Supporting Information

Substrate preferences establish the order of cell wall assembly in *Staphylococcus aureus*

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Materials and Methods

Materials. All reagents and chemicals were purchased from Sigma-Aldrich, unless indicated otherwise. UDP-[^{14}C]-GlcNAc was purchased from American Radiolabeled Chemicals, Inc (ARC). Luria-Bertani broth was used to grow Novablue(DE3) and BL21(DE3) *E. coli* strains. Synthetic Lipid II and the synthetic WTA precursor, LI^{WTA}, were synthesized as reported.[1] *S. aureus* wild-type SgtB and SgtB*, containing the point mutation Y181D, were expressed and purified as previously described (Table S4).[2] *S. aureus* PBP4, wild-type PBP2, and a PBP2 construct with a point mutation S398G were expressed and purified as previously described (Table S4).[3] Lipid II was extracted, isolated, and quantified as reported previously.[3a] PCR primers were obtained from Integrated DNA Technologies and Eton Bioscience. Oligosaccharides were purchased from Megazyme. For cloning, gibson master mix was 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. *S. aureus* RN4200 was grown in tryptic soy broth. NovaBlue and BL21(DE3) *E. coli* strains were grown with shaking in Luria-Bertani broth (VWR) at 37 °C and 16 °C. Kanamycin was used at 50 µg/mL.

Instrumentation and Software. Agilent LC/MS chromatograms were obtained on an Agilent Technologies 1100 series LC-MSD instrument using electrospray ionization (ESI). Kinetics and quantitative ligase activity assays were analyzed using Graphpad Prism version 7.0b. Amersham Biosciences Typhoon 9210 instrument was used to visualize autoradiographs, and images were analyzed using ImageJ software. Diffraction data were processed using structural biology software accessed through SBGrid consortium. Data were indexed and integrated using Mosflm[4]

scaled using the CCP4 suite program AIMLESS[5] Phases for crystals of both complexes were calculated by molecular replacement using Phaser[6] with the ΔTM-TagT-octaprenyl-pyrophosphate structure (PBD ascension number 4DE9) as the search model. The structures were iteratively refined and manually improved using phenix.refine[7] and Coot[8] respectively. Restraints for ligands were generated using the AM1 setting in eLBOW[9] and ligands were then placed into unmodeled density early in refinement using Coot. Placement of the ligands and determination of the correct orientation of the sugar moieties was aided by use of the polder maps tool in Phenix.[10] Figures were generated using PyMOL

Methods

Cloning LcpB and TagT constructs

A soluble, wild-type TagT construct lacking the transmembrane helix (ΔTM-TagT construct, amino acids 46-323) was prepared with a C-terminal His_6-tag in a pET28(b)(+) vector as previously reported.[11] To obtain mutant TagT constructs, the plasmid was amplified with a quickchange, primer set as detailed in Table S2. After PCR amplification, the PCR product was digested with Dpn1 in cutsmart buffer (NEB, #R0176S), and then transformed into Novablue *E. coli*. Colonies were sequenced using T7 and T7t primers to confirm the respective single amino acid mutation. For expression, the pET_28_*tagT* (*ywtF*) was transformed into BL21(DE3) *E. coli*. Strain and plasmid table are found in Table S4.

Purification of TagT constructs and co-crystallization with WTA precursors

Wild-type ΔTM-TagT (amino acids 46-323, also annotated as *ywtF*) and the respective mutant TagT constructs were purified using similar conditions previously published,[11] and wild-type TagT was crystallized using similar conditions previously published.[11] An overnight culture

of BL21(DE3) containing pET28b-ΔTM-*tagT*-His_6 was diluted 1:100 into LB media with 50 μg/ml kanamycin and grown at 37 ˚C while shaking at 220 rpm. At OD_{600}=0.6, the temperature was reduced to 16 ˚C and 1mM IPTG was subsequently added to induce protein expression. The culture was grown overnight (~16hrs), and cells were pelleted by centrifugation at 5250x*g* at 4 ˚C for 15 minutes. Cells were resuspended in buffer A (50mM Tris-HCl (pH 8), 300 mM NaCl, and 10 mM imidazole), supplemented with 10 μg/ml lysozyme, and 100 μM PMSF. The cell suspension was mixed using an Ultra Turrax T10 homogenizer, and then lysed by 4x passage through a cell disruptor (EmulsiFlex C3, Avestin) at 15,000 psi. Following lysis, cell debris were pelleted at 8,500*g* for 8 minutes at 4˚C and the supernatant (cell lysate) then clarified by centrifugation at 100,000*g* at 4˚C for 30 minutes. The clarified lysate was then rocked for 30 minutes at 4˚C with 2.5 mL Ni-NTA Superflow resin (Qiagen) beads pre-equilibrated with the resuspension buffer. After flowing through the supernatant, the nickel was washed with 40 ml resuspension buffer. Protein was eluted with 5 mL of the resuspension buffer except with 250 mM imidazole. After concentrating with a 10-kDa MWCO Amicon concentrator, the protein was purified by size-exclusion chromatography with a Superdex S75 10/300 GL using an ÄKTA Pure system (GE Healthcare). Fractions corresponding to monomeric ΔTM-TagT were collected and concentrated to 25 mg/ml again using a 10-kDa MWCO Amicon concentrator.

To obtain co-crystals of the wild-type, ΔTM-TagT with WTA precursor, purified, concentrated ΔTM-TagT was aliquoted and used to resuspend 2x molar excess of dried LI^{WTA} or LII_A^{WTA}, and protein and lipid-linked substrate were co-incubated on ice for 1-2 hours. Crystals were obtained by mixing 1 µL of protein solution in sitting drops with 1 µL of reservoir containing 100 mM HEPES pH 7.0-7.5, 20-22% w/v PEG 3350, and 200 mM $MgCl_2$. Although large, well diffracting crystals of apo ΔTM-TagT were readily obtained and reproducible, ΔTM-

TagT-LI^{WTA} and ΔTM-TagT-LII$_A^{WTA}$ complexes tended to form small, irregularly shaped crystals, or did not nucleate at all. Streak seeding from apo crystals into drops with ΔTM-TagT-substrate complexes improved crystal morphology and later diffraction quality. Crystals were after harvested 1-2 weeks of growth, cryoprotected in reservoir solution supplemented with 10% w/v PEG400, and then flash frozen in liquid nitrogen. X-ray diffraction data were collected at wavelength 0.97918Å on beamlines 24-ID-C and 24-ID-E of the Advanced Photon Source at Argonne National Laboratory. Both ΔTM-TagT-LI^{WTA} and ΔTM-TagT-LII$_A^{WTA}$ containing crystals belonged to space group $P4_12_12$ and had similar unit cell dimensions (Table S5).

Diffraction data were processed using structural biology software accessed through the SBGrid consortium.[12] Data were indexed and integrated using Mosflm[4] scaled using the CCP4 suite program AIMLESS[5] Phases for crystals of both complexes were calculated by molecular replacement using Phaser[6] with the ΔTM-TagT-octaprenyl-pyrophosphate structure (PBD ascension number 4DE9) as the search model. The structures were iteratively refined and manually improved using phenix.refine[7] and Coot[8] respectively. Restraints for ligands were generated using the AM1 setting in eLBOW[9] and ligands were then placed into unmodeled density early in refinement using Coot. Placement of the ligands and determination of the correct orientation of the sugar moieties was aided by use of the polder maps tool in Phenix.[10] Figures were generated using PyMOL.[26]

Preparation of radiolabeled LII$_A^{WTA}$

LI^{WTA}, hexaprenyl-pyrophospho-GlcNAc, was prepared previously from published chemical routes.[13] LI^{WTA} (100 μM) was incubated with TagA (15 μM), UDP-[^{14}C]-GlcNAc (specific activity= 300 nCi nmol^{-1}), and MnaA (3 μM) in previously described conditions.[14]

LII_A^{WTA} was purified using a Bakerbond™ spe Octadecyl (C18) disposable column (Catalog number # 7020-01). The C18 column was equilibrated with 2x 750 μl MeOH/0.1% NH_4OH and 2x 750 μl H_20/0.1% NH_4OH. The column was washed 4x with 500 μl H_2O/0.1% NH_4OH and eluted with 3x 500 μl MeOH/0.1% NH_4OH. Product yield was obtained by measuring [^{14}C] counts using a liquid scintillation counter (Beckman, LS6500), and using a standard curve of the UDP-[^{14}C]-GlcNAc to assess product conversion.

