additional 10 μM aliquot of PBP was added and the reaction incubated for another 1 h. The reactions were heat quenched at 95 °C for 10 min and allowed to cool to room temperature. SgtB (0.2 μM) was added and the mixture incubated for 30 min at room temperature. To digest the polymerized material, reactions were treated with 4 U mutanolysin (from *Streptomyces globisporus*, Sigma-Aldrich) for 1.5 h at 37 °C followed by another 4 U aliquot with incubation for an additional 1.5 h. Sodium borohydride (10 mg/mL, 30 μL) was added to reduce the cleaved muropeptides and the reactions incubated at room temperature for 30 min. The pH of the solution was then adjusted to ~4 by adding 20% phosphoric acid (approximately 5 μL) and the reactions lyophilized to dryness. The residue was then suspended in 18 μL water and subjected to LC-MS analysis as described below.

LC-MS was conducted with the MS operating in positive ion mode. The muropeptide fragments were separated on a Waters Symmetry Shield RP18 column (5 μm, 3.9 x 150 mm) using the following method: 0.5 mL/min solvent A (H_2O, 0.1% formic acid) for 5 min followed by a linear gradient of 0% solvent B (acetonitrile, 0.1% formic acid) to 40% B over 25 min. Molecular ions corresponding to the expected muropeptide fragments were extracted from the total ion chromatogram.

We note that we observed small decreases in labeling conversion after Lipid II stocks (in DMSO) had been freeze/thawed many times. To minimize this effect, aliquots of Lipid II were prepared at an appropriate working concentration and discarded after approximately 5 freeze/thaws.

MS/MS analysis of Lipid II stem peptides. Lipid II (20 μM) was polymerized with SgtB (0.2 μM) in reaction buffer (50 mM HEPES, 10 mM $CaCl_2$, pH 7.5) at room temperature for 30 min (total volume 30 μL, 10% DMSO). The resulting peptidoglycan strands were digested with mutanolysin and the muropeptide fragments reduced with $NaBH_4$ as above. The pH of the reactions was then adjusted to ~4 by adding 20% phosphoric acid (approximately 5 μL) and the reactions lyophilized to dryness. The residue was then suspended in 18 μL water and subjected to LC-MS analysis. Muropeptides were separated via the same HPLC method as above. MS/MS was conducted with the MS operating in positive ion mode and targeting the $[M]^{+2}$ ions for fragmentation. Ions were fragmented by

collision-induced dissociation (CID) with the collision energy calculated by the MS according to the following formula: CID energy = (slope · m/z) / (100 + offset), where slope = 3.1 and offset = 2.

Radiolabeling of Lipid II. Lipid II (~40 μM) was incubated with [^{14}C]-D-Ala (1.5 mM) and *Ef*PBPX (30 μM) in reaction buffer (12.5 mM HEPES, 2 mM $MnCl_2$, 0.25 mM Tween-80, pH 7.5) at room temperature for 1.5 h (40 μL reaction volume, 20% DMSO). The reaction was quenched by adding 900 μL H_2O/0.1% NH_4OH. The reaction mixture was loaded onto a Bakerbond SPE C18 disposable silica column (J. T. Baker) by gravity flow. The column was washed 6× with 600 μL H_2O/0.1% NH_4OH, 2× with 500 μL H_2O/0.1% NH_4OH/5% methanol, and 2× with 500 μL H_2O/0.1% NH_4OH/10% methanol. The Lipid II was eluted with 3× 500 μL methanol/0.1% NH_4OH. Product yield was estimated by measuring [^{14}C] counts via liquid scintillation and using a standard curve of [^{14}C]-D-Ala to assess the amount of radiolabel incorporated. The elution fractions were then pooled and the solvents removed *in vacuo*. A 40 μM stock of the [^{14}C]-D-Ala-Lipid II was prepared in DMSO.

To generate peptidoglycan oligomers, radiolabeled Lipid II (2 μL) was incubated with SgtB^{Y181D} (5 μM) in reaction buffer (12.5 mM HEPES, 2 mM $MnCl_2$, 0.25 mM Tween-80, pH 7.5) at room temperature for 30 min (20 μL reaction volume, 20% DMSO). The reactions were quenched by heating at 95 °C for 5 min and the solvents removed *in vacuo*. The residue was suspended in 10 μL 2× SDS loading dye and loaded into the wells of a 20 cm × 16 cm 10% acrylamide-Tris gel (prepared using Protogel).[11] The gel was run at 30 mA for 5 h with an anode buffer consisting of 100 mM Tris (pH 8.8) and cathode buffer consisting of 100 mM Tris, 100 mM tricine (pH 8.25), 0.1% SDS.[11] The gels were dried and then exposed to a phosphor screen for 48 h. The autoradiograph was imaged using a Typhoon scanner.

***E. faecalis* and *S. gordonii* peptidoglycan crosslinking assay.** Lipid II extracted from *E. faecalis* (**2**, 40 μM) was polymerized with SgtB (1 μM) in reaction buffer (50 mM HEPES, 10 mM $CaCl_2$, pH 7.5) for 30 min at room temperature (total volume 30 μL, 10% DMSO). *Ef*PBPX or *Sg*PBPX (5 μM) was added and the reaction incubated at room temperature for 1 h. As a positive control for crosslinking, analogous reactions were conducted with a high molecular weight, class A PBP from *E. faecalis*, PBP2a.[3] *E. faecalis* PBP2a (5 μM) was incubated with Lipid II (400 μM) in reaction buffer for 1.5 h at room temperature (total volume 30 μL, 10% DMSO). The reactions were heat quenched at 95 °C for 10 min and allowed to cool to room temperature before digestion with mutanolysin and LC-MS analysis via the same protocol as for Lipid II labeling reactions, above.

Construction of *E. faecalis* Tn::*EFpbpX* and Tn::*murN*. *E. faecalis* strain MMH594[12] was transformed with pZXL5Tet, a tetracycline resistant derivative of the nisin-inducible *mariner* transposon delivery vector pZXL5[13] (see the complementation protocol, below, for the methods for electroporation). Tetracycline-resistant electrotransformants were initially selected at 28 °C in BHI broth supplemented with 5 μg/mL tetracycline and 10 μg/mL chloramphenicol, then diluted 1:1000 into BHI broth pre-warmed to 37 °C, and supplemented with 5 μg/mL tetracycline and 25 ng/mL nisin to induce transposition. Putative *mariner* insertion mutants were recovered, arrayed, and sequenced (Kenneth Beckman, University of Minnesota Genomics Center), to identify insertions in *EFpbpX*

(MMH594 *ukw_2981*::*tet*; termed Tn::*EFpbpX*) and *murN* (MMH594 *ukw_2089*::*tet*; termed Tn::*murN*). Tn::*EFpbpX* possesses the *mariner* transposon between bases 781 and 782 in *ukw_2981*, which would be predicted to truncate the protein in a way that removes 232 of 493 amino acids from the C-terminus. Tn::*murN* possesses the *mariner* transposon insertion between bases 1027 and 1028 in *ukw_2089*, which would be predicted to truncate the protein in a way that removes 83 of 426 amino acids from the C-terminus.

Complementation of *Ef*PBPX. The *ef_3129* gene, along with approximately 150 bp of the upstream promoter region, was amplified from *E. faecalis* V583 genomic DNA using the primers (5'-ATGATTACGAATTCGAGCTCCTGCAATTTTCAAGACTTTCTTGG -3') and (5'-CGACTCTAGAGGATCCTTAGAATAAAAGAAACAAGCCAACG -3'). The purified DNA fragment was ligated into BamHI- and SacI-cut pAT28 via isothermal assembly to generate plasmid pMW1255. The *ef_3129* insert was confirmed by sequencing.

Plasmids were introduced into *E. faecalis* MMH594 Tn::*EFpbpX* via electroporation according to a previously published protocol.[14] To generate electrocompetent cells, 50 mL of BHI broth supplemented with 0.5 M sucrose/8% glycine (w/v) was inoculated with 500 µL of a stationary phase overnight culture of *E. faecalis* Tn::*EFpbpX*. The culture was grown with shaking at 37 °C for 20 h (final OD_{600} 0.9-1.0). The culture was centrifuged at 2,500 × *g* for 20 min at 4 °C and the pellet washed 2× with 40 mL ice cold 0.5 M sucrose/10% glycerol (v/v). After the final wash, cells were suspended in residual buffer, aliquoted, and stored at -80 °C. Before transformation, 50 µL of electrocompetent cells were thawed on ice, 1 µg of plasmid DNA added, and the cells incubated on ice for 30 min. The cells were then transferred to an ice cold 2 mm electroporation cuvette and pulsed at 2 kV. Immediately after pulsing, 1 mL of ice cold BHI with 0.5 M sucrose, 10 mM $MgCl_2$, and 10 mM $CaCl_2$ was added. The suspension was chilled on ice for 5 min and then incubated static at 37 °C for 2 h. Transformants were selected on BHI plates supplemented with 500 µg/mL spectinomycin and 4 µg/mL tetracycline.

Digestion and LC-MS analysis of *E. faecalis and S. gordonii* cell walls. The protocol for isolation and mutanolysin digestion of *E. faecalis and S. gordonii* peptidoglycan was adapted from a previously published protocol.[15] BHI broth or THB (10 mL, supplemented with any required antibiotics) was inoculated with a single colony and the culture grown for at least 16 h at 37 °C. The final culture OD_{600} was normalized to 1.6 and cells from a 2 mL aliquot harvested via centrifugation at 18,000 × *g* for 1 min at room temperature. The cell pellet was suspended in 1 mL 0.1 M Tris (pH 6.8)/0.25% sodium dodecylsulfate (w/v) and boiled at 100 °C for 20 min. The suspension was centrifuged at 18,000 × *g* for 1 min and the pellet washed 3× with 1 mL water. The washed pellet was suspended in 1 mL water and sonicated in a water bath for 30 min. To this suspension, 500 µL 0.1 M Tris (pH 6.8) with 15 µg/mL DNase and 60 µg/mL RNase was added and the mixture incubated at 37 °C with shaking for 2 h. After heat denaturing the enzymes at 95 °C for 5 min, the suspension was centrifuged at 18,000 × *g* for 3 min and the resulting pellet washed 1× with 1 mL water. The pellet was then suspended in 500 µL 1 M HCl and incubated at 37 °C for 4 h. The suspension was centrifuged at 18,000 × *g* for 3 min and the pellet washed with 1 mL aliquots of water until the supernatant pH was > 5. The peptidoglycan pellet was suspended in 100 µL 12.5 mM $NaHPO_4$ (pH

5.5) and 10 μL 4000 U/mL mutanolysin added. The peptidoglycan was digested at 37 °C with vigorous shaking for 16 h. The sample was heated at 95 °C for 5 min to denature the mutanolysin and the undigested/insoluble material pelleted by centrifugation at 18,000 × *g* for 5 min. The supernatant was transferred to a new microcentrifuge tube before adding 50 μL 10 mg/mL $NaBH_4$ and incubating for 30 min at room temperature. The final pH was then adjusted to ~4 with 20% H_3PO_4 before LC-MS analysis.

LC-MS analysis of peptidoglycan digests was conducted with the MS operating in positive ion mode. The muropeptide fragments were separated on a Waters Symmetry Shield RP18 column (5 μm, 4.6 x 250 mm) using the following method: 0.5 mL/min solvent A (H_2O, 0.1% formic acid) for 10 min followed by a linear gradient of 0% solvent B (acetonitrile, 0.1% formic acid) to 20% B over 90 min.

***In vitro* labeling of *E. faecalis* peptidoglycan with Lysine and Boc-Lysine.** To generate *E. faecalis* peptidoglycan, *E. faecalis* Lipid II (**2**, 10 μM) was incubated with SgtB (1 μM) and 10 mM lysine in 1× reaction buffer (50 mM HEPES, 10 mM $CaCl_2$, pH 7.5) for 20 min at room temperature (total volume 30 μL, 5% DMSO). *E. faecalis* PBPX (10 μM) was added and the reaction incubated for 1 hour at room temperature. The proteins were heat denatured at 95 °C for 10 min. Peptidoglycan strands were digested with mutanolysin and samples prepared for LC-MS as in the protocol for Lipid II labeling reactions, above.

ACKNOWLEDGEMENTS

We thank Prof. David Rudner for donating *S. pneumoniae* strains and Josue Flores-Kim for assistance with *S. pneumoniae* culture and genetics. We thank Elizabeth M. Selleck Fiore for technical advice in generating *E. faecalis* transposon mutants. We thank Yuan Qiao and Veerasak Srisuknimit for assistance with Lipid II extraction and technical advice in labeling experiments.

Table S1: Bacterial strains and plasmids used in this study.

Strain or plasmid	Description*	Reference
Enterococcus faecalis		
V583	Wild-type; Van^R	16
MMH594	Wild-type	12
Tn::*EFpbpX*	MMH594 *ukw_2981*::*tet*; Tet^R	This study
Tn::*murN*	MMH594 *ukw_2089*::*tet*; Tet^R	This study
Streptococcus gordonii		
V288 (Challis)	Wild-type	17
Δ*SGpbpX*	V288 Δ*sgo_1585*::*erm*; Erm^R	This study
Streptococcus pneumoniae		
R6	Wild-type	18
D39 Δ*cps* Δ*bgaA*	Δ*cps*, Δ*bgaA*::*erm*; Erm^R	8
Δ*murMN*	R6 Δ*murMN*::*erm*; Erm^R	This study
Escherichia coli		
XL1 blue	*endA1 gyrA96*(nal^R) *thi-1 recA1 relA1 lac glnV44 F'*[*::Tn10 proAB*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] *hsdR17*(r_K^- m_K^+)	Stratagene
BL21(DE3)	*F*$^-$ *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λDE3[*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)	19
Plasmids		
pET28b(+)	IPTG-inducible protein expression vector; Kan^R	Novagen
pMW1010	pET28b(+) carrying His_6-EF_3129[T36-P429], *Ef*PBPX expression vector; Kan^R	This study
pAT1	pET28b(+) carrying His_6-SGO_1585[E24-S392], *Sg*PBPX expression vector; Kan^R	This study
pPBP4[Y21-Q383]	pET28b(+) carrying His_6-PBP4[Y21-Q383], *Sa*PBP4 expression vector; Kan^R	6
pPBP5[D30-N383]	pET22b carrying His_6-PBP5[D30-N383], *Ec*PBP5 expression vector; $Carb^R$	5
pWTS645	pET28b(+) carrying His_6-PBP2a[A68-N728], *E. faecalis* PBP2a expression vector; Kan^R	3
pMgt1†	*S. aureus* SgtB-His_6 expression vector; $Carb^R$	20
pET24bSgtBY181D‡	*S. aureus* $SgtB^{Y181D}$-His_6 (SgtB*) expression vector; Kan^R	4
pAT28	Broad range shuttle vector; $Spec^R$	21
pMW1255	pAT28 carrying *ef_3129*; *Ef*PBPX complementation vector; $Spec^R$	This study
pZXL5Tet⊗	Nisin-inducible *mariner* transposon delivery vector; Cm^R, Tet^R	13

*Abbreviations: $Carb^R$, carbenicillin resistance; Cm^R, chloramphenicol resistance; Erm^R, erythromycin resistance; Kan^R, kanamycin resistance; $Spec^R$, spectinomycin resistance; Tet^R, tetracycline resistance; Van^R, vancomycin resistance.
† SgtB is also known as Mgt1.
‡ $SgtB^{Y181D}$ is abbreviated as SgtB* in the main text.
⊗ Full details of this plasmid will be disclosed in a subsequent publication.

