Detection of Transport Intermediates in the Peptidoglycan Flippase MurJ Identifies Residues Essential for Conformational Cycling

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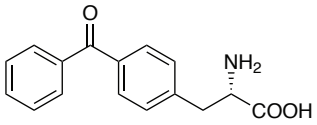
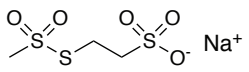
SUPPLEMENTARY FIGURES AND TABLES

SUPPLEMENTARY TEXT

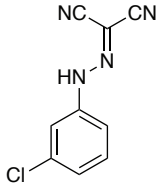
Supplementary figure S1. Chemical structures of compounds used in this study.



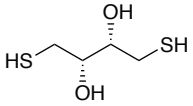
***p*-benzoyl-L-phenylalanine (*p*Bpa)**



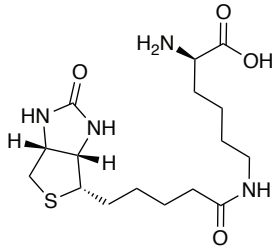
Sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES)



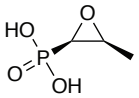
carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP)



DL-dithiothreitol (DTT)

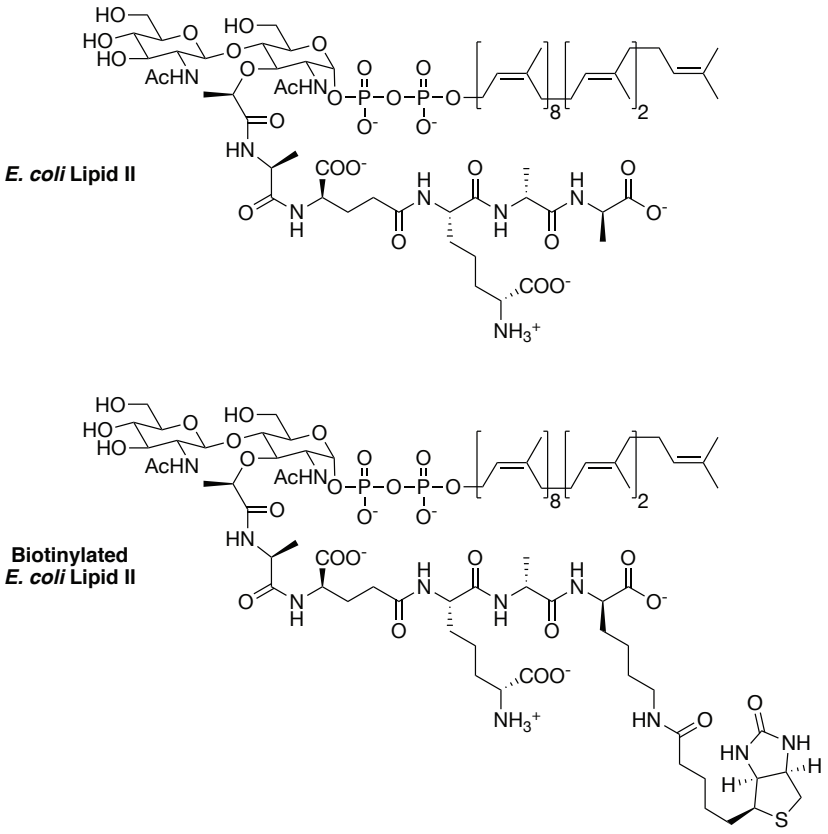


biotin-D-Lysine (BDL)

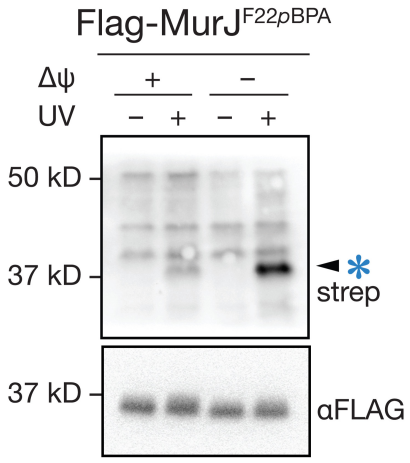


fosfomycin

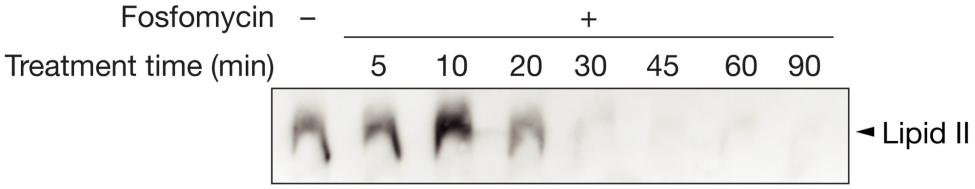
Supplementary figure S2. Chemical structures of native and biotinylated *E. coli* Lipid II.