LcpB reactions with native PG polymers for PAGE autoradiography

Native Lipid II (20 μM; DMSO) was incubated with SgtB* (800 nM) in 1X TGase buffer (50 mM HEPES (pH 7.5), 10 mM $CaCl_2$) and 20% DMSO in a large batch. The reaction was mixed by flicking the tube and spinning it down to avoid any loss of material. After a 30 minute incubation at room temperature, SgtB* was subsequently heat-inactivated (10 minutes at 95 ˚C). The batch of peptidoglycan oligomers were split in two; to obtain crosslinked PG, ΔTM-PBP4 (4 μM) was added to one portion and incubated for an additional 1 hour. The PBP4-reaction was then heat-inactivated (10 minutes at 95 ˚C). Subsequently, both mixtures (uncrosslinked and crosslinked batches) were placed on ice for 5 minutes and then the protein precipitate was pelleted at 16,000xg for 5 minutes. The supernatant was removed and dried down using a nitrogen stream. PG oligomers (crosslinked and uncrosslinked; both prepared from 20 μM Lipid II) were added to a reaction tube with LcpB (400 nM) and radiolabeled LII_A^{WTA} (4 μM; specific activity=300 nCi/nmol) in 20% DMSO. Reactions proceeded for 1 hour at room temperature. LcpB was heat-inactivated (10 minutes at 95 ˚C). To test crosslinking after WTA-attachment, PBP4 (4 μM) was added to the respective reactions and incubated for 1 hour at room temperature. For reactions treated with lysostaphin, 1 mg/ml lysostaphin was added and reactions

were incubated at 37 ˚C, 220 rpm for 1 hour.[15] Lysostaphin was heat-inactivated (10 minutes at 95 ˚C). After completion, all reactions were dried using a speed vacuum. Dried reactions were resuspended in 10 μl of 2X SDS.

Determining kinetic constants for TagT

Synthetic Lipid II (40 μM) was incubated with wild-type SgtB (500 nM) for 30 minutes in the presence of a PGT buffer (50 mM HEPES (7.5), 0.25 mM Tween-80, 2 mM $MnCl_2$). Reactions were heat-quenched at 100 ˚C for 10 minutes. The precipitated protein was subsequently removed, and the collected supernatant was dried using a N_2 stream at 40 ˚C. To determine kinetic constants of wild-type TagT, a reaction (10 μl total volume) containing TagT (200 nM), PG oligomers (from 40 μM Lipid II; in PGT buffer), and LII_A^{WTA} (0.5 μM-4 μM; in DMSO) with 20% total DMSO was incubated at room temperature for twenty minutes. Reactions were directly quenched with equal volume of methanol. Reactions were then spotted on individual, 20-cm cellulose-based chromatography paper strips (Sigma Aldrich, Catalog # WHA3030861) and using a previously developed separation method.[1a] Using a 5:3 isobutyric acid: ammonium hydroxide co-solvent in a saturated chamber, the paper was incubated with solvent at room temperature overnight. The dried paper strips were then cut into 2.5 cm fragments from the origin and analyzed in 10 ml of liquid scintillation fluid (EcoScint XR, National Diagnostics, Catalog # LS-272). Using a standard curve of [^{14}C]-UDP-GlcNAc, moles of the WTA-disaccharide unit (GlcNAc-[^{14}C]-ManNAc) incorporated into the peptidoglycan were calculated. The % conversion was determined as moles of WTA-disaccharide (GlcNAc-[^{14}C]-ManNAc) attached to PG/moles of LII_A^{WTA} used. Velocity (μM/min) was plotted against

the WTA substrate (μM). Km and V_{max} values were determined in GraphPad Prism 7.0 using a best-fit analysis.

LcpB reactions with native, long PG polymers prepared from SgtB and PBP4

To prepare long uncrosslinked PG polymers, a large batch of native Lipid II (20 μM) was incubated with wild-type SgtB (800 nM) at room temperature for 1 hour in buffer A (50 mM HEPES (pH 7.5), 10 mM $CaCl_2$). The reaction was quenched at 95 ˚C for 10 minutes, and precipitated SgtB protein as removed. The batch was split in two portions; to obtain crosslinked peptidoglycan, PBP4 (4 μM) was added to one portion, and the PBP4 reaction was incubated at room temperature for an additional 1 hour. The PBP4-reaction was quenched at 95 ˚C for 10 minutes. Precipitated protein was then removed after centrifugation at 16,000xg for 10 minutes. Supernatant was collected, and dried under a N_2 stream at 40 ˚C. Both polymer batches (each prepared from 20 μM Lipid II) were incubated with LII_A^{WTA} (2 μM), LcpB (400 nM), in the presence of 20% DMSO (note: dried PG polymer contains buffer A). At each time point (10, 20, 30, and 60 minutes), the reaction was directly quenched in 10 μl of methanol (equal reaction volume). Reactions were then spotted on individual, 20-cm cellulose-based chromatography paper strips (Sigma Aldrich, Catalog # WHA3030861) and using a previously developed separation method.[1a] Using a 5:3 isobutyric acid: ammonium hydroxide co-solvent in a saturated chamber, the paper was incubated with solvent at room temperature overnight. The dried paper strips were then cut into 2.5 cm fragments from the origin and analyzed in 10 ml of liquid scintillation fluid (EcoScint XR, National Diagnostics, Catalog # LS-272). The % conversion was calculated as moles of WTA-disaccharide (GlcNAc-[^{14}C]-ManNAc) attached to PG/initial moles of LII_A^{WTA} used.

LcpB reactions with peptidoglycan polymers prepared from wild-type PBP2 and $PBP2^{S398G}$

To prepare long uncrosslinked PG polymers, native Lipid II (20 μM) was incubated with $PBP2^{S398G}$ (4 μM) at room temperature for 2 hours in buffer A (50 mM HEPES (pH 7.5), 10 mM $CaCl_2$) in 20% DMSO. Reaction was quenched at 95 ˚C for 10 minutes. To obtain crosslinked PG polymer, native Lipid II (20 μM) was incubated with $PBP2^{WT}$ (4 μM) at room temperature for 2 hours in buffer A. Reactions were quenched at 95 ˚C for 10 minutes. Precipitated protein was removed and the supernatant containing the PG polymer was dried under a N_2 stream at 40 ˚C. Subsequently, LII_A^{WTA} (2 μM) and LcpB (400 nM) were added to each PG polymer in the presence of 20% DMSO (nascent and crosslinked). At 60 minutes, the reactions were directly quenched in 10 μl of methanol (equal reaction volume). Reactions were analyzed using paper strip chromatography as described above. Reactions were repeated in biological and technical triplicate.