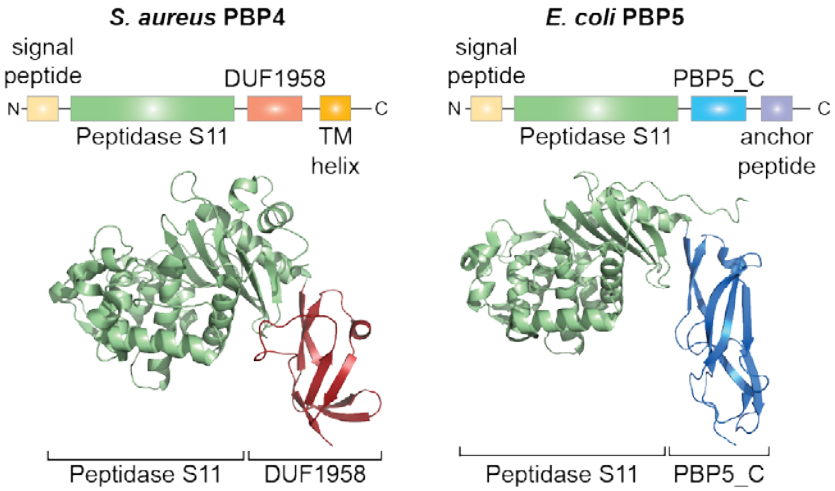


Figure S1: Structure and domain organization of the low molecular weight PBPs *S. aureus* PBP4 (PDB, 1TVF) and *E. coli* PBP5 (PDB, 1NZO). Low molecular weight PBPs are often annotated as carboxypeptidases based on the presence of the Peptidase S11 catalytic domain (Pfam, PF00768).[22] PBPs with the widely distributed PBP5_C domain (Pfam, PF07943) are anchored to the plasma membrane via an amphipathic peptide.[23] In contrast, PBPs with a DUF1958 domain (Pfam, PF09211) are always anchored via a putative 1-2 pass α-helical transmembrane domain.

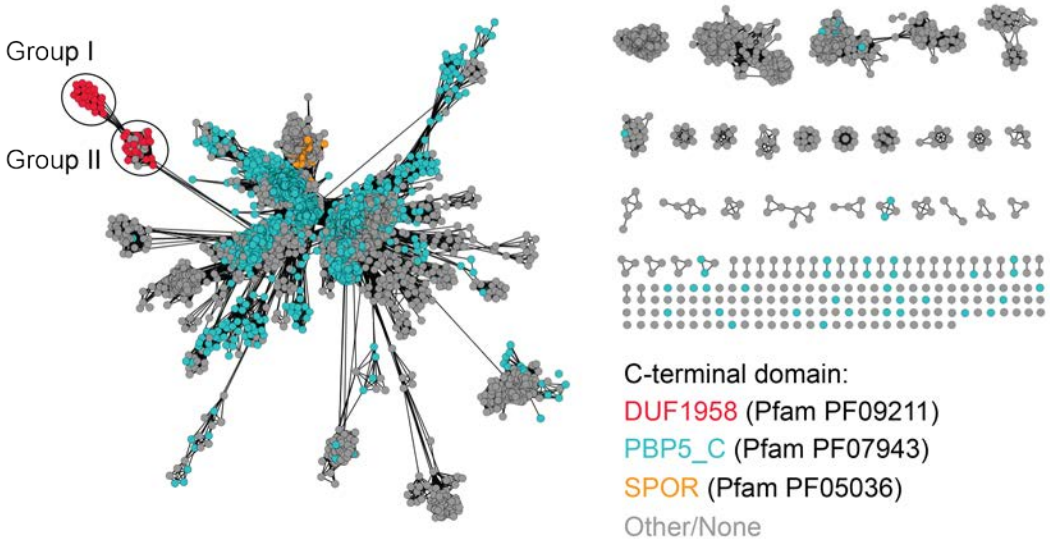


Figure S2: Full sequence similarity network of the Peptidase S11 Pfam family. "Edges" (lines) are drawn between PBP nodes with ≥ 40% sequence identity in the catalytic domain. PBP nodes are colored if they possess an annotated C-terminal domain. Candidate transpeptidases (those containing DUF1958 domains) cluster into two groups. Group I contains staphylococcal PBPs (including *Sa*PBP4), and Group II contains primarily enterococcal/streptococcal PBPs (including *Ef*PBPX and *Sg*PBPX).

We note that groups I and II contain no PBPs with a PBP5_C domain at the C-terminus. PBPs with a DUF1958 C-terminal domain are only found in groups I and II. In building the SSN, our alignment analysis was restricted to only the Peptidase S11 catalytic domain; thus, the PBPs cluster only due to sequence similarity in the catalytic domain (i.e. independently of their C-terminal domain).

Table S2: Bacteria carrying PBPs with a DUF1958 C-terminal domain.[a]

Species	Strain	NCBI Accession Number (Genome Locus Tag)
Enterococcus avium	ATCC 14025	WP_049222131
Enterococcus caccae	ATCC BAA-1240	WP_010770427
Enterococcus faecalis	V583	NP_816737 (*EF_3129*)
Enterococcus gilvus	ATCC BAA-350	WP_010781795
Enterococcus haemoperoxidus	ATCC BAA-382	WP_010761910
Enterococcus malodoratus	ATCC 43197	WP_010743085
Enterococcus moraviensis	ATCC BAA-383	WP_010766468
Enterococcus pallens	ATCC BAA-351	WP_010760028
Enterococcus raffinosus	ATCC 49464	EOH74842
Enterococcus saccharolyticus	ATCC 43076	WP_016173926
Enterococcus silesiacus	LMG 23085	ALS02237 (*ATZ33_12835*)
Granulicatella elegans	ATCC 700633	EEW93669
Granulicatella adiacens	ATCC 49175	WP_005605320
Micrococcus caseolyticus	JCSC5402	BAH16867 (*MCCL_0160*)
Ruminococcus gnavus	AGR2154	WP_024852514
Streptococcus anginosus	C1051	AGU81318 (*SAIN_0565*)
Streptococcus constellatus	subsp. *pharyngis* C818	AGU74190 (*SCR2_0583*)
Streptococcus cristatus	AS 1.3089	AGK71514 (*I872_07150*)
Streptococcus gordonii	V288 (Challis)	ABV11076 (*SGO_1585*)
Streptococcus intermedius	JTH08	BAM23168 (*SCIM_0514*)
Streptococcus parasanguinis	ATCC 15912	AEH55220 (*HMPREF0833_10189*)
Streptococcus salivarius	CCHSS3	CCB93371 (*SALIVB_1083*)
Streptococcus sanguinis	SK36	YP_001034730 (*SSA_0747*)
Streptococcus sinensis	HKU4	WP_037614491
Streptococcus sp. DD10	DD10	KXT74061
Streptococcus sp. DD11	DD11	KXT82656
Staphylococcus aureus	Newman	BAF66884 (*NWMN_0612*)
Staphylococcus carnosus	TM300	CAL27204 (*SCA_0291*)
Staphylococcus haemolyticus	JCSC1435	BAE05563
Staphylococcus hyicus	ATCC 11249	AJC96827 (*SHYC_10495*)
Staphylococcus lugdunensis	N920143	CCB54680 (*SLUG_21560*)
Staphylococcus massiliensis	S46	WP_009384485
Staphylococcus microti	DSM 22147	WP_044360342
Staphylococcus pasteuri	SP1	AGZ26026 (*STP1_1728*)
Staphylococcus pettenkoferi	VCU012	WP_049408040
Staphylococcus pseudintermedius	HKU10-03	ADV04881 (*SPSINT_0352*)
Staphylococcus saprophyticus	ATCC 15305	BAE19222 (*SSP2077*)
Staphylococcus simiae	CCM 7213	WP_002465256
Staphylococcus warneri	SG1	AGC91338 (*A284_10115*)
Vagococcus lutrae	LBD1	WP_023606004

[a] Proteins were identified from the DUF1958 Pfam entry (Pfam PF09211) or via BLAST search using the *S. aureus* PBP4, *E. faecalis* PBPX, or *S. gordonii* PBPX DUF1958 domain as the query sequence.

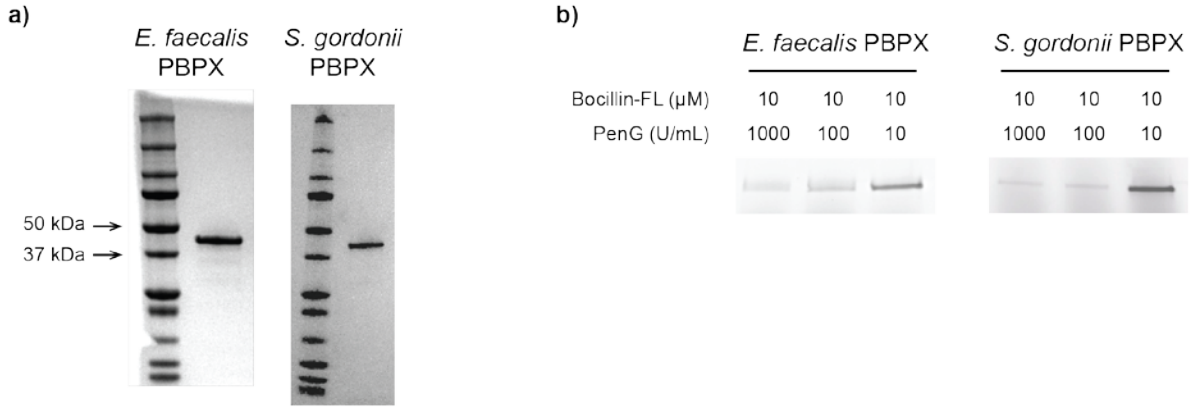


Figure S3: Expression and purification of *E. faecalis* EF_3129 (*Ef*PBPX) and *S. gordonii* SGO_1585 (*Sg*PBPX). (a) Coomassie-stained gels of purified protein with 1 μg total loading. (b) Bocillin-FL-binding assays to confirm proper PBP folding. PBPs (2.5 μM) were incubated with Bocillin-FL and PenG in assay buffer (see Methods). After SDS-PAGE, Bocillin-FL was imaged by fluorescence scanning via Typhoon. The competitive binding of Bocillin-FL indicates that the PBP catalytic domain is folded.

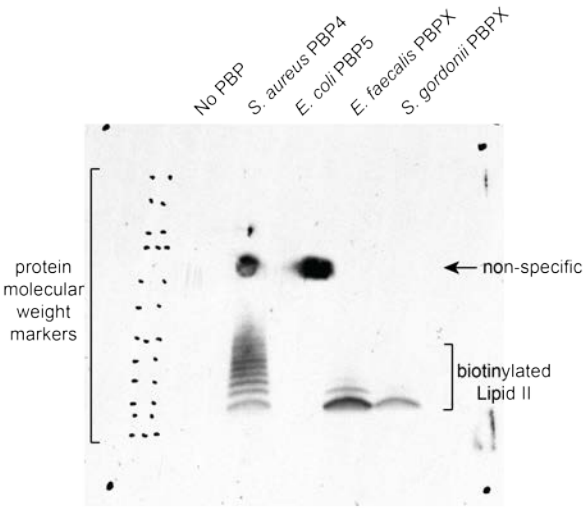


Figure S4: Full western blot from Figure 3a showing biotinylation of *S. aureus* Lipid II. *S. aureus* Lipid II was incubated with BDL and the indicated PBP for 1 h before SDS-PAGE and blotting with streptavidin-HRP.

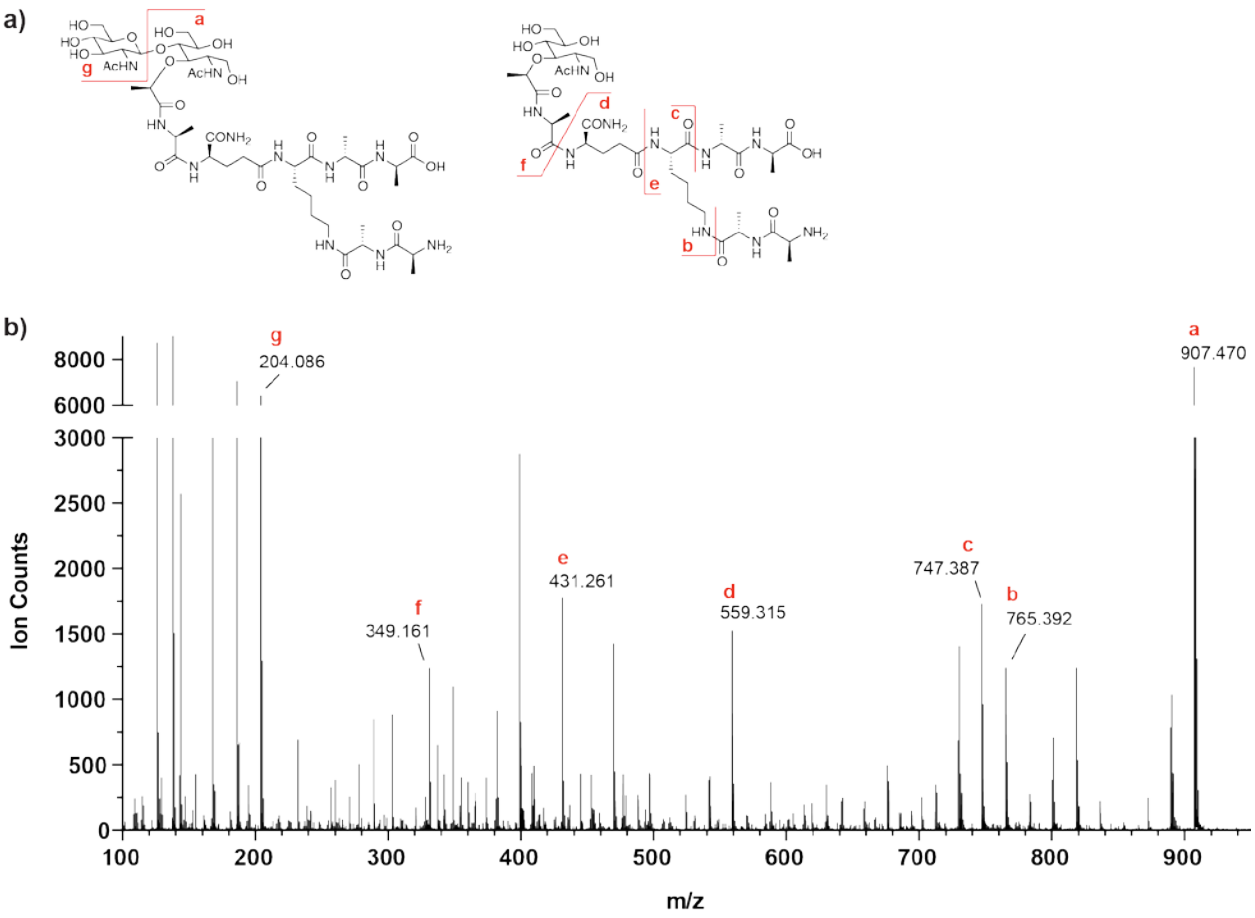


Figure S5: Targeted MS/MS of Lipid II extracted from *E. faecalis* (**2**, Figure 1a). Lipid II was polymerized with SgtB and digested with mutanolysin before LC-MS/MS analysis. The $[M]^{+2}$ ion was targeted for fragmentation.