LC/MS analysis to determine the percent crosslinking after PBP4 and PBP2

To determine % crosslinking after PBP4-treatment, native Lipid II (20 μM) was incubated with $SgtB^{Y181D}$ (800 nM) at room temperature for 30 minutes in buffer A (50 mM HEPES (pH 7.5), 10 mM $CaCl_2$) and 20% DMSO. The reaction was quenched at 95 ˚C for 10 minutes. PBP4 (4 μM) was added and then the reaction was incubated at room temperature for an additional 2 hours. To determine % crosslinking after wild-type PBP2 treatment, native Lipid II (20 μM) was incubated with $PBP2^{WT}$ (4 μM) at room temperature for 2 hours in buffer A. The reaction was quenched at 95 ˚C for 10 minutes.

The reactions were quenched at 95 ˚C for 10 min, and treated with mutanolysin (from *Streptomyces globisporus*, Sigma, 1 U) for 1.5 h at 37˚C followed by another 1 U aliquot for 1.5 h. The cleaved muropeptides were then reduced with 10 μl of 10 mg/ml $NaBH_4$ (prepared in water before immediate use). The reduced muropeptides were quenched with phosphoric acid (20%, 1.4 μL) to adjust the pH to 4. The reaction mixture was then flash-frozen, lyophilized, redissolved in 20 μL H_2O and subjected to LC/HRMS analysis as described previously.[15] Using a Bruker qTOF in ESI-MS mode, the sample was separated on a Waters Symmetry Shield RP18 column (5 µM, 3.9 × 150 mm) with a matching column guard. The muropeptides 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 40% ACN (0.1% formic acid)/H_2O (0.1% formic acid) over 25 min. Molecular ions corresponding to expected muropeptides (i.e., pentapeptide-containing monomer, tetrapeptide-containing monomer, dimer, and trimer) were extracted (see Figure S3). The M+2/2 peaks for species observed were integrated to determine the abundance of muropeptide species, and the % of crosslinks was determined using an equation from Glauner *et. al*: the sum of (dimer abundance/2) + (trimer abundance/3) divided total abundance of all muropeptides observed.[16]

Comparing ligase activities of wild-type TagT, mutant TagT constructs, wild-type LcpB, and LcpB^{D70A}

Synthetic Lipid II (40 μM) was incubated with wild-type SgtB (500 nM) for 30 minutes in the presence of a PGT buffer (50 mM HEPES (7.5), 0.25 mM Tween-80, 2 mM $MnCl_2$). Reactions were heat-quenched at 100 ˚C for 10 minutes. The precipitated protein was subsequently removed, and the collected supernatant was dried using a N_2 stream at 40 ˚C. To compare the ligase activities of wild-type TagT, wild-type LcpB, and mutant LCP constructs, the

respective LCP protein (400 nM) was incubated with PG oligomers (from 40 μM Lipid II; in PGT buffer), and [^{14}C]-LII_A^{WTA} (2 μM; in DMSO) was incubated at room temperature for twenty minutes. Reactions were directly quenched with equal volume of methanol. Reactions were then spotted on individual, 20-cm cellulose-based chromatography paper strips (Sigma Aldrich, Catalog # WHA3030861) and using a previously developed separation method.[1a] Using a 5:3 isobutyric acid: ammonium hydroxide co-solvent in a saturated chamber, the paper was incubated with solvent at room temperature overnight. The dried paper strips were then cut into 2.5 cm fragments from the origin and analyzed in 10 ml of liquid scintillation fluid (EcoScint XR, National Diagnostics, Catalog # LS-272). A standard curve of [^{14}C]-UDP-GlcNAc was made to calculate the moles of the WTA-disaccharide unit (GlcNAc-[^{14}C]-ManNAc) incorporated into the peptidoglycan. The % conversion was calculated as moles of WTA-disaccharide (GlcNAc-[^{14}C]-ManNAc) attached to PG divided by initial moles of [^{14}C]-LII_A^{WTA}. Graphs were determined in GraphPad Prism 7.0 using a best-fit analysis.

LCP reactions with different divalent cations

Peptidoglycan polymer was prepared from 20 μM synthetic Lipid II and wild-type SgtB (2 μM) in the presence of 50 mM HEPES (pH 7.5) and 200 mM NaCl for 2 hours. TagT (500 nM) was incubated with [^{14}C]-LII_A^{WTA} (4 μM) with synthetic PG polymer with the addition of 15 mM of various cations ($CaCl_2$, $MnCl_2$, $Zn(OAc)_2$) or 2 mM EDTA with NaCl present. Reactions were quenched after 30 minutes and subjected to paper strip chromatography. The % conversion was determined from moles of radiolabeled WTA incorporated into PG/moles of [^{14}C]-LII_A^{WTA}.

LCP reactions with oligosaccharides

LcpB or TagT (500 nM) were incubated with 4 μM [^{14}C]-LII_A^{WTA} (specific activity=300 nCi/nmol), 32 μM of the oligosaccharide, and 1X TGase buffer (50 mM HEPES (pH 7.5), 10 mM $CaCl_2$). Reactions were mixture, incubated at room temperature for five minutes, and then quenched directly with equal volume methanol. The oligosaccharides were purchased from Megazyme with ≥ 95% purity. The oligosaccharides included Diacetyl-chitobiose (NAG2, Part Number: O-CHI2), Tri-acetyl chitotriose (NAG3, Part number: O-CHI3), Tetraacetyl-chitotetrose (NAG4, Part Number: O-CHI4), Pentaacetyl-chitopentose (NAG5, Part Number: O-CHI5), Hexaacetyl-chitohexose (NAG6, O-CHI6), Cellopentose (Part Number: O-CPE). De-acetylated chitopentaose was purchased from Carbosynth LLC (Part Number: OC09269). After a five-minute incubation at room temperature, reactions were directly quenched with 10 μl of methanol (equal reaction volume). Reactions were subsequently dried down and resuspended in 10 μl of 2X SDS buffer for PAGE analysis.

For end point assays, the chitin assays ran for 1 hour at room temperature. Diacetyl-chitobiose and triacetyl-chitotriose were also tested under non-catalytic conditions. LcpB (32 μM) was incubated with [^{14C}]- LII_A^{WTA} (4 μM) and unlabeled chitin-based tri- or di- saccharide (960 μM) for 2 hours.

PAGE analysis

The acrylamide gels used were similar to those described previously[17]. Gels (20 cm x 16 cm (H x W); 1.0 mM thickness) were constructed with 10% acrylamide/Tris.[17] The gels were electrophoresed with anode and cathode buffer as previously described.[17-18] The anode buffer consisted of 100 mM Tris (pH 8.8), and the cathode buffer consisted of 100 mM Tris (pH 8.25),

100 mM Tricine, and 0.1% SDS. At a 30 mA voltage, gels ran for approximately 5 hours. The gels were dried with a gel dryer (Labconco, Catalog #4330100). Dried gels were exposed to a general purpose storage phosphor screen for approximately 12-24 hours. An autoradiograph was imaged using a Typhoon 9400 imager and analyzed using the software ImageQuant TL.

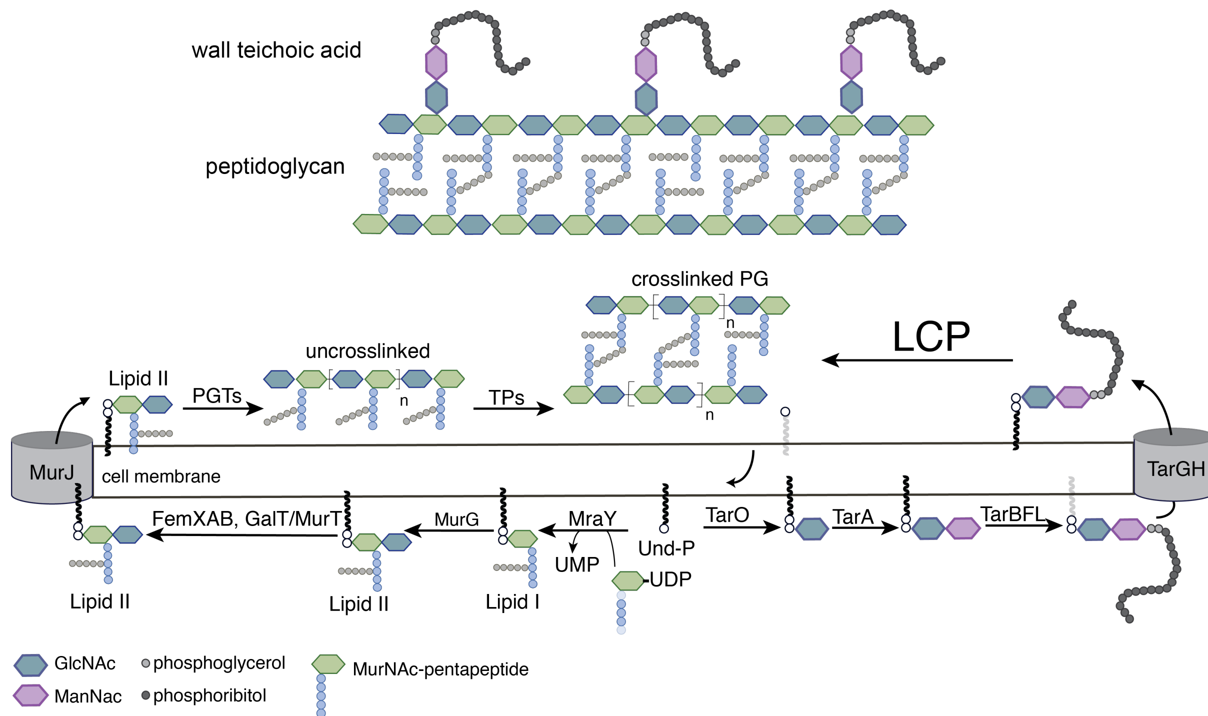

Figure S1. Schematic representing PG and WTA biosynthesis in *Staphylococcus aureus* (*S. aureus*). It has not been established if LCP ligases transfer WTA substrates to peptidoglycan before or after crosslinking.

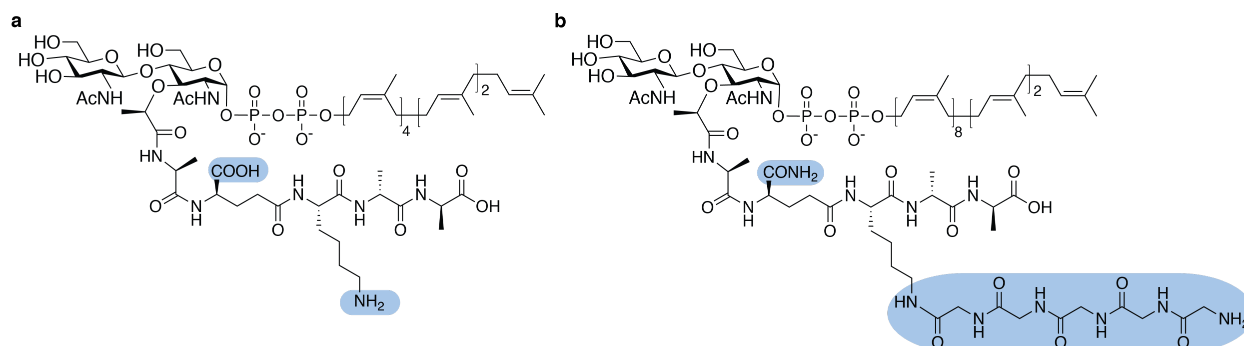

Figure S2. Chemical structures of synthetic Lipid II and native Lipid II purified from *S. aureus*. Synthetic Lipid II (left) is not capable of forming crosslinks because it lacks key features found on native *S. aureus* Lipid II (right). (a) Synthetic Lipid II contains a carboxylate and a free lysine on the third position of the stem peptide (highlighted in blue).[1a] (b) In contrast, native *S. aureus* Lipid II contains a pentaglycine crossbridge (highlighted in blue) that is required to synthesize crosslinked peptidoglycan.[3a]

Active PBP	$PBP2^{WT}$	PBP4	$PBP2^{S398G}$
Hydrolyzed monomer	9%	n.s.	4%
Crosslinked dimer	17%	25%	1%
Crosslinked Trimer	n.s.	n.s.	n.s.

Table S1. LC/MS analysis of peptidoglycan polymers prepared from PBP4 and $PBP2^{WT}$, and $PBP2^{S398}$ reactions. Peptidoglycan polymers were prepared as described in the methods section, dried, and resuspended in deionized water for LC/MS analysis.[15] Extracted ion chromatograms were generated and peaks corresponding to different muropeptide species (Figure S3) were integrated to determine the overall abundance. Percentage of crosslinks was determined using from the standard equation: Sum of (Dimer abundance/2 + Trimer abundance/3) divided by the total abundance of all species observed.[16] n.s. indicates a non-significant abundance.