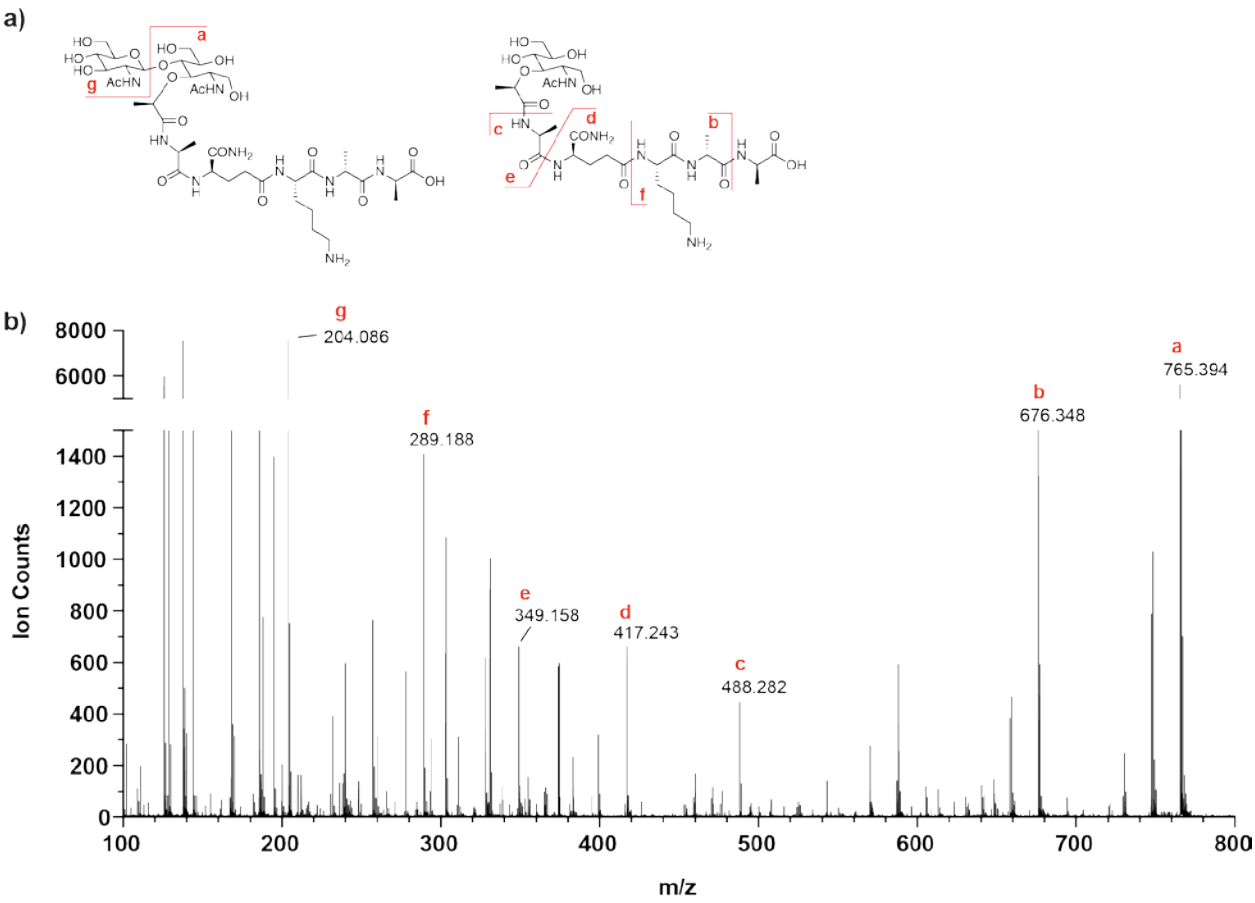
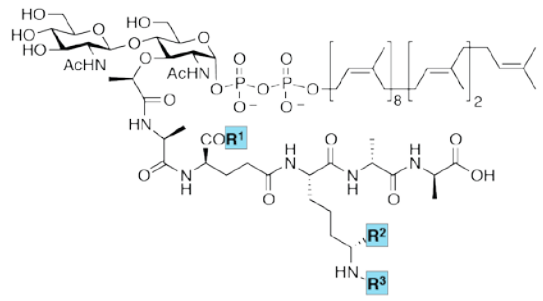


Figure S6: Targeted MS/MS of Lipid II extracted from *S. pneumoniae* Δ*murMN* (**3**, Figure 1a). Lipid II was polymerized with SgtB and digested with mutanolysin before LC-MS/MS analysis. The $[M]^{+2}$ ion was targeted for fragmentation.

a)



Entry	Species	Genotype	R^1	R^2	R^3
1	*S. aureus*	WT	$-NH_2$	-H	Gly_5
2	*E. faecalis*	WT	$-NH_2$	-H	L-Ala-L-Ala
3	*S. pneumoniae*	*murMN*	$-NH_2$	-H	-H
4	Synthetic	–	-OH	-H	-H
5	*E. coli*	WT	-OH	-COOH	-H
6	*B. subtilis*	WT	-OH	$-CONH_2$	-H

b)

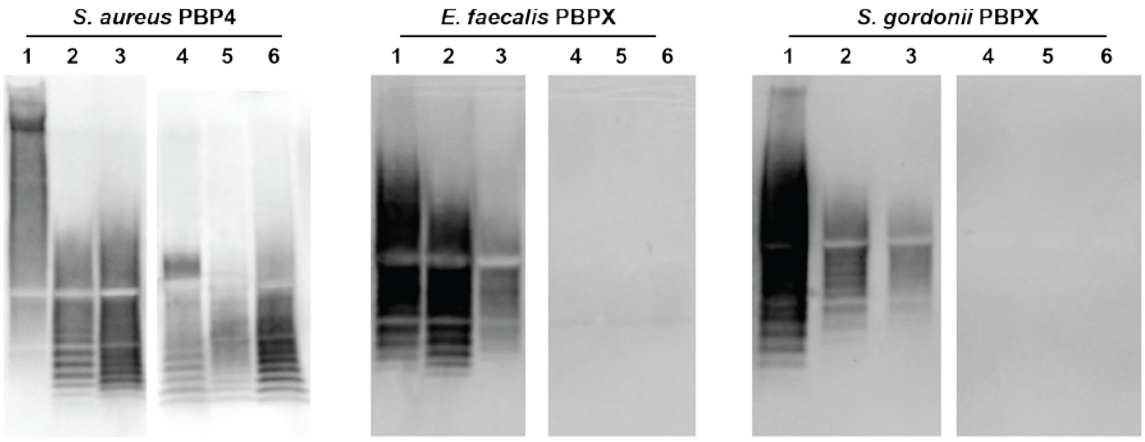


Figure S7: The low molecular weight PBPs are promiscuous toward peptidoglycan substrates. (a) Structures of the Lipid IIs tested. (b) Western blots showing biotinylation of peptidoglycan substrates. Lipid II (3 μM of **1**, **2**, **3** or 20 μM of **4**, **5**, **6**) was polymerized with $SgtB^{Y181D}$ (5 μM) for 30 min before incubation with BDL (3 mM) and the indicated PBP (5 μM). The reaction products were separated via SDS-PAGE and detected by blotting with streptavidin-HRP.

We note that when *S. aureus* Lipid II (**1**) is labeled using *Sa*PBP4, crosslinking of peptidoglycan competes with BDL labeling. As a result, the BDL-labeled peptidoglycan runs higher in the gel and with ill-defined bands.

The starting concentration of Lipid II used in labeling polymer generated from **4**, **5**, and **6** was raised to enable detection via western blot. *Sa*PBP4 will label polymer generated from these substrates, which bear iso-Glu at the second position of the stem peptide, albeit at a lower level than **1**, **2**, or **3**, which contain iso-Gln at the second position. We did not detect biotinylation of polymer generated from **4**, **5**, or **6** using *Ef*PBPX or *Sg*PBPX even at higher Lipid II concentrations.

*Ef*PBPX and *Sg*PBPX also appear to prefer substrates bearing branch residues on lysine (R^3, above), as evidenced by the weaker labeling of **3**, which has no branch, relative to **1** and **2**. These PBPs evidently recognize the stem peptide branch even though these residues are over 8 bonds away from the terminal D-Ala, the site of reactivity.

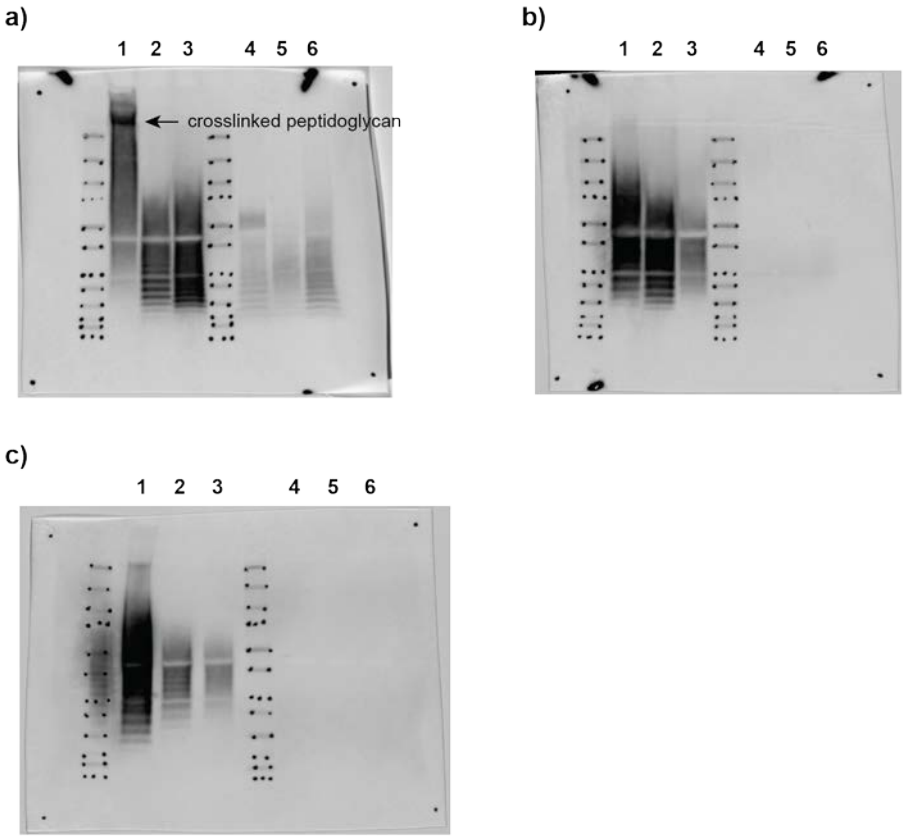


Figure S8: Full western blots from Figure S7 showing biotinylation of peptidoglycan with varying stem peptide structures. Lipid II was labeled using (a) *S. aureus* PBP4, (b) *E. faecalis* PBPX, or (c) *S. gordonii* PBPX. Blots were exposed for 10 s (a) or 1 min (b and c). Lane numbers indicate the Lipid II structure used (see main text Figure 1a or Figure S7).

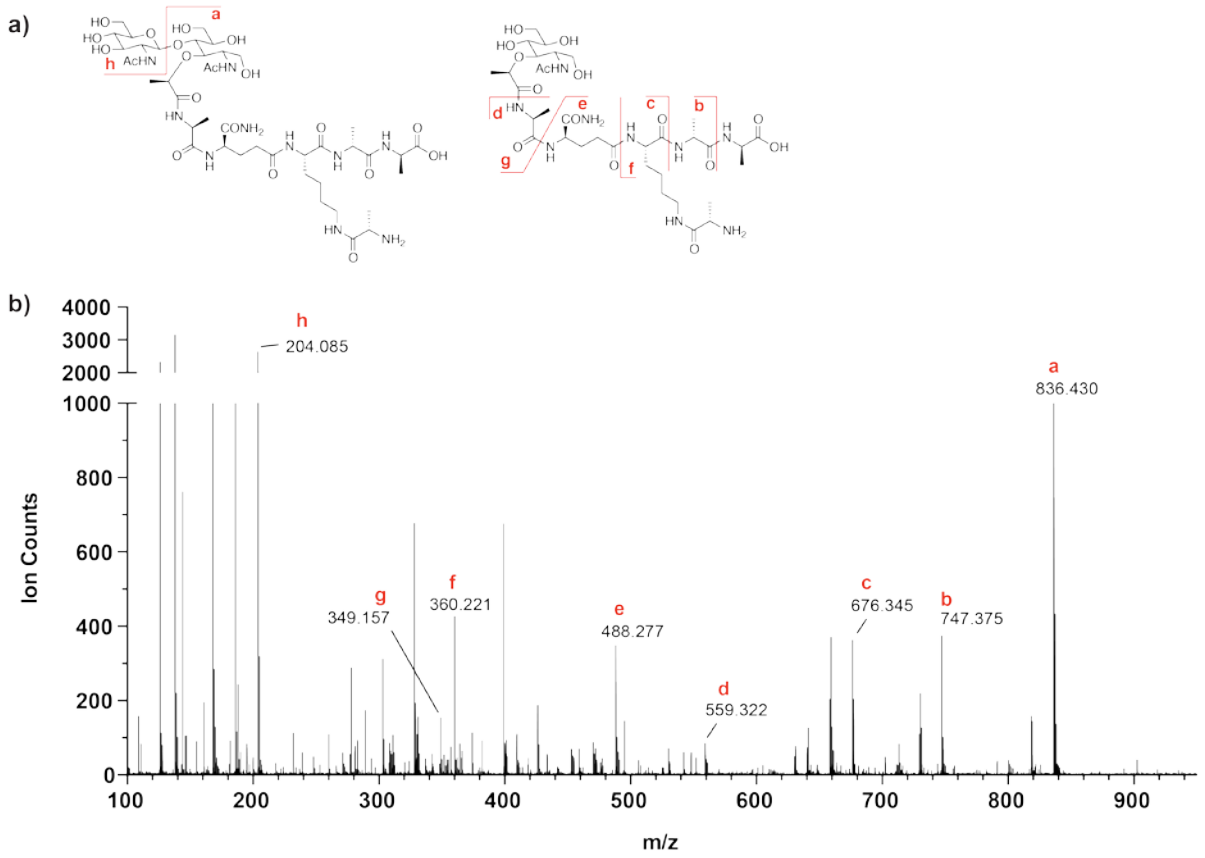
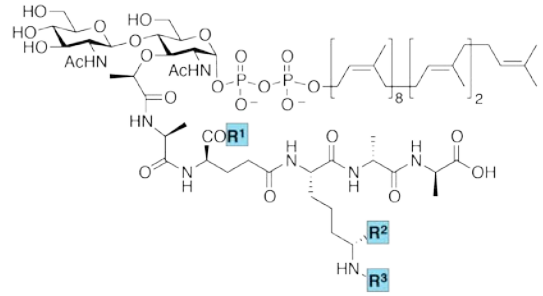