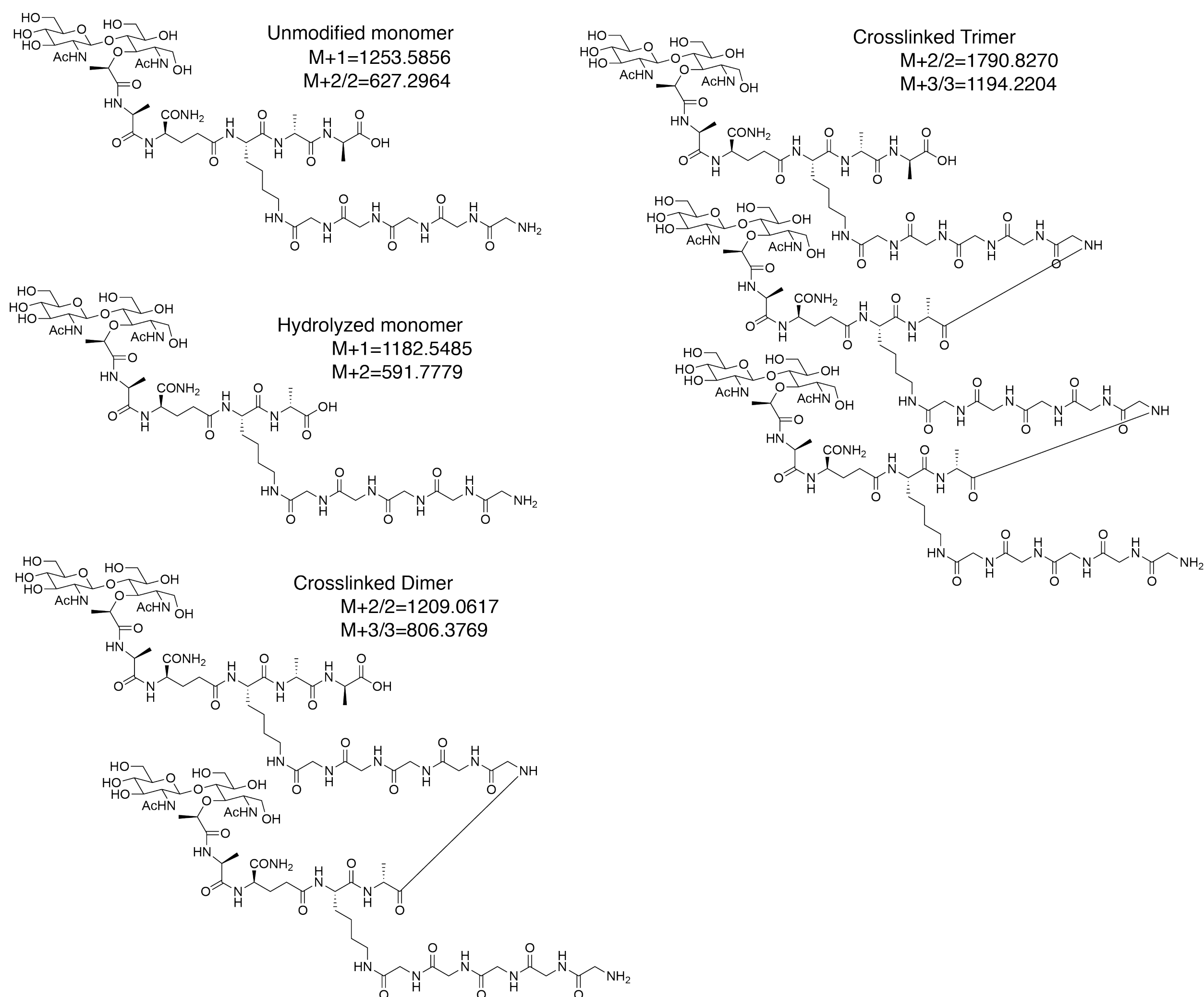

Figure S3. Different muropeptide species observed under LC/MS analysis of PBP4 and PBP2 reactions. Extracted ion chromatograms were analyzed for the mass ions (shown above) for each muropeptide species, similar as described previously.[15]

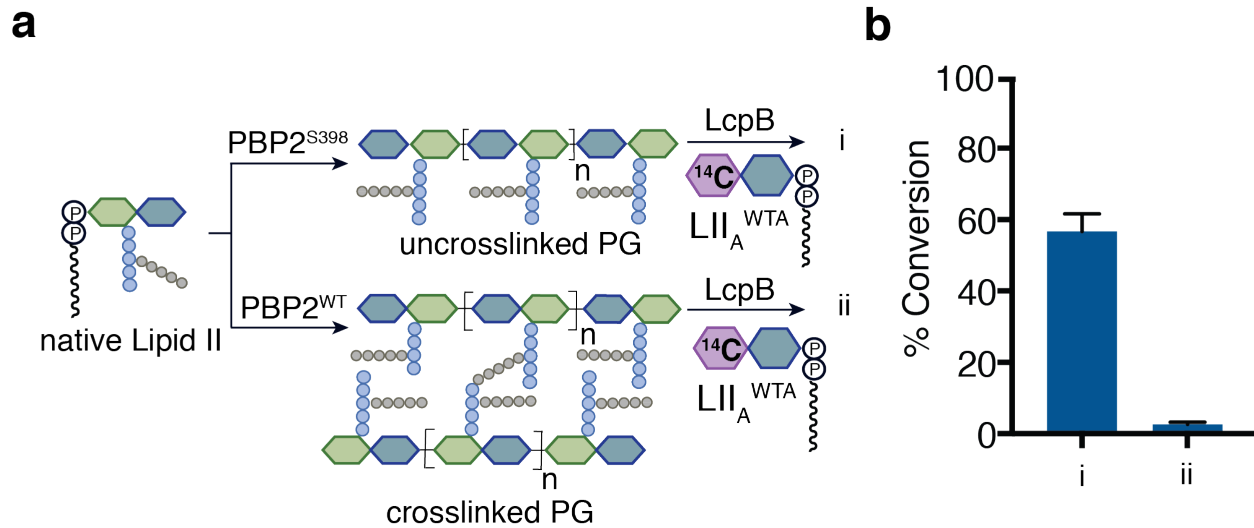

Figure S4. LcpB does not ligate to crosslinked peptidoglycan prepared from wild-type PBP2. Uncrosslinked peptidoglycan was prepared from the TP inactive PBP^{S398G} (i) and crosslinked peptidoglycan was prepared with wild-type PBP2 that contains both an active PGT and TP domain (ii). Either PG polymer (i. or ii) was incubated with LcpB and [^{14}C]-LII_A^{WTA}. Reactions were quenched after a 1 hour incubation, and analyzed using paper chromatography. (b) % conversion was calculated from moles of WTA incorporated into PG/moles of LII_A^{WTA} used. Reactions were repeated in biological and technical triplicate. All points are the mean ± s.e.m. (n=3).

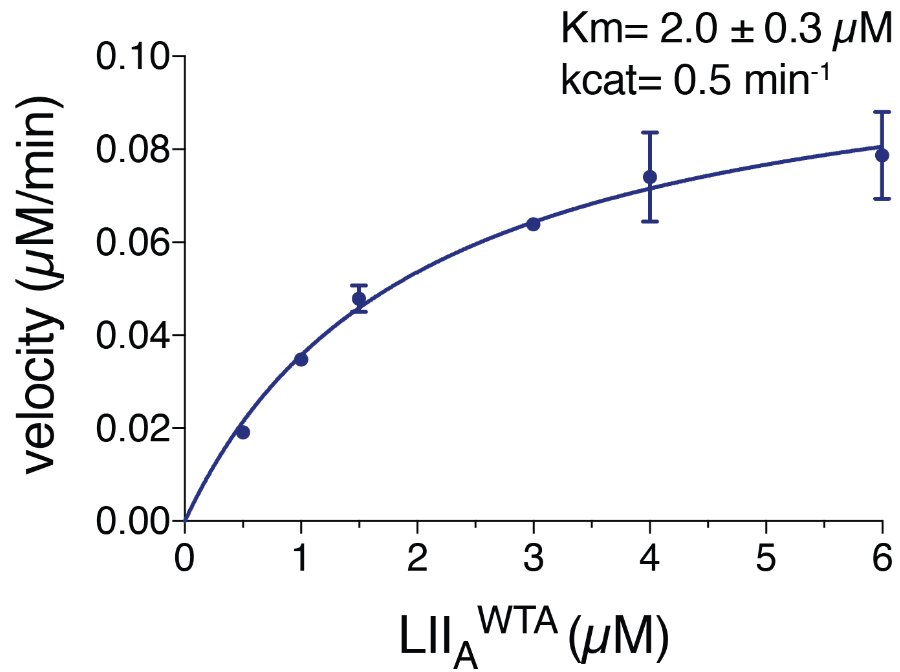

Figure S5. In the presence of peptidoglycan polymer and [^{14}C]- LII_A^{WTA}, TagT transfers the radiolabeled WTA sugars onto the PG. Reactions were analyzed using paper strip chromatography. Kinetic constants were determined after analysis in Graphpad Prism 7.0b. All points are the mean ± s.e.m. (n=3).

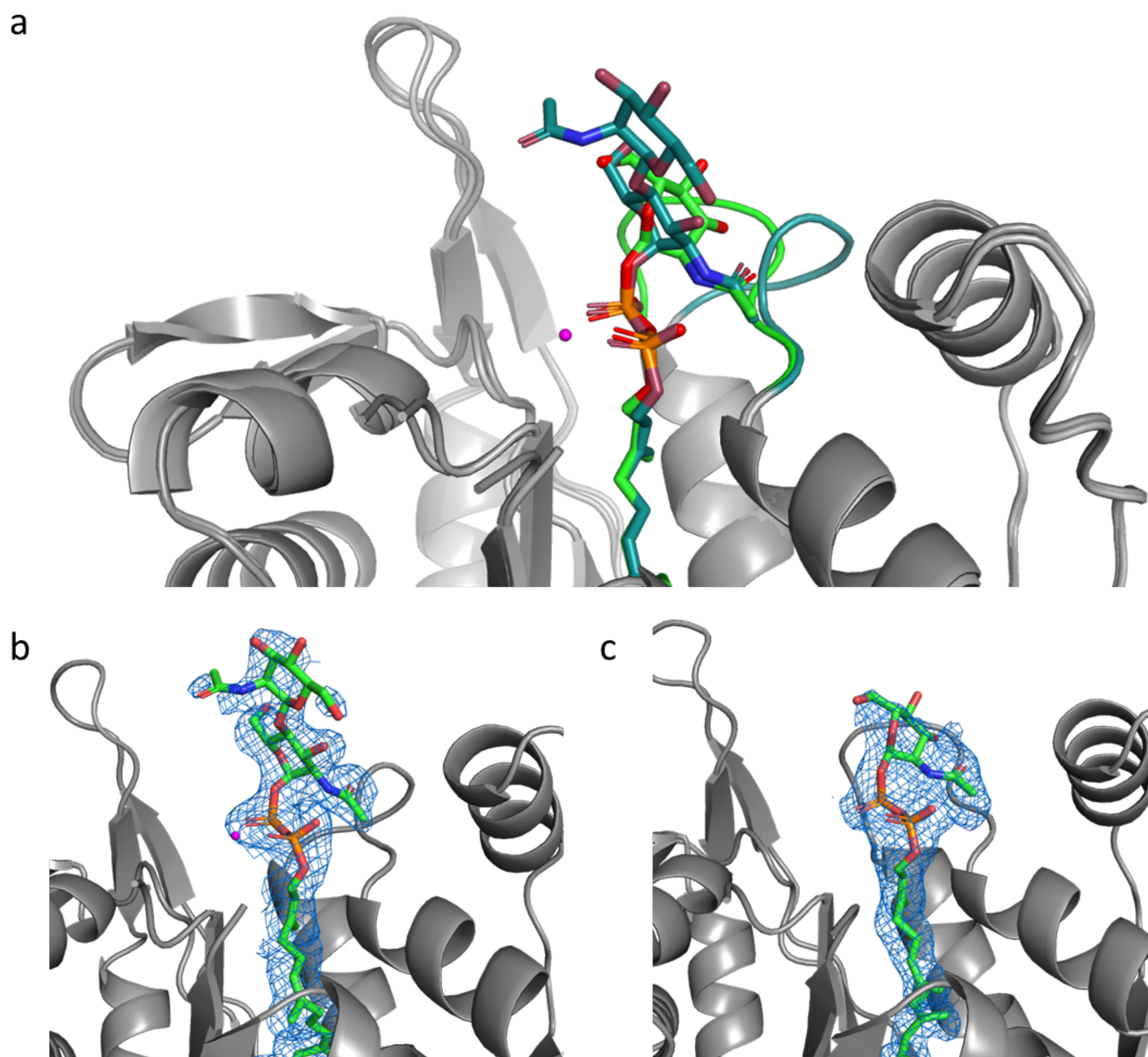

Figure S6. Alignment of the LI^{WTA}-TagT and LII_A^{WTA}-TagT structures shows significant differences in the orientation of the WTA precursors' pyrophosphate, which influences the coordination of the divalent metal cation**.** (a) TagT bound to LI^{WTA} (green carbon atoms, red oxygen atoms), which contains only one sugar (GlcNAc), is overlaid with the TagT structure bound to LII_A^{WTA} (teal carbon atoms, purple oxygen atoms), which contains two sugars (GlcNAc-ß(1,4)-ManNAc). A Mg^{2+} cation (magenta) is observed in the TagT bound to LII_A^{WTA} but not in the TagT complex with LI^{WTA}. (b) TagT bound to LII_A^{WTA} with F_O - F_C omit map around LII_A^{WTA} contoured at 1.0σ. (c) TagT bound to LI^{WTA} with F_O - F_C omit map around LI^{WTA} contoured at 1.5σ.

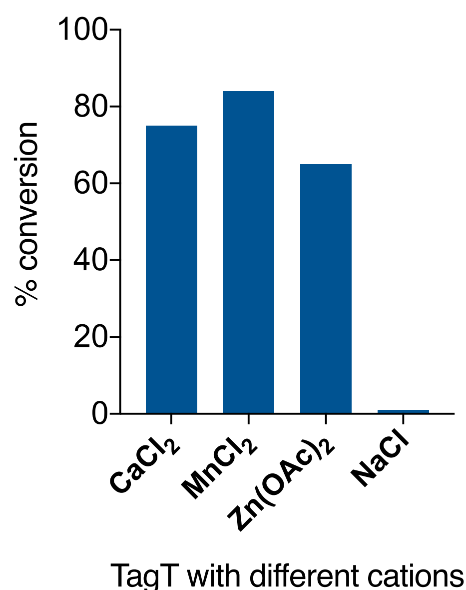

Figure S7. TagT transfers radiolabeled LII_A^{WTA} to synthetic, long peptidoglycan oligomers in the presence of divalent cations (i.e., Ca^{2+}, Mn^{2+}, Zn^{2+}). TagT (500 nM) was incubated with $[^{14}C]$-LII_A^{WTA} (4 μM) with synthetic PG polymer (prepared from 20 μM synthetic Lipid II and wild-type SgtB (2 μM) in the presence of 50 mM HEPES (pH 7.5) and 200 mM NaCl) and 15 mM of various cations ($CaCl_2$, $MnCl_2$, $Zn(OAc)_2$). Reactions were quenched after 30 minutes and subjected to paper strip chromatography. % conversion was determined from moles of radiolabeled WTA incorporated into PG/moles of $[^{14}C]$-LII_A^{WTA}.

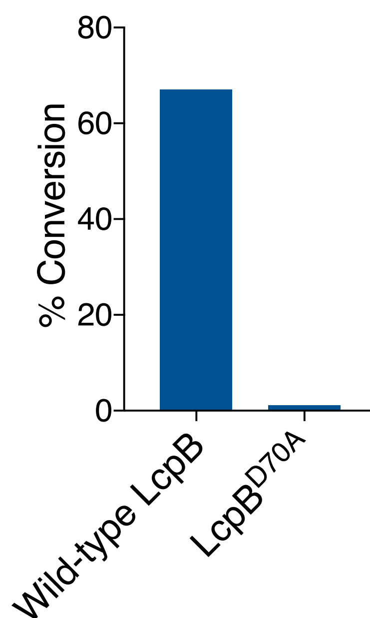

Figure S8. Mutating conserved aspartate, D70, in LcpB completely abolishes its ability to ligate WTA onto PG. D70, corresponding to D82 in TagT, was mutated to an alanine residue. In the presence of long PG polymer and [^{14}C]-LII_A^{WTA}, wild-type LcpB transfers WTA onto PG (incubation time 20 minutes) and $LcpB^{D70A}$ does not transfer at all.

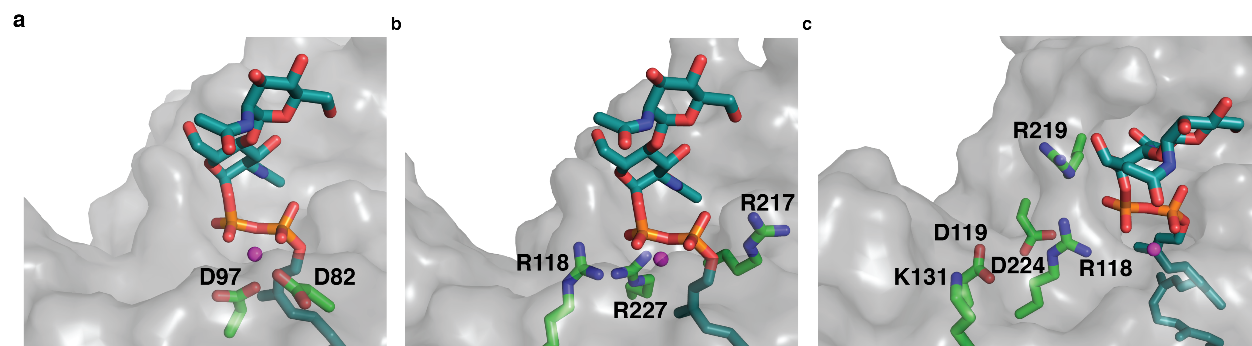

Figure S9. Expanded views of the TagT: LII_A^{WTA} co-complex with annotated, strictly-conserved residues show differences in their proximity to the WTA substrate. (a) D97 and D82 coordinate the Mg^{2+} cation (violet sphere), (b) R217, R227, and R118 are within hydrogen bond distance to the pyrophosphate linkage, (c) K131, D119, D224, R118, and R219 are all within the proposed peptidoglycan binding groove.