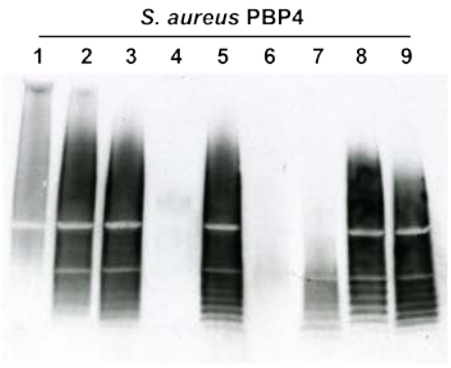
Figure S9: Targeted MS/MS of Lipid II extracted from *E. faecalis* Tn::*murN* (**9**, Figure S10). Lipid II was polymerized with SgtB and digested with mutanolysin before LC-MS/MS analysis. The $[M]^{+2}$ ion was targeted for fragmentation.

a)

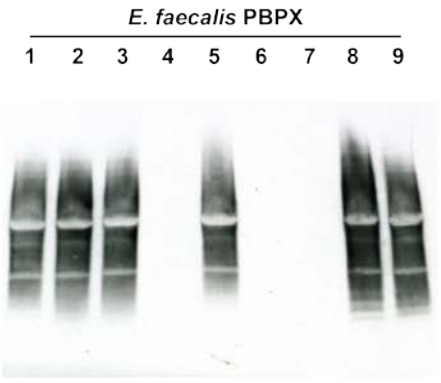


Entry	Species	Genotype	R^1	R^2	R^3
1	*S. aureus*	WT	NH_2	H	Gly_5
2	*S. aureus*	*femB*	NH_2	H	Gly_3
3	*S. aureus*	*femAB*	NH_2	H	Gly
4	Synthetic	–	OH	H	H
5	*S. pneumoniae*	*murMN*	NH_2	H	H
6	*E. coli*	WT	OH	COOH	H
7	*B. subtilis*	WT	OH	$CONH_2$	H
8*	*E. faecalis*	WT	NH_2	H	L-Ala-L-Ala
9	*E. faecalis*	*murN*	NH_2	H	L-Ala

b)



c)



d)

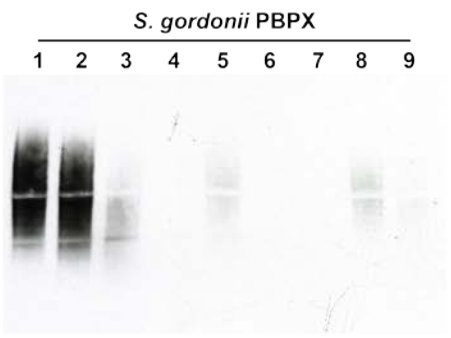


Figure S10: *Sa*PBP4 and *Ef*PBPX can label a variety Lipid II substrates. a) The expanded set of Lipid II variants used to test the substrate scope of the low molecular weight PBP transpeptidases. Note: the compound numbering differs from the main text in this figure. The numbering here applies only to Figure S10. We have previously described the isolation of **2** and **3** from *S. aureus* mutants.[1b] *This substrate is also the native Lipid II in *S. gordonii*. Lipid II (3 μM) was polymerized with $SgtB^{Y181D}$ (5 μM) for 30 min before incubation with BDL (3 mM) and 5 μM of *Sa*PBP4 (b), *Ef*PBPX (c), or *Sg*PBPX (d).

We note that we observed reduced labeling of polymers generated from synthetic (**4**), *E. coli* (**6**), and *B. subtilis* (**7**) Lipid II using *Sa*PBP4 in this experiment because only 3 μM Lipid II was used in the labeling reaction rather than 20 μM as in Figure S7. Higher concentrations of Lipid II were required to detect substantial labeling of these substrates, which contain iso-Glu in the stem peptide, with *Sa*PBP4.

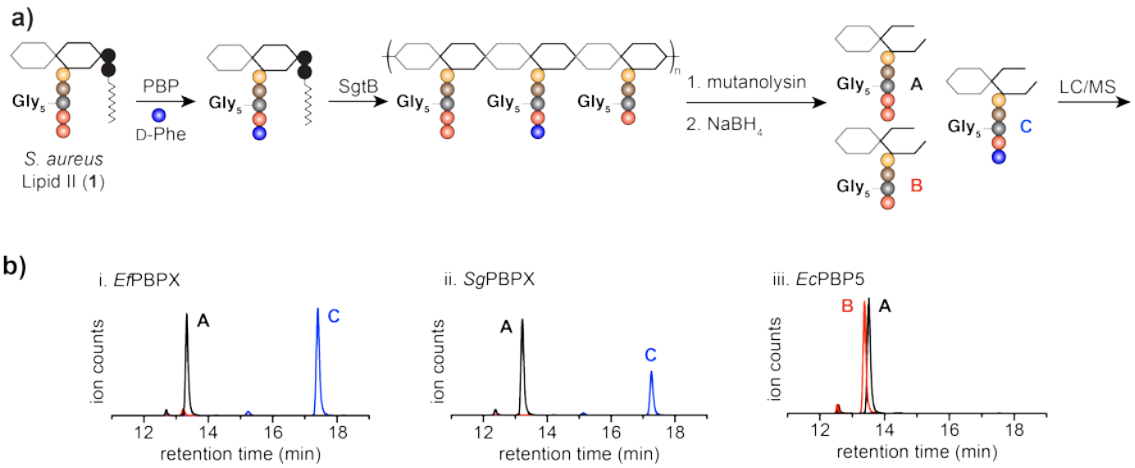


Figure S11: *S. aureus* Lipid II labeling reactions with D-Phe. (a) Schematic of Lipid II labeling reactions. *S. aureus* Lipid II (**1**, Figure 1a, 20 μM) and D-Phe (4 mM) were incubated with 20 μM *Ef*PBPX, 20 μM *Sg*PBPX, or 5 μM *Ec*PBP5 for 1 h. (b) LC-MS extracted ion chromatograms of the D-Phe labeling reactions.

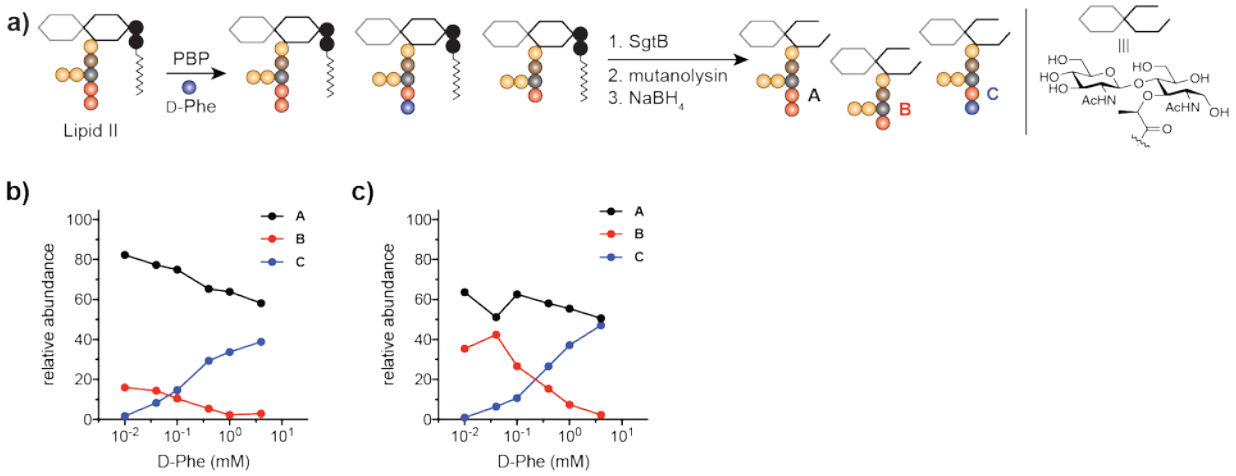


Figure S12: Millimolar levels of exogenous D-amino acid are sufficient to suppress hydrolysis. (a) Schematic of D-Phe labeling reactions. Lipid II extracted from *E. faecalis* (**2**, Figure 1a, 20 μM) was incubated with 5 μM *Ef*PBPX (b) or *Sa*PBP4 (c) and varying concentrations of D-Phe for 1 h. The reaction products were analyzed by LC-MS and the product masses extracted from the total ion chromatogram. Data points indicate the relative amount of each product as determined by integrating the resulting EICs.

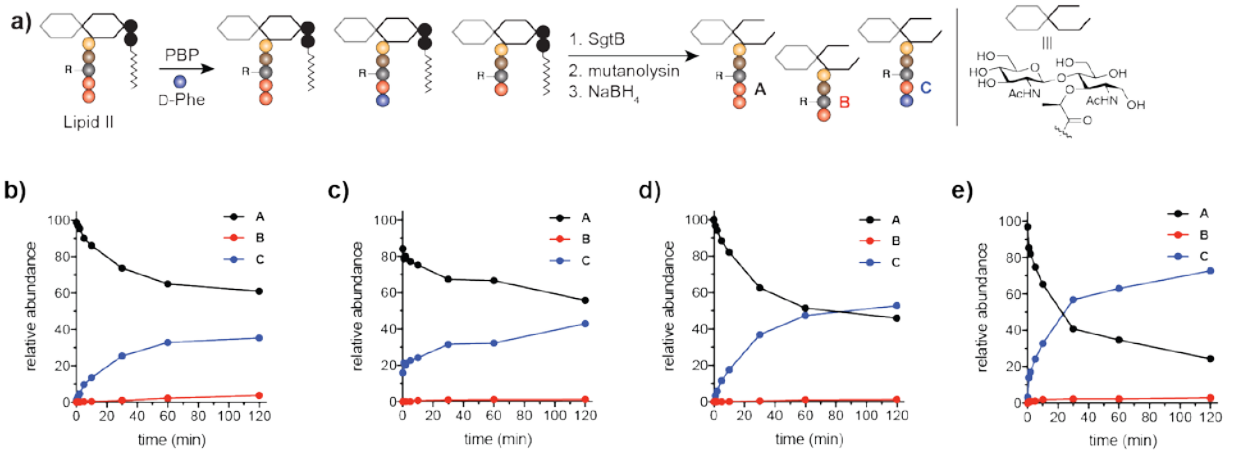


Figure S13. Low molecular weight PBPs rapidly exchange D-Phe into Lipid II. (a) Schematic of D-Phe labeling reactions. Lipid II extracted from *S. aureus* (**1**, Figure 1a, 20 μM) and D-Phe (4 mM) were incubated with 5 μM *Ef*PBPX (b) or *Sg*PBPX (c) for the indicated time. Analogous reactions were performed with Lipid II extracted from *E. faecalis* (**2**, Figure 1a) and *Ef*PBPX (d) or *Sa*PBP4 (e). The reaction products were analyzed by LC-MS and the product masses extracted from the total ion chromatogram. Data points indicate the relative amount of each product as determined by integrating the resulting extracted ion chromatograms.

Table S3: *S. aureus* Lipid II (1) Labeling Reactions[a]

D-amino acid	*E. faecalis* PBPX			*S. gordonii* PBPX		
	pentapeptide	tetrapeptide	+ label	pentapeptide	tetrapeptide	+ label
BDL	ND	ND	ND	ND	ND	ND
D-PG[b]	7	1	92	11	0	89
F_3-D-Ala	8	3	89	3	1	97
D-Ala-d_3	7	0	93	11	0	89

[a] *S. aureus* Lipid II (**1**, Figure 1A, 8 μM) was incubated with PBP (20 μM) and the indicated D-amino acid (25 mM) for 1 h. See Methods for full conditions. Values indicate relative abundance of each product as determined by integrating the LC-MS EICs. ND = not determined
[b] D-propargyl glycine

Table S4: *E. faecalis* Lipid II (2) Labeling Reactions[a]

D-amino acid label	*S. aureus* PBP4			*E. faecalis* PBPX		
	pentapeptide	tetrapeptide	+ label	pentapeptide	tetrapeptide	+ label
BDL	19	1	80	31	4	65
D-PG[b]	39	1	60	36	3	61
F_3-D-Ala	45	0	55	33	9	58
D-Ala-d_3	23	0	77	45	6	49

[a] *E. faecalis* Lipid II (**2**, Figure 1a, 10 μM) was incubated with PBPs (10 μM) and the indicated D-amino acid (10 mM) for 1 h. See Methods for full conditions. Values indicate relative abundance of each product as determined by integrating the LC-MS EICs.
[b] D-propargyl glycine

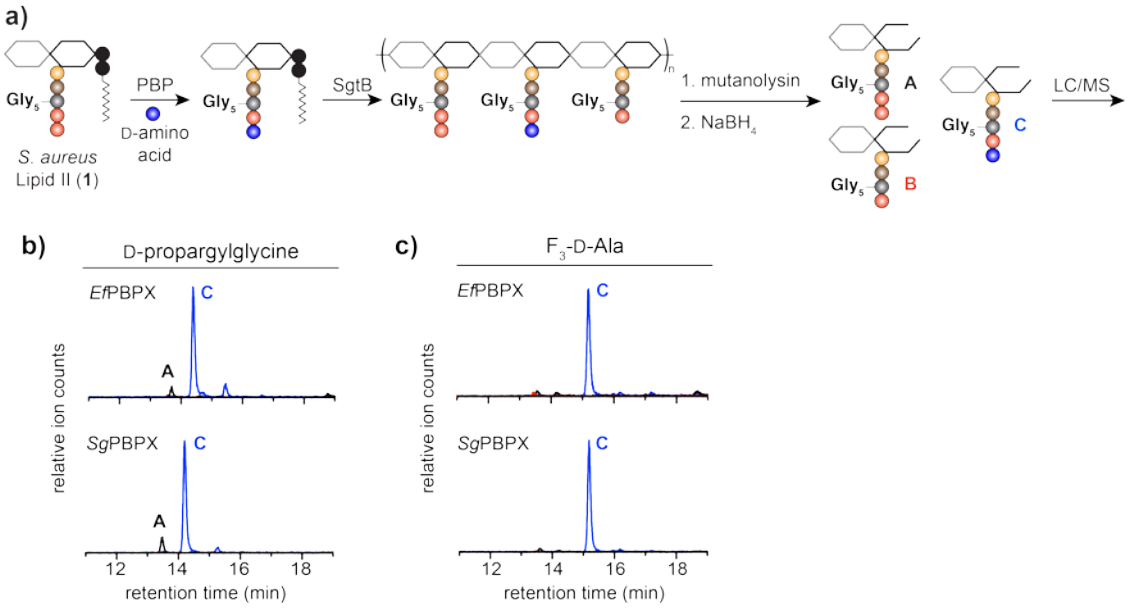


Figure S14: Labeling of *S. aureus* Lipid II (**1**) with D-propargylglycine, and trifluoro-D-Ala. a) Schematic of labeling reactions. See Methods for reaction conditions for each PBP. See Figure S18 for product characterization. LC-MS extracted ion chromatograms of the products of D-propargylglycine (b) and trifluoro-D-Ala (c) labeling reactions.