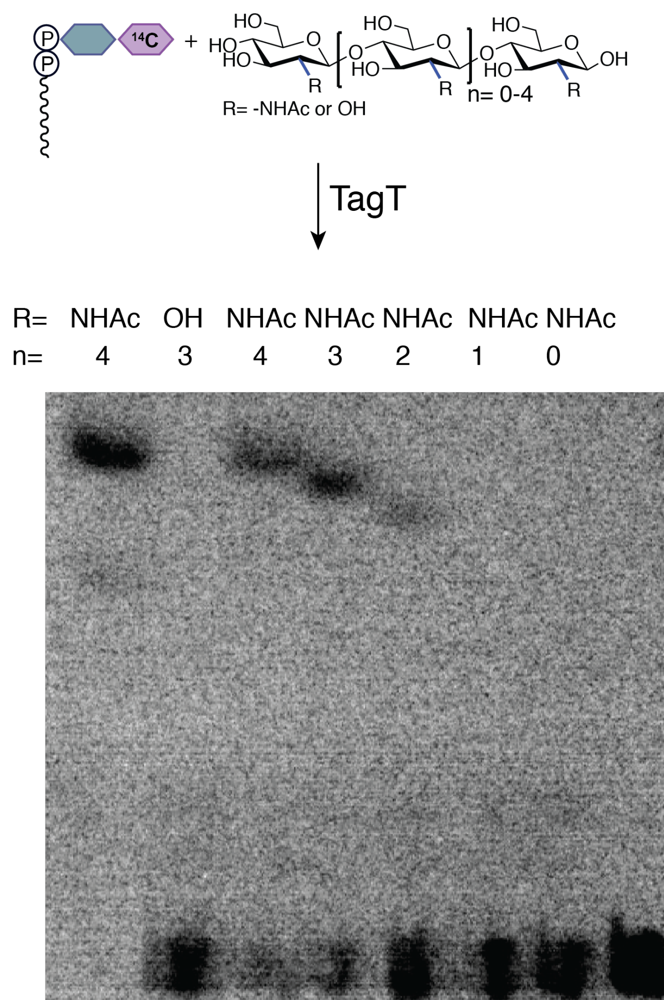

Figure S10. TagT transfers radiolabeled WTA to chitin-based oligomers with at least four saccharide units as visualized by PAGE autoradiography. TagT was incubated with [^{14}C]-LII$_A^{WTA}$ and unlabeled oligosaccharides for 5 minutes at room temperature before reactions were directly quenched with methanol.

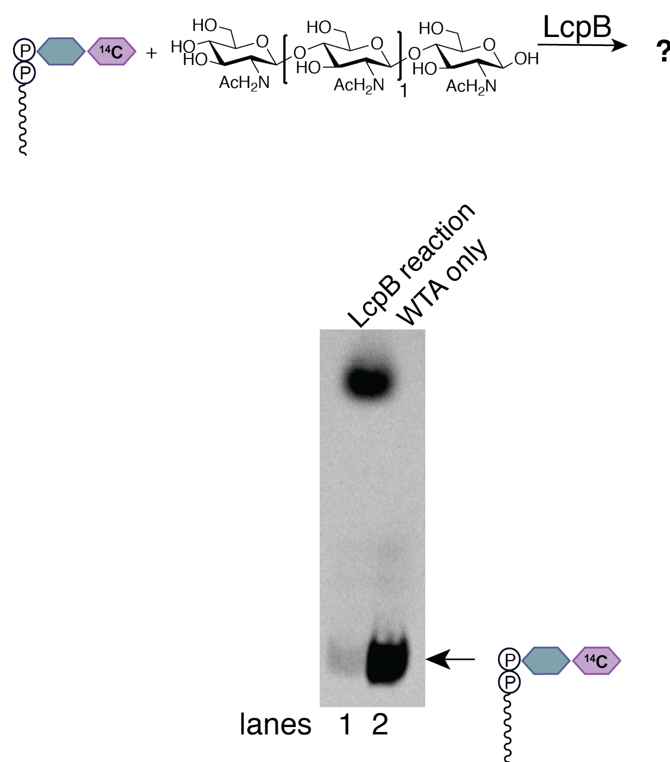

Figure S11. Under non-catalytic conditions, LcpB transfers radiolabeled LII_A^{WTA} to the chitin-based tri-saccharide. LcpB (32 μM) was incubated with $^{[14C]}$]- LII_A^{WTA} (4 μM) and unlabeled chitin-based tri-saccharide (960 μM) for 2 hours.

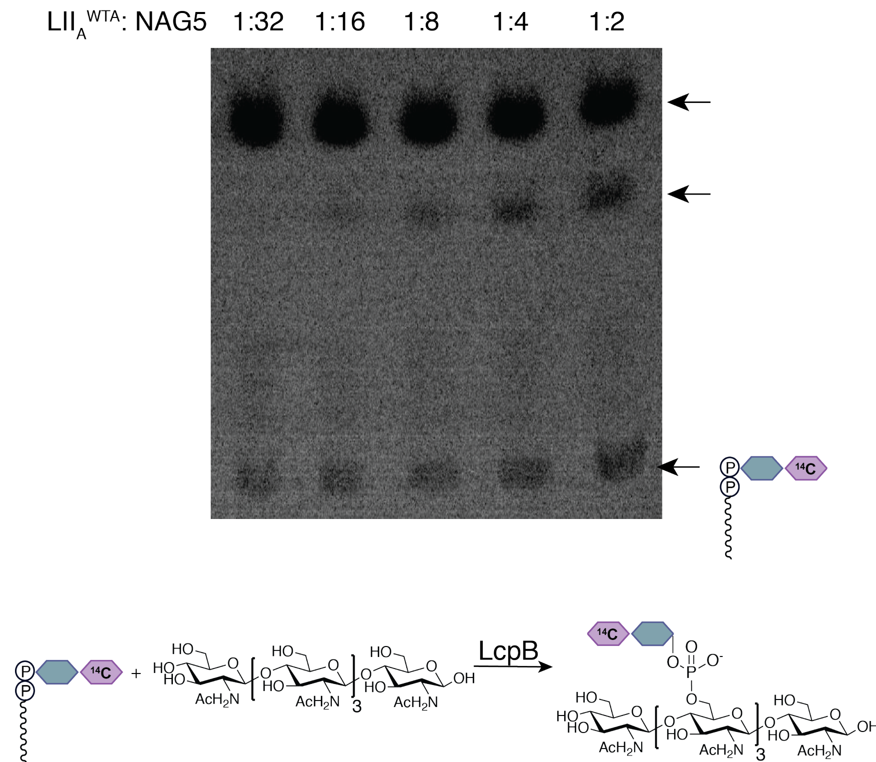

Figure S12. Bands corresponding to WTA-chitin oligosaccharide products are dependent on the ratio of LII$_A^{WTA}$:oligosaccharide. [^{14}C]-LII$_A^{WTA}$ (2 μM) was incubated with the chitin-based penta-oligosaccharide (NAG5; 4-64 μM) and LcpB (500 nM) for 20 minutes. A dominant product band is observed; a second, faint band is observed (indicated by arrows) with less penta-oligosaccharide present.