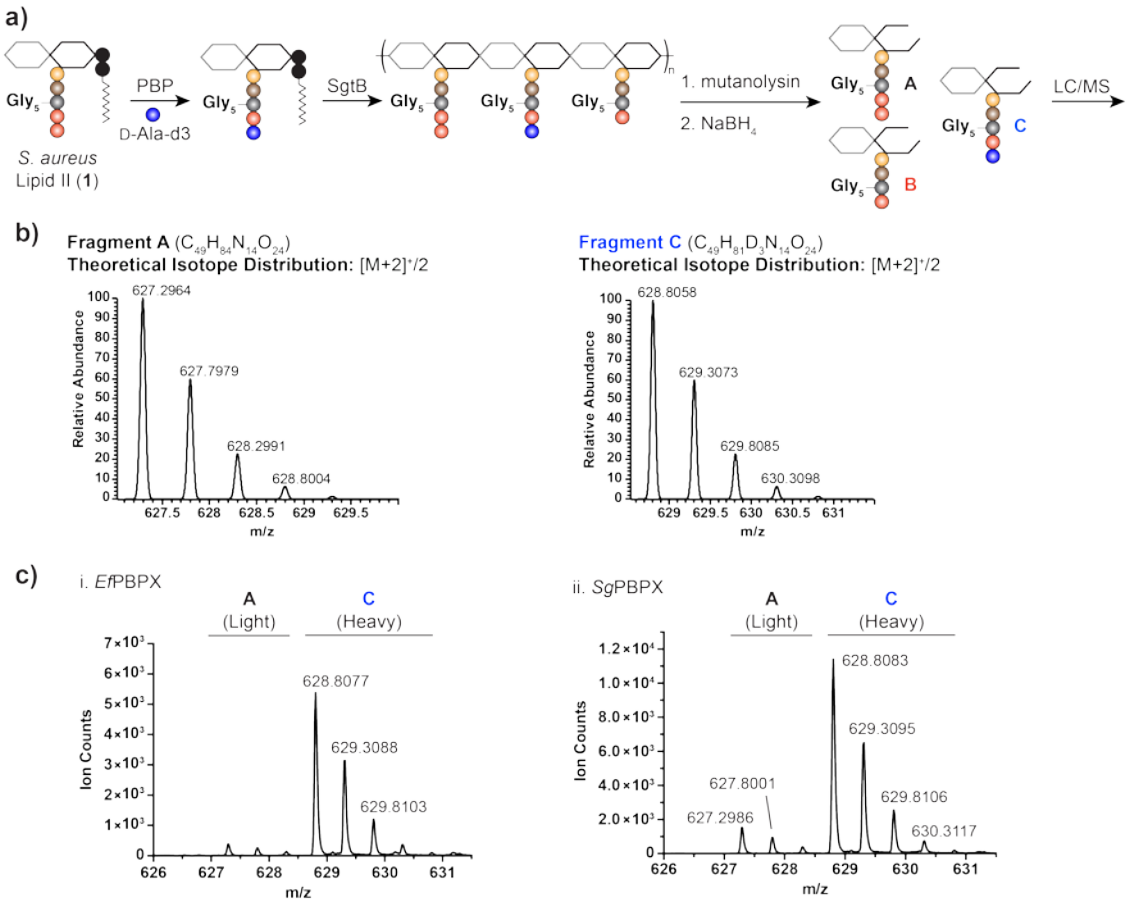


Figure S15: Labeling of *S. aureus* Lipid II (**2**) with D-Ala-d_3. a) Schematic of labeling reactions. See Methods for reaction conditions. b) Theoretical isotope distributions for unlabeled Lipid II (Fragment A) and deuterated Lipid II (Fragment C). c) MS spectra of reaction products after incubation with *Ef*PBPX (i) or *Sg*PBPX (ii).

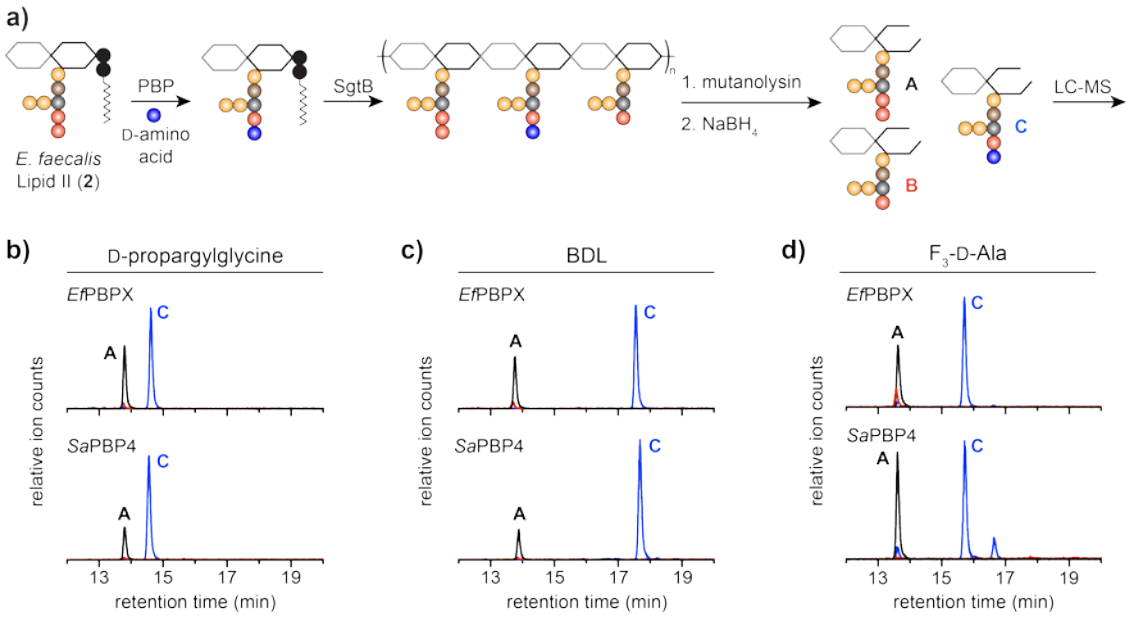


Figure S16: Labeling of *E. faecalis* Lipid II (**2**) with BDL, D-propargylglycine, and trifluoro-D-Ala. a) Schematic of labeling reactions. See Methods for reaction conditions for each PBP. See Figure S19 for product characterization. LC-MS extracted ion chromatograms (EICs) of the products of D-propargylglycine (b), BDL (c), and trifluoro-D-Ala (d) labeling reactions.

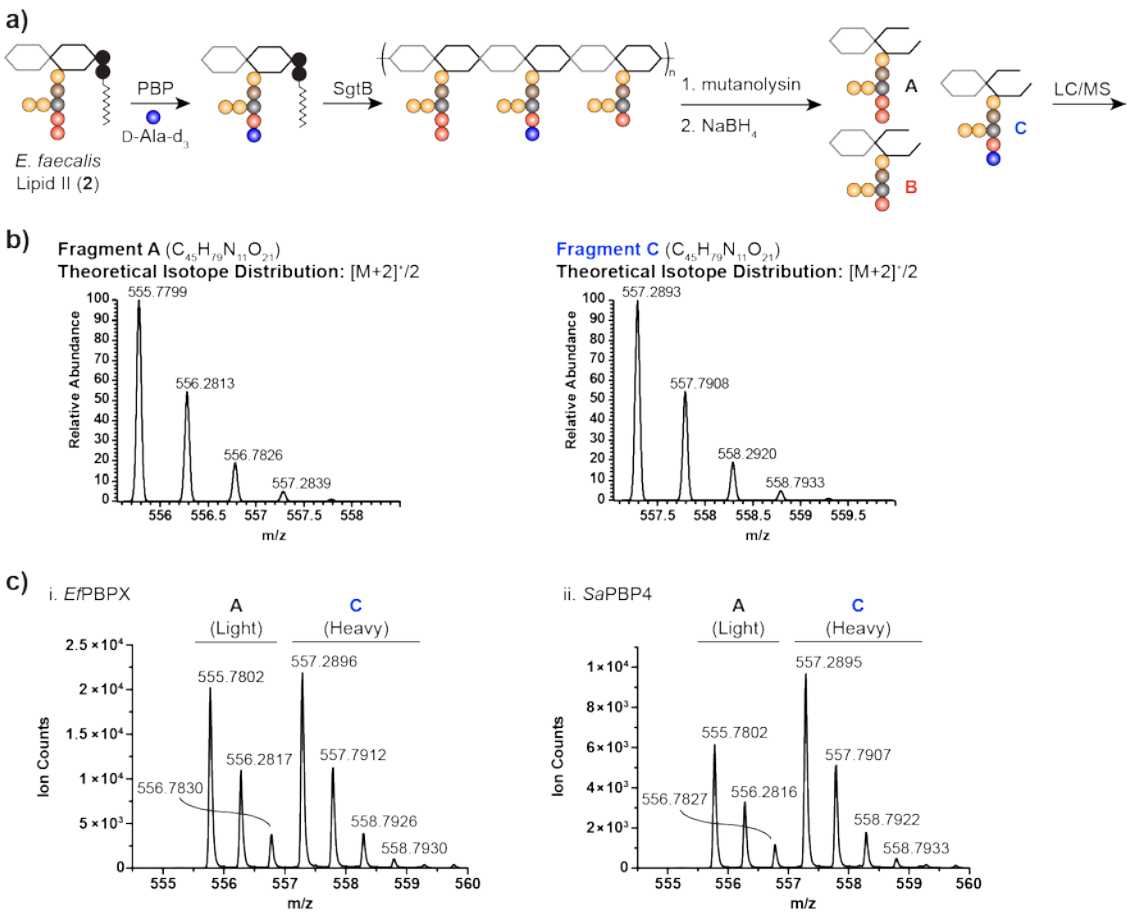


Figure S17: Labeling of *E. faecalis* Lipid II (**2**) with D-Ala-d_3. a) Schematic of labeling reactions. See Methods for reaction conditions for each PBP. b) Theoretical isotope distributions for unlabeled Lipid II (Fragment A) and deuterated Lipid II (Fragment C). c) MS spectra of reaction products after incubation with *Ef*PBPX (i) or *Sa*PBP4 (ii).

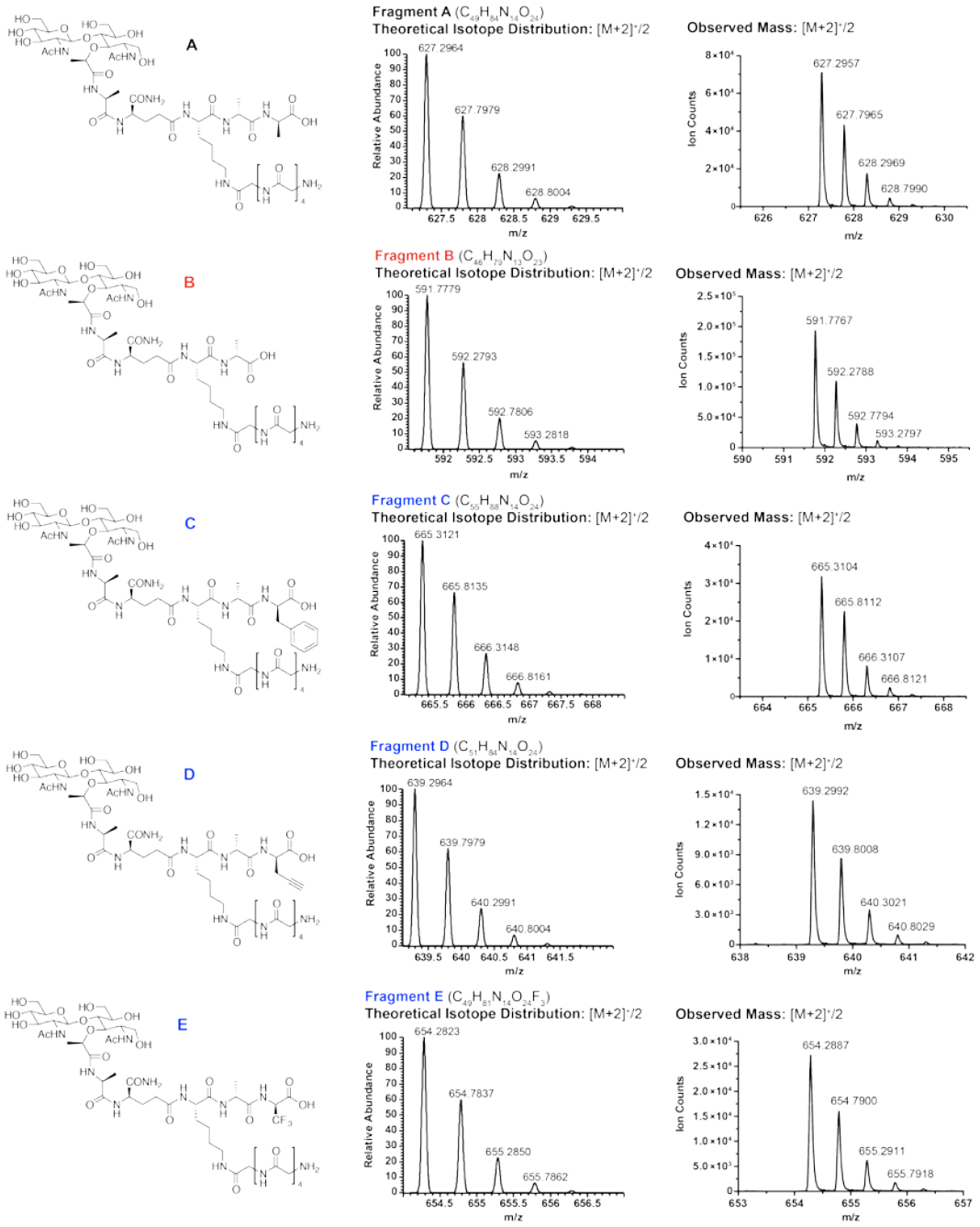


Figure S18: Mass spectra of the muropeptide products of *S. aureus* Lipid II (**1**) labeling reactions.

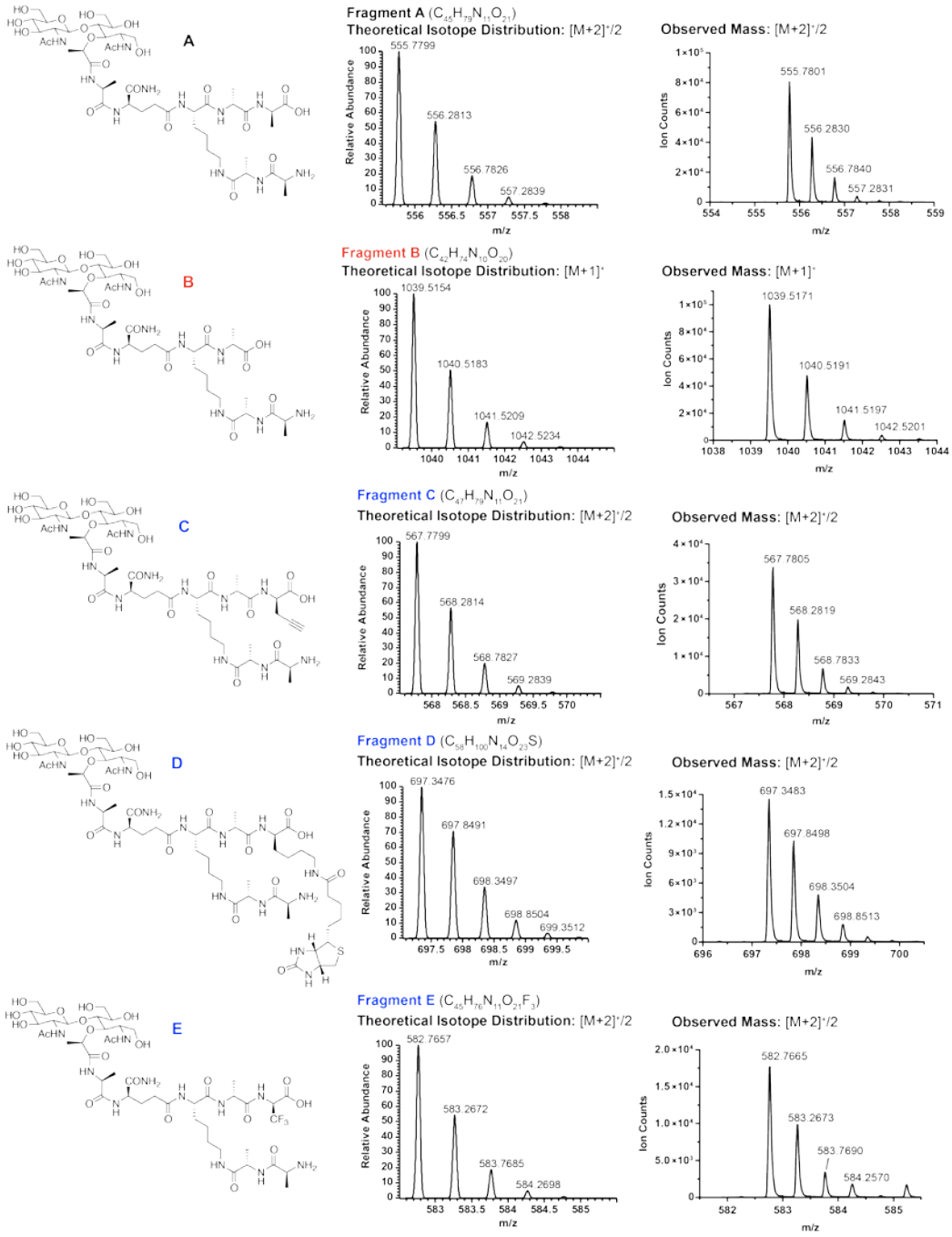


Figure S19: Mass spectra of the muropeptide products of *E. faecalis* Lipid II (**2**) labeling reactions.

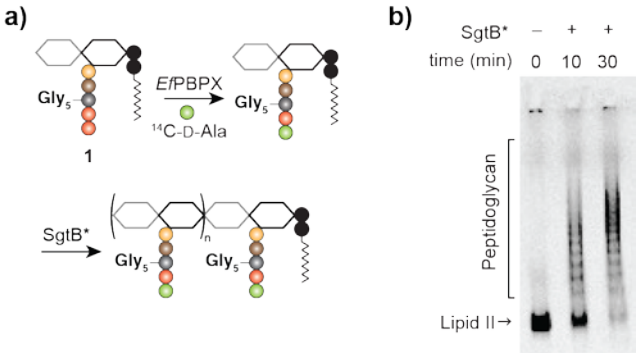


Figure S20: *Ef*PBPX can be used to generate radiolabeled *S. aureus* Lipid II and uncrosslinked peptidoglycan oligomers. a) Schematic of exchange of [^{14}C]-D-Ala into *S. aureus* Lipid II (**1**). See Methods for conditions. Radiolabeled products were purified from the excess [^{14}C]-D-Ala using a C18 gravity column before polymerization with SgtB*. b) PAGE-autoradiograph of labeled Lipid II and peptidoglycan oligomers with repeating disaccharide units ranging from 1 to approximately 10.

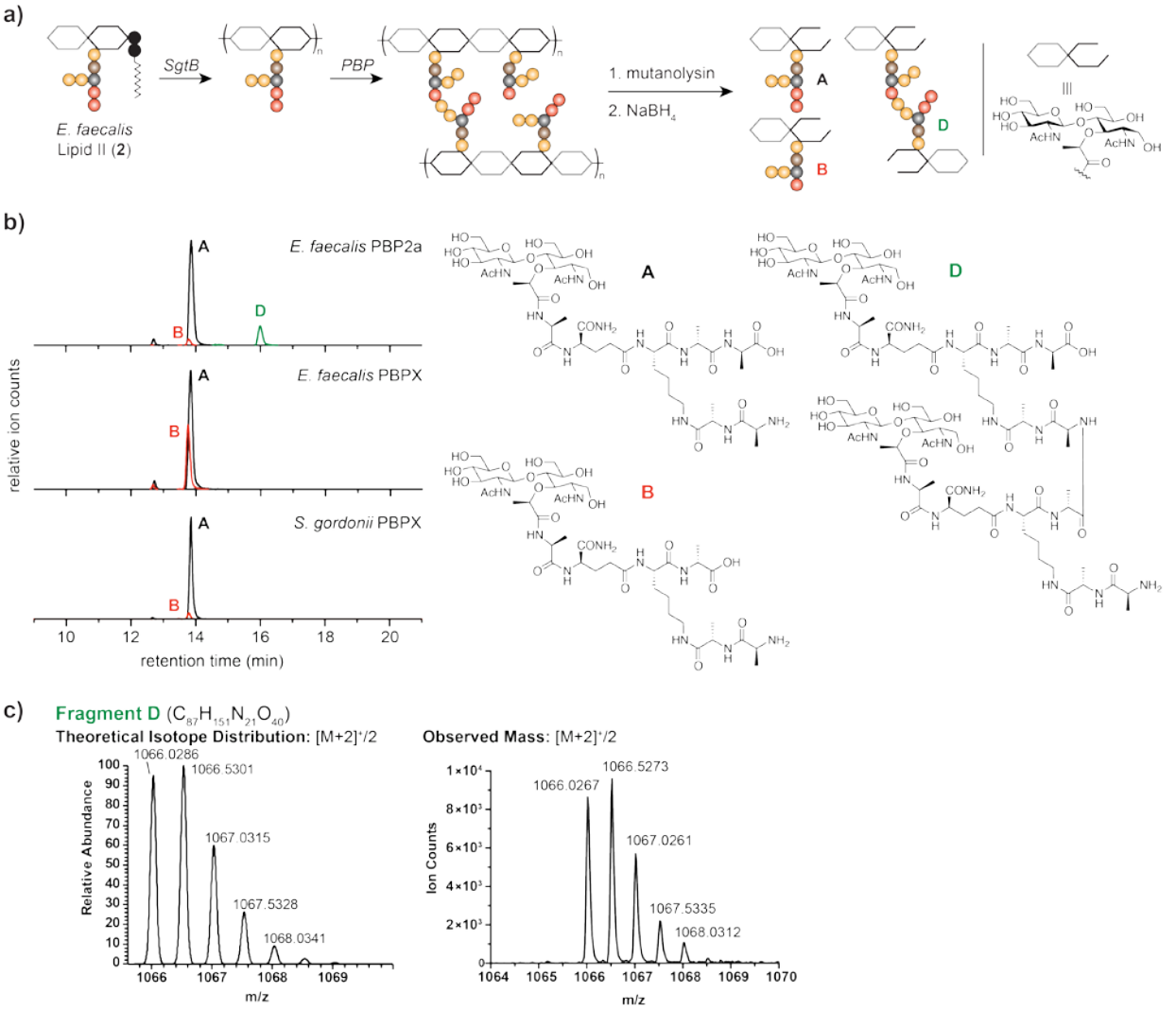


Figure S21: *E. faecalis* PBPX and *S. gordonii* PBPX do not crosslink their native substrate. (a) Schematic of peptidoglycan crosslinking reactions. See Methods for reaction conditions. (b) LC-MS extracted ion chromatograms (EICs) of the reaction products after incubating *E. faecalis* peptidoglycan with the indicated PBP. The structures corresponding the extracted masses are indicated. (c) MS spectrum of the crosslinked product **D**. See Figure S19 for MS spectra of products **A** and **B**.

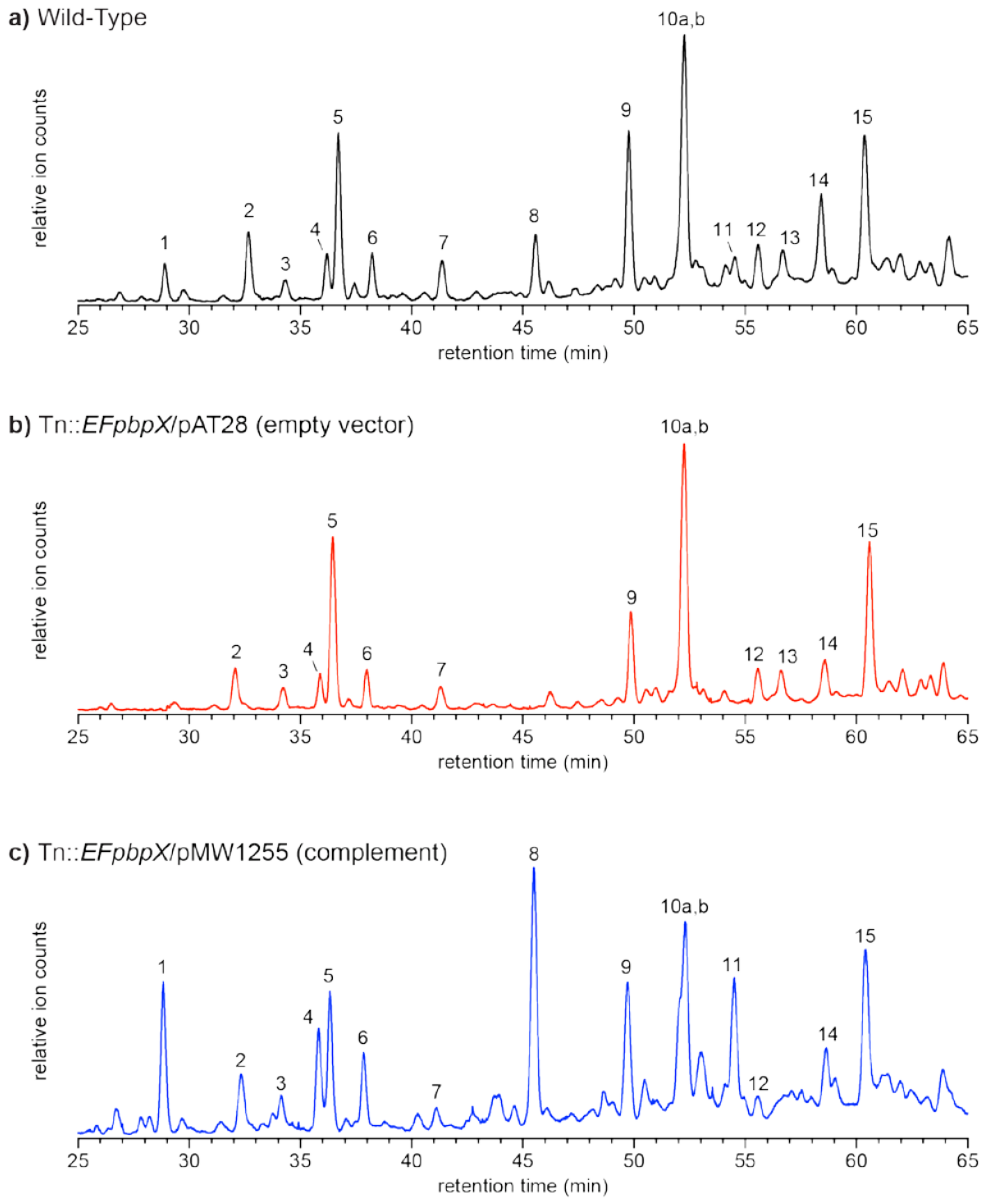


Figure S22: Analysis of *E. faecalis* peptidoglycan indicates that *Ef*PBPX adds lysine to the cell wall. Peptidoglycan was isolated from wild-type *E. faecalis* MMH594 (a), Tn::*EFpbpX*/pAT28 (empty vector) (b), and Tn::*EFpbpX* /pMW1255 (complement) (c) and digested with mutanolysin. Muropeptides were analyzed by LC-MS. See Table S5 for masses and proposed structures of each peak and Table S6 for relative peak areas within the sample.

The only significant change in the cell wall composition in the absence of *Ef*PBPX is the loss of peaks 1, 8, and 11. These peaks are muropeptide monomers, dimers, and trimers, respectively, with masses consistent with exchange of the terminal D-Ala on the stem peptide with lysine (Table S5). We did not observe substantial changes in the relative amount of crosslinking when *Ef*PBPX is deleted (Table S6).