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Primer Name	5'-3' Nucleotide Sequence
F'D70A_LcpB	CTTTTCTTAGGTATCGCGGATAACGATGGAAGAAG
R'D70A_LcpB	CTTCTTCCATCGTTATCCGCGATACCTAAGAAAAG
F'D82A_TagT	GTATTGCTCTTAGGAATTGCCGCCAGGGAGAAAAACGGCG
R'D82A_TagT	CGCCGTTTTTCTCCCTGGCGGCAATTCCTAAGAGCAATAC
F'D97A_TagT	CCGTCGATCAGGCAAGAAGTGCCGCGAATGTGCTGGTTACATTT
R'D97A_TagT	AAATGTAACCAGCACATTCGCGGCACTTCTTGCCTGATCGACGG
F'R118A_TagT	CAGCTAAAATGCTGAGCATTCCGGCCGACGCCTATGTAAACATC
R'R118A_TagT	GATGTTTACATAGGCGTCGGCCGGAATGCTCAGCATTTTAGCTG
F'D119T_TagT	CAGCTAAAATGCTGAGCATTCCGCGTACCGCCTATGTAAACATCC CAGGCCACG
R'D119T_TagT	CGTGGCCTGGGATGTTTACATAGGCGGTACGCGGAATGCTCAGCA TTTTAGCTG
F'K131A_TagT	CATCCCAGGCCACGGGTATGATGCCTTCACACATGCTCACGCTTA C
R'K131A_TagT	GTAAGCGTGAGCATGTGTGAAGGCATCATACCCGTGGCCTGGGAT G
F'R217A_TagT	GAAGAAGCGCTCGCTTACGTAGCCACACGTAAAGCGGACAGTG
R'R217A_TagT	CACTGTCCGCTTTACGTGTGGCTACGTAAGCGAGCGCTTCTTC
F'R219A_TagT	GCGCTCGCTTACGTAAGAACACGTAAAGCGGACAGTGACCTTTTA C
R'219A_TagT	GCGCTCGCTTACGTAAGAACACGTAAAGCGGACAGTGACCTTTTA C
F'D224A_TagT	GAACACGTAAAGCGGACAGTGCCCTTTTACGCGGGCAAAGACAA
R'D224A_TagT	TTGTCTTTGCCCGCGTAAAAGGGCACTGTCCGCTTTACGTGTTC
F'R227A_TagT	GTAAAGCGGACAGTGACCTTTTAGCCGGGCAAAGACAAATGGAA GTG
R'R227A_TagT	CACTTCCATTTGTCTTTGCCCGGCTAAAAGGTCACTGTCCGCTTTA C

Table S2. Primers used in this study.

Data Set	WT TagT + LII_A^{WTA}	WT TagT + LI^{WTA}
Space Group	**P 41 21 2**	**P 41 21 2**
Unit Cell		
Dimensions (a, b, c), Å	66.41 66.41 145.15	66.23 66.23 140.04
Angles (α, β, γ), °	90, 90, 90	90, 90, 90
Data Collection*		
Wavelength, Å	0.979	0.979
Resolution Range	60.39 – 1.86 (1.90-1.86)	59.87 - 1.65 (1.71-1.65)
Rmerge	0.019 (1.49)	0.046 (1.43)
Completeness, %	99.1 (99.6)	99.4 (98.2)
Mean I / σ(I)	13.1 (0.6)	16.2 (0.8)
Unique Reflections	27947	38209
Multiplicity	4.2 (4.2)	4.9 (3.7)
Refinement		
Rwork, % / Rfree, %	0.2156 / 0.2346	0.1998 / 0.2215
Average B ($Å^2$), protein	48.9	37.8
Average B ($Å^2$), lipid	62.2	59.6
Ramachandran plot		
Favored/disallowed %	96.79 / 0	97.57 / 0.46
Rmsd from ideal geometry		
Bond lengths, Å	0.0027	0.0192
Bond angel, °	0.55	1.58

Table S3. Data collection and refinement statistics for co-crystals of wild-type, ΔTM-TagT with LII_A^{WTA} and LI^{WTA}. Data collection statistics for highest resolution shells are shown in parentheses.

Strain or plasmid	Description[*]	Reference
Staphylococcus aureus		
RN4220 (NCTC8325)	Wild-type *S. aureus* strain	(3a)
Escherichia coli		
NovaBlue (DE3)	*endA1 hsdR17* (r_{k12}- m_{k12}+) *supE44thi-1 recA1 gyrA96 relA1 lac* (DE3) *F' proAB lacI*^{Q}Z*ΔM15::Tn10* (Tet^{R}) *gyrA96*(nal^{R}) *thi-1 recA1 relA1 lac glnV44 F'*[*::Tn10 proAB*$^{+}$ *lacI*^{q} Δ(*lacZ*)*M15*] *hsdR17*(r_K^- m_K^+)	Novagen
BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) λDE3[*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^{+}$]$_{K-12}$($λ^S$)	Novagen
Plasmids		
pET28b(+)	IPTG-inducible protein expression vector; Kan^R	Novagen
pET24b(+)	IPTG-inducible protein expression vector; Kan^R	Novagen
pET_42a(+)	IPTG-inducible protein expression vector; Kan^R	Novagen
pLcpB	pET28b(+) carrying LcpB[31-405]_His_6, Kan^R	(1c)
pLcpB^{D70A}	pET28b(+) carrying LcpB[31-405]_D70A_His_6, Kan^R	This study
pTagT	pET28b(+) carrying TagT[46-323]_His_6, Kan^R	(11)
pTagT^{D82A}	pET28b(+) carrying TagT[46-323]_D82A_His_6, Kan^R	This study
pTagT^{D97A}	pET28b(+) carrying TagT[46-323]_D97A_His_6, Kan^R	This study
pTagT^{R118A}	pET28b(+) carrying TagT[46-323]_R118A_His_6, Kan^R	This study
pTagT^{D119T}	pET28b(+) carrying TagT[46-323]_D119T_His_6, Kan^R	This study
pTagT^{K131A}	pET28b(+) carrying TagT[46-323]_K131A_His_6, Kan^R	This study
pTagT^{R219A}	pET28b(+) carrying TagT[46-323]_R219A)His_6, Kan^R	This study
pTagT^{D224A}	pET28b(+) carrying TagT[46-323]_D224A_His_6, Kan^R	This study
pTagT^{R227A}	pET28b(+) carrying TagT[46-323]_R227A_His_6, Kan^R	This study
pET24bSgtB^{Y181D}	*S. aureus* SgtB^{Y181D}-His_6 (SgtB*) expression vector; Kan^R	(2)
pMgt1	*S. aureus* SgtB-His_6 expression vector; $Carb^R$	(2)
pPBP4	pET28b(+) carrying PBP4[21-383] _His_6, Kan^R	(3b)
pPBP2	pET28b(+) carrying PBP2[59-716] _His_6, Kan^R	(3a)
pPBP2^{S398G}	pET28b(+) carrying PBP2[21-383]_S398G_His_6, Kan^R	(3a)

[*]Abbreviations: $Carb^R$, carbenicillin resistance; Kan^R, kanamycin resistance.
[†] SgtB is also known as Mgt1.
[‡] SgtB^{Y181D} is abbreviated as SgtB* in the main text.

Table S4: Bacterial strains and plasmids used in this study.

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