Table S5: Muropeptide masses detected in *E. faecalis* cell wall digests

Peak	Observed Mass (charge)	Order	Proposed Stem Peptide Sequence
1	1167.61 (1), 584.31 (2)	Monomer	L-Ala-D-isoGln-L-Lys-(L-Ala-L-Ala)-D-Ala-**Lys**
2	968.48 (1), 484.74 (2)	Monomer	peak 5 lacking two alanines
3	1039.52 (1), 520.26 (2)	Monomer	isomer of peak 4
4	1039.52 (1), 520.26 (2)	Monomer	L-Ala-D-isoGln-L-Lys-(L-Ala-L-Ala)-D-Ala
5	1110.55 (1), 555.78 (2)	Monomer	L-Ala-D-isoGln-L-Lys-(L-Ala-L-Ala)-D-Ala-D-Ala
6	1110.55 (1), 555.78 (2)	Monomer	isomer of peak 5
7	1111.54 (1), 556.27 (2)	Monomer	L-Ala-**D-isoGlu**-L-Lys-(L-Ala-L-Ala)-D-Ala-D-Ala
8	1094.56 (2), 730.04 (3)	Dimer	crosslink of peaks 1 and 5
9	994.99 (2), 663.66 (3)	Dimer	crosslink of peaks 2 and 5
10a	1030.51 (2), 687.34 (3)	Dimer	crosslink of peaks 4 and 5
10b	1066.03 (2), 711.02 (3)	Dimer	crosslinked dimer of peak 5
11	1070.21 (3), 802.91 (4)	Trimer	crosslink of peaks 5, 5, and 1
12	1066.52 (2), 711.35 (3)	Dimer	crosslink of peaks 5 and 7
13	1066.52 (2), 711.35 (3)	Dimer	isomer of peak 12
14	1505.24 (2), 1003.83 (3), 753.13 (4)	Trimer	crosslink of peaks 5, 5, and 2
15	1576.28 (2), 1051.19 (3), 788.64 (4)	Trimer	crosslinked trimer of peak 5

Table S6: Relative abundance of muropeptide peaks in the *E. faecalis* cell wall[a]

Monomers			Dimers			Trimers		
Peak	**Wild-Type**	**Tn::*EFpbpX***	**Peak**	**Wild-Type**	**Tn::*EFpbpX***	**Peak**	**Wild-Type**	**Tn::*EFpbpX***
1	5	0	**8**	6	0	**11**	2	0
4	6	5	**10a**	28	34	**15**	8	10
5	30	37	**10b**	3	2			
7	5	4	**12**	4	4			
			13	3	4			
Total	46	46	**Total**	44	44	**Total**	10	10

[a]Values were calculated by first extracting the peak mass from the total ion chromatogram, integrating the resulting EIC peak area, and dividing by the sum of all the muropeptide peak areas within each sample. Only peaks that could be unequivocally assigned as monomers/dimers/trimers (not derived from cleavage of crosslinks) were included in the analysis. We also found no reduction in crosslinking in the absence of *Ef*PBPX when total peptidoglycan crosslinking was calculated using the method of Glauner.[24]

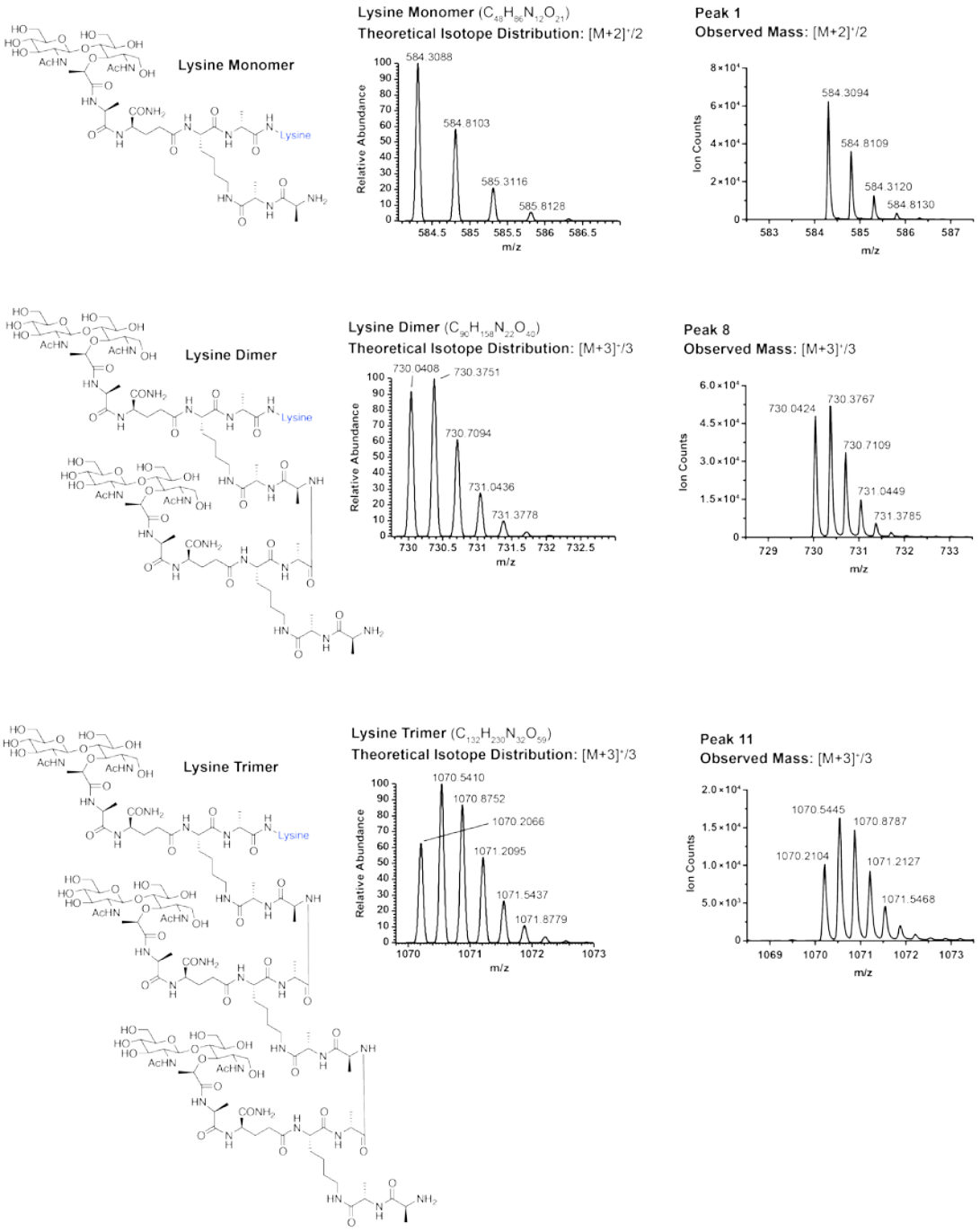


Figure S23. MS spectra of the muropeptide-lysine adducts from *E. faecalis* peptidoglycan. See Figure S22 for total ion chromatograms.

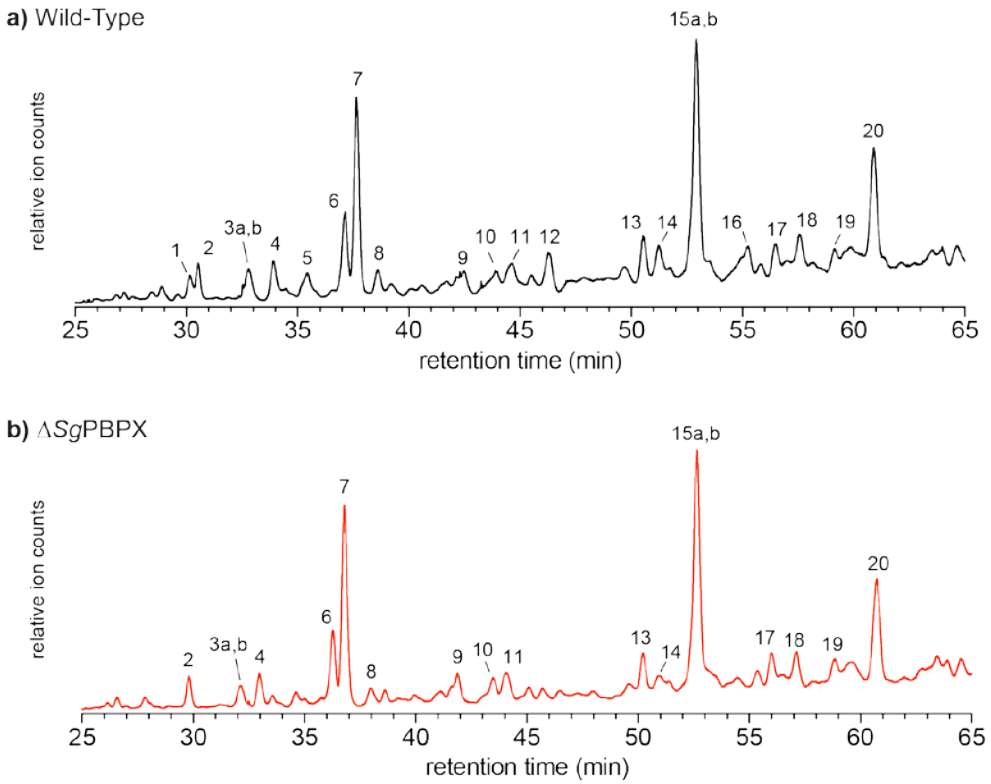


Figure S24. LC-MS analysis of *S. gordonii* peptidoglycan. Peptidoglycan was isolated from wild-type *S. gordonii* V288 (a) and Δ*Sg*PBPX cells (b) and digested with mutanolysin. Muropeptides were analyzed by LC-MS. See Table S7 for masses and proposed structures of each peak and Table S8 for relative peak areas within the sample.

Table S7: Muropeptide masses detected in *S. gordonii* cell wall digests

Peak	Observed Mass (charge)	Order	Proposed Stem Peptide
1	1167.61 (1), 584.31 (2)	Monomer	L-Ala-D-isoGln-L-Lys-(L-Ala-L-Ala)-D-Ala-**Lys**
2	927.45 (1), 464.23 (2)	Monomer	
3a	698.31 (1)		
3b	720.29 (1)		
4	968.48 (1), 484.74 (2)	Monomer	peak 7 lacking two alanines
5	1096.54 (1), 584.77 (2)		
6	1039.52 (1), 520.26 (2)	Monomer	L-Ala-D-isoGln-L-Lys-(L-Ala-L-Ala)-D-Ala
7	1110.55 (1), 555.78 (2)	Monomer	L-Ala-D-isoGln-L-Lys-(L-Ala-L-Ala)-D-Ala-D-Ala
8	969.47 (1), 485.24 (2)		
9	1111.54 (1), 556.27 (2)	Monomer	L-Ala-**D-isoGlu**-L-Lys-(L-Ala-L-Ala)-D-Ala-D-Ala
10	1045.02 (2), 697.02 (3)	Dimer	
11	1066.03 (2), 711.02 (3)		
12	1094.56 (2), 730.04 (3)	Dimer	crosslink of peaks 1 and 7
13	994.99 (2), 663.66 (3)	Dimer	crosslink of peaks 4 and 7
14	1059.02 (2), 706.35 (3)		
15a	1030.51 (2), 687.34 (3)	Dimer	crosslink of peaks 6 and 7
15b	1066.03 (2), 711.02 (3)	Dimer	crosslinked dimer of peak 7
16	1070.21 (3), 802.91 (4)	Trimer	crosslink of peaks 7, 7, and 1
17	1066.52 (2), 711.35 (3)	Dimer	crosslink of peaks 7 and 9
18	1066.52 (2), 711.35 (3)	Dimer	isomer of peak 17
19	1505.24 (2), 1003.84 (3), 753.13 (4)	Trimer	crosslink of peaks 7, 7, and 4
20	1576.28 (2), 1051.19 (3), 788.64 (4)	Trimer	crosslinked trimer of peak 5

Table S8: Relative abundance of muropeptide peaks in the *S. gordonii* cell wall[a]

Monomers			Dimers			Trimers		
Peak	**Wild-Type**	**Δ*SGpbpX***	**Peak**	**Wild-Type**	**Δ*SGpbpX***	**Peak**	**Wild-Type**	**Δ*SGpbpX***
1	4	0	**12**	7	0	**16**	3	0
4	5	5	**15a**	5	5	**20**	5	6
6	11	12	**15b**	21	23			
7	32	39	**17**	2	3			
9	3	5	**18**	2	2			
Total	55	61	**Total**	37	33	**Total**	8	6

[a]Values were calculated by first extracting the peak mass from the total ion chromatogram, integrating the resulting EIC peak area, and dividing by the sum of all the muropeptide peak areas within each sample. Only peaks that could be unequivocally assigned as monomers/dimers/trimers (not derived from cleavage of crosslinks) were included in the analysis. We also found no reduction in crosslinking in the absence of *Sg*PBPX when total peptidoglycan crosslinking was calculated using the method of Glauner.[24]

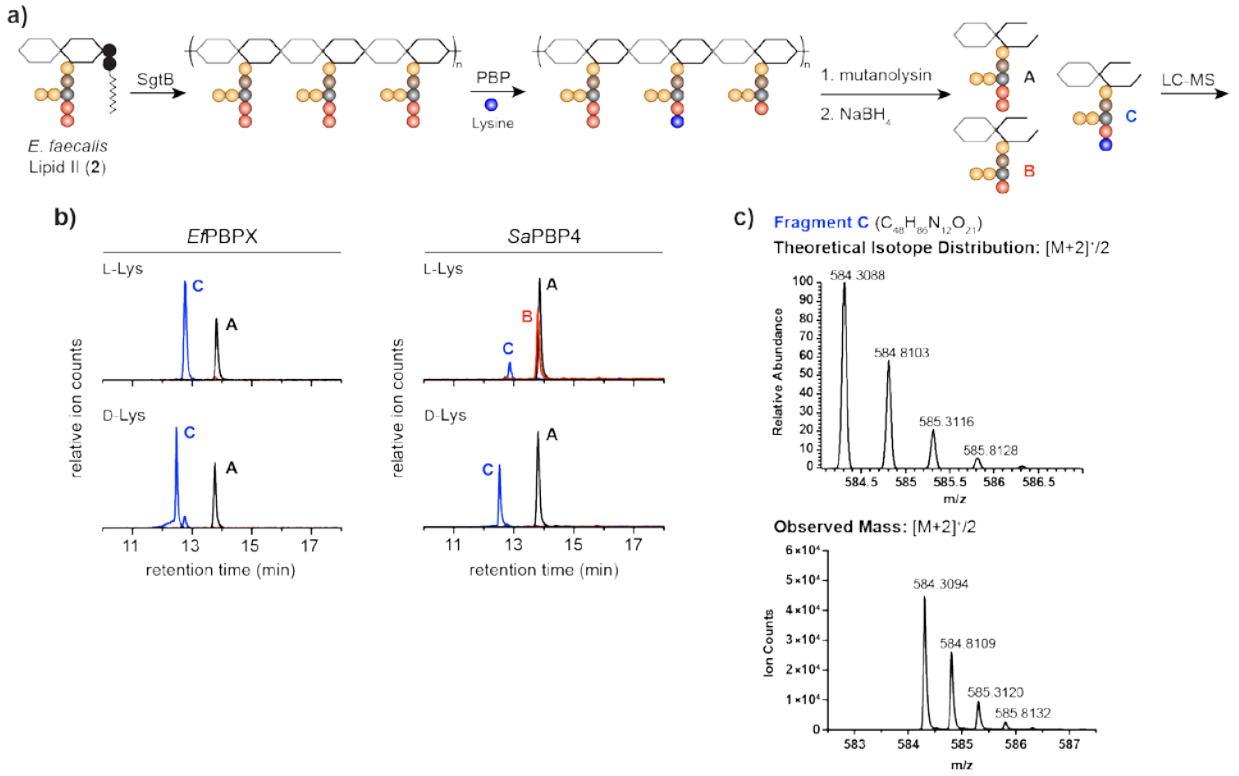


Figure S25: *Ef*PBPX efficiently transfers both D- and L-lysine to *E. faecalis* peptidoglycan *in vitro*. a) Schematic of *E. faecalis* peptidoglycan labeling reactions. See Methods for reaction conditions. b) LC-MS extracted ion chromatograms of the products of D- and L-Lys labeling reactions using *Ef*PBPX or *Sa*PBP4. c) MS spectrum of the lysine-containing product **C**. See Figure S19 for MS spectra of products **A** and **B**.

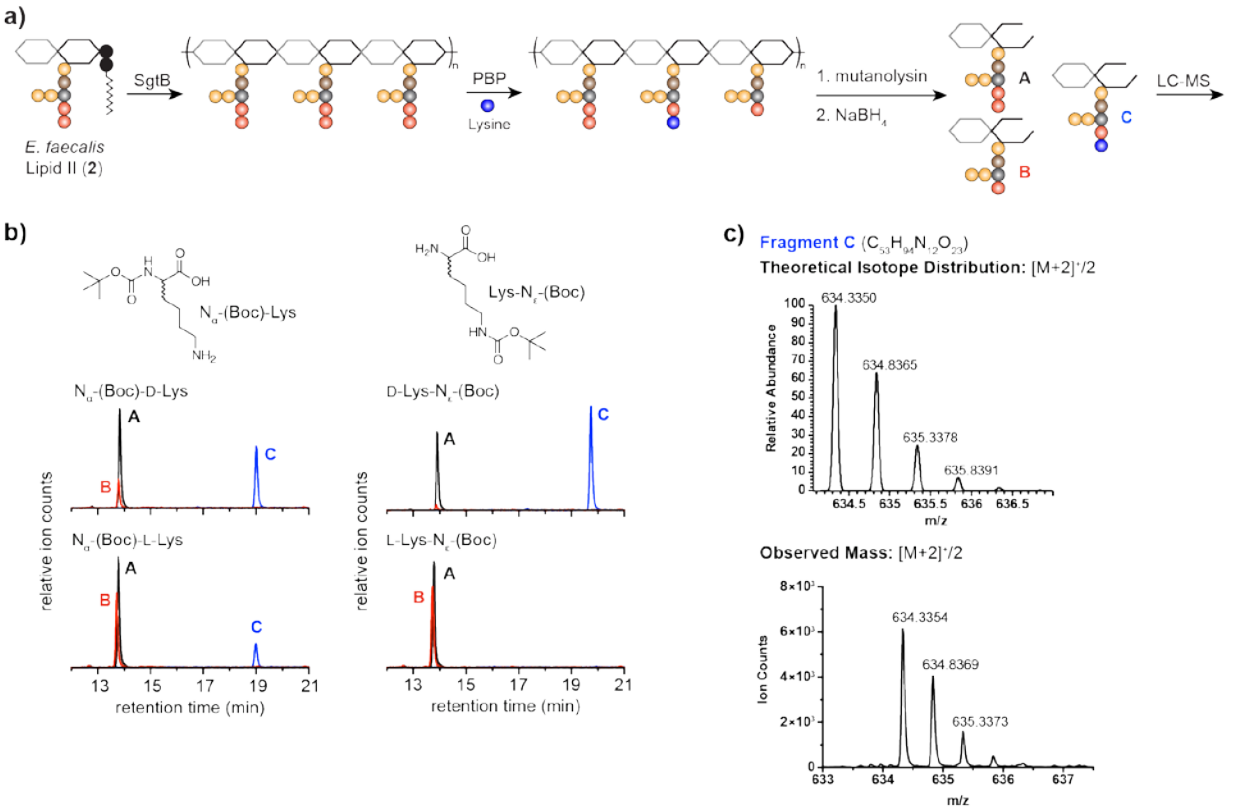


Figure S26: *Ef*PBPX can add Lys through the side chain amine. a) Schematic of *E. faecalis* peptidoglycan labeling reactions. See Methods for reaction conditions. b) LC-MS extracted ion chromatograms of the products of Boc-Lys labeling reactions using *Ef*PBPX. c) MS spectrum of the Boc-Lys-containing product **C**. See Figure S19 for MS spectra of products **A** and **B**.

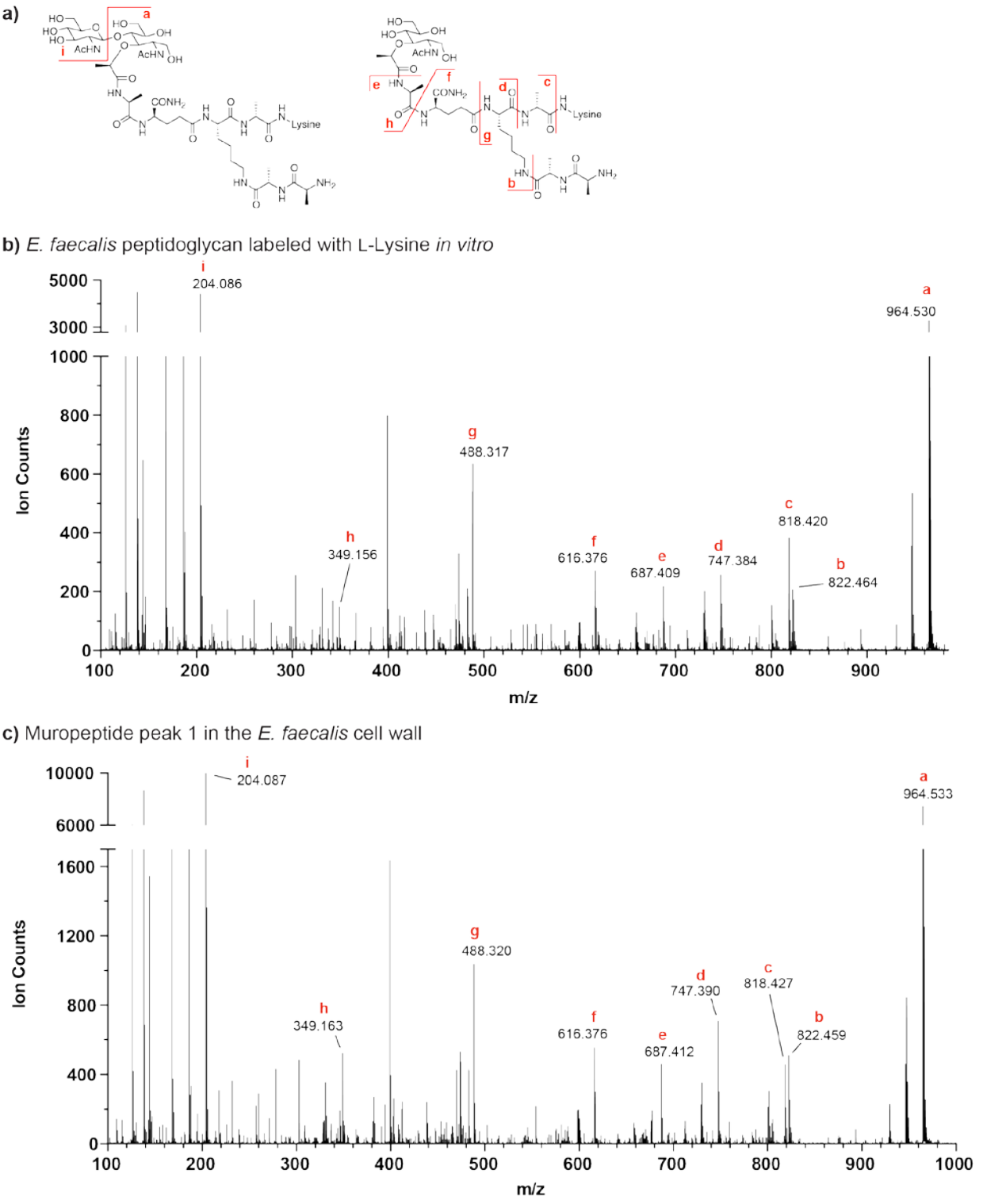


Figure S27: LC-MS/MS analysis of the cell wall-lysine adduct matches a sample prepared *in vitro*. a) Fragmentation pattern observed in MS/MS spectra. b) MS/MS spectrum of the product of *E. faecalis* peptidoglycan, generated from **2**, labeled with L-Lys using *Ef*PBPX. c) MS/MS spectrum of the lysine monomer adduct, peak 1 (see Figure S22), from wild-type *E. faecalis* peptidoglycan.

References

(1) (a) Qiao, Y.; Srisuknimit, V.; Rubino, F.; Schaefer, K.; Ruiz, N.; Walker, S.; Kahne, D. *Nat. Chem. Biol.* **2017,** *13*, 793-798; (b) Srisuknimit, V.; Qiao, Y.; Schaefer, K.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2017**.

(2) (a) Lupoli, T. J.; Taniguchi, T.; Wang, T.-S.; Perlstein, D. L.; Walker, S.; Kahne, D. E. *J. Am. Chem. Soc.* **2009,** *131*, 18230-18231; (b) Tsukamoto, H.; Kahne, D. *Bioorg. Med. Chem. Lett.* **2011,** *21*, 5050-5053.

(3) Wang, T.-S. A.; Manning, S. A.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2008,** *130*, 14068-14069.

(4) Rebets, Y.; Lupoli, T.; Qiao, Y.; Schirner, K.; Villet, R.; Hooper, D.; Kahne, D.; Walker, S. *ACS Chem. Biol.* **2014,** *9*, 459-467.

(5) Lupoli, T. J.; Tsukamoto, H.; Doud, E. H.; Wang, T.-S. A.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2011,** *133*, 10748-10751.

(6) Qiao, Y.; Lebar, M. D.; Schirner, K.; Schaefer, K.; Tsukamoto, H.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2014,** *136*, 14678-14681.

(7) Gerlt, J. A. *Biochemistry* **2017,** *56*, 4293-4308.

(8) Fenton, A. K.; Mortaji, L. E.; Lau, D. T. C.; Rudner, D. Z.; Bernhardt, T. G. *Nat. Microbiol.* **2016,** *2*, 16237.

(9) Yoshida, Y.; Yang, J.; Peaker, P.-E.; Kato, H.; Bush, C. A.; Cisar, J. O. *J. Biol. Chem.* **2008,** *283*, 12654-12664.

(10) Lebar, M. D.; May, J. M.; Meeske, A. J.; Leiman, S. A.; Lupoli, T. J.; Tsukamoto, H.; Losick, R.; Rudner, D. Z.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2014,** *136*, 10874-10877.

(11) (a) Barrett, D.; Wang, T.-S. A.; Yuan, Y.; Zhang, Y.; Kahne, D.; Walker, S. *J. Biol. Chem.* **2007,** *282*, 31964-31971; (b) Wang, T.-S. A.; Lupoli, T. J.; Sumida, Y.; Tsukamoto, H.; Wu, Y.; Rebets, Y.; Kahne, D. E.; Walker, S. *J. Am. Chem. Soc.* **2011,** *133*, 8528-8530.

(12) Huycke, M. M.; Spiegel, C. A.; Gilmore, M. S. *Antimicrob. Agents Chemother.* **1991,** *35*, 1626-1634.

(13) Zhang, X.; Paganelli, F. L.; Bierschenk, D.; Kuipers, A.; Bonten, M. J. M.; Willems, R. J. L.; van Schaik, W. *PLoS Genet.* **2012,** *8*, e1002804.

(14) Cruz-Rodz, A. L.; Gilmore, M. S. *Mol. Gen. Genet.* **1990,** *224*, 152-154.

(15) Kühner, D.; Stahl, M.; Demircioglu, D. D.; Bertsche, U. *Sci. Rep.* **2014,** *4*, 7494-7497.

(16) Sahm, D. F.; Kissinger, J.; Gilmore, M. S.; Murray, P. R.; Mulder, R.; Solliday, J.; Clarke, B. *Antimicrob. Agents Chemother.* **1989,** *33*, 1588-1591.

(17) Macrina, F. L.; Tobian, J. A.; Jones, K. R.; Evans, R. P.; Clewell, D. B. *Gene* **1982,** *19*, 345-353.

(18) Hoskins, J.; Alborn, W. E.; Arnold, J.; Blaszczak, L. C.; Burgett, S.; DeHoff, B. S.; Estrem, S. T.; Fritz, L.; Fu, D. J.; Fuller, W.; Geringer, C.; Gilmour, R.; Glass, J. S.; Khoja, H.; Kraft, A. R.; Lagace, R. E.; LeBlanc, D. J.; Lee, L. N.; Lefkowitz, E. J.; Lu, J.; Matsushima, P.; McAhren, S. M.; McHenney, M.; McLeaster, K.; Mundy, C. W.; Nicas, T. I.; Norris, F. H.; O'Gara, M.; Peery, R. B.; Robertson, G. T.; Rockey, P.; Sun, P. M.; Winkler, M. E.; Yang, Y.; Young-Bellido, M.; Zhao, G.; Zook, C. A.; Baltz, R. H.; Jaskunas, S. R.; Rosteck, P. R.; Skatrud, P. L.; Glass, J. I. *J. Bacteriol.* **2001,** *183*, 5709-5717.

(19) Studier, F. W.; Moffatt, B. A. *J. Mol. Biol.* **1986,** *189*, 113-130.

(20) Heaslet, H.; Shaw, B.; Mistry, A.; Miller, A. A. *J. Struct. Biol.* **2009,** *167*, 129-135.

(21) Trieu-Cuot, P.; Carlier, C.; Poyart-Salmeron, C.; Courvalin, P. *Nucleic Acids Res.* **1990,** *18*, 4296.

(22) Finn, R. D.; Coggill, P.; Eberhardt, R. Y.; Eddy, S. R.; Mistry, J.; Mitchell, A. L.; Potter, S. C.; Punta, M.; Qureshi, M.; Sangrador-Vegas, A.; Salazar, G. A.; Tate, J.; Bateman, A. *Nucleic Acids Res.* **2016,** *44*, D279-85.

(23) Kumarasiri, M.; Fisher, J. F.; Mobashery, S., Penicillin-binding Protein 5 of *Escherichia coli*. In *Handbook of Proteolytic Enzymes*, Elsevier Ltd: 2012; pp 3474-3480.

(24) Glauner, B. *Anal. Biochem.* **1988,** *172*, 451-464.