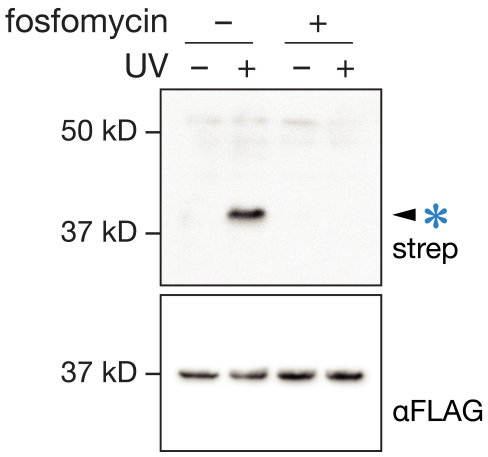
Supplementary figure S3. Uncropped western blots from **Figure 1e**.



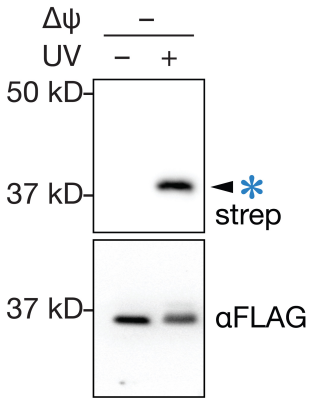
Supplementary figure S4. Fosfomycin treatment depletes Lipid II levels. A culture of NR5684 was grown to OD 0.3 in M63 minimal medium and resuspended to OD 1.0. Sucrose was added to 0.23 M final concentration, followed by 100 μg/mL fosfomycin, and the cells incubated at 37 °C, 550 rpm on Thermomixer C®. 100 μL aliquots were taken after the specified time periods for Lipid II quantification.



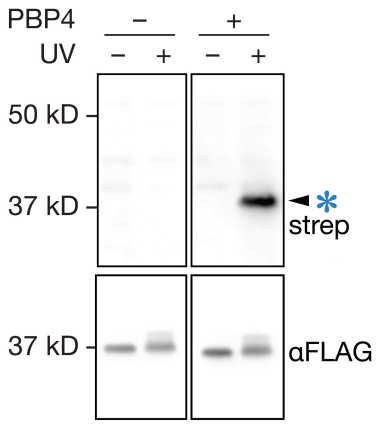
Supplementary figure S5. Uncropped western blots from **Figure 2a**.



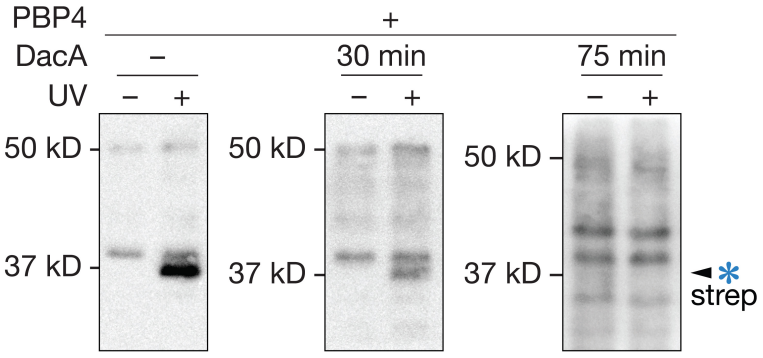
Supplementary figure S6. Uncropped western blots from **Figure 2b**.



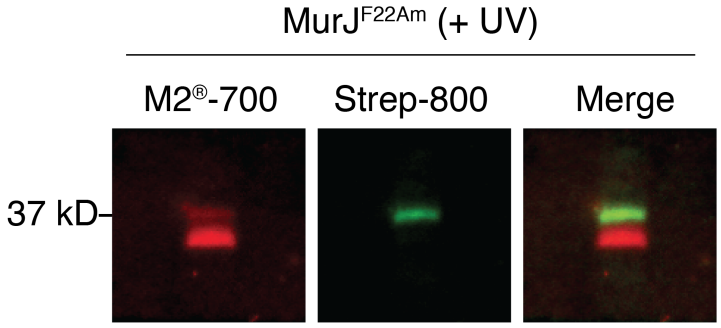
Supplementary figure S7. Uncropped western blots from **Figure 2c**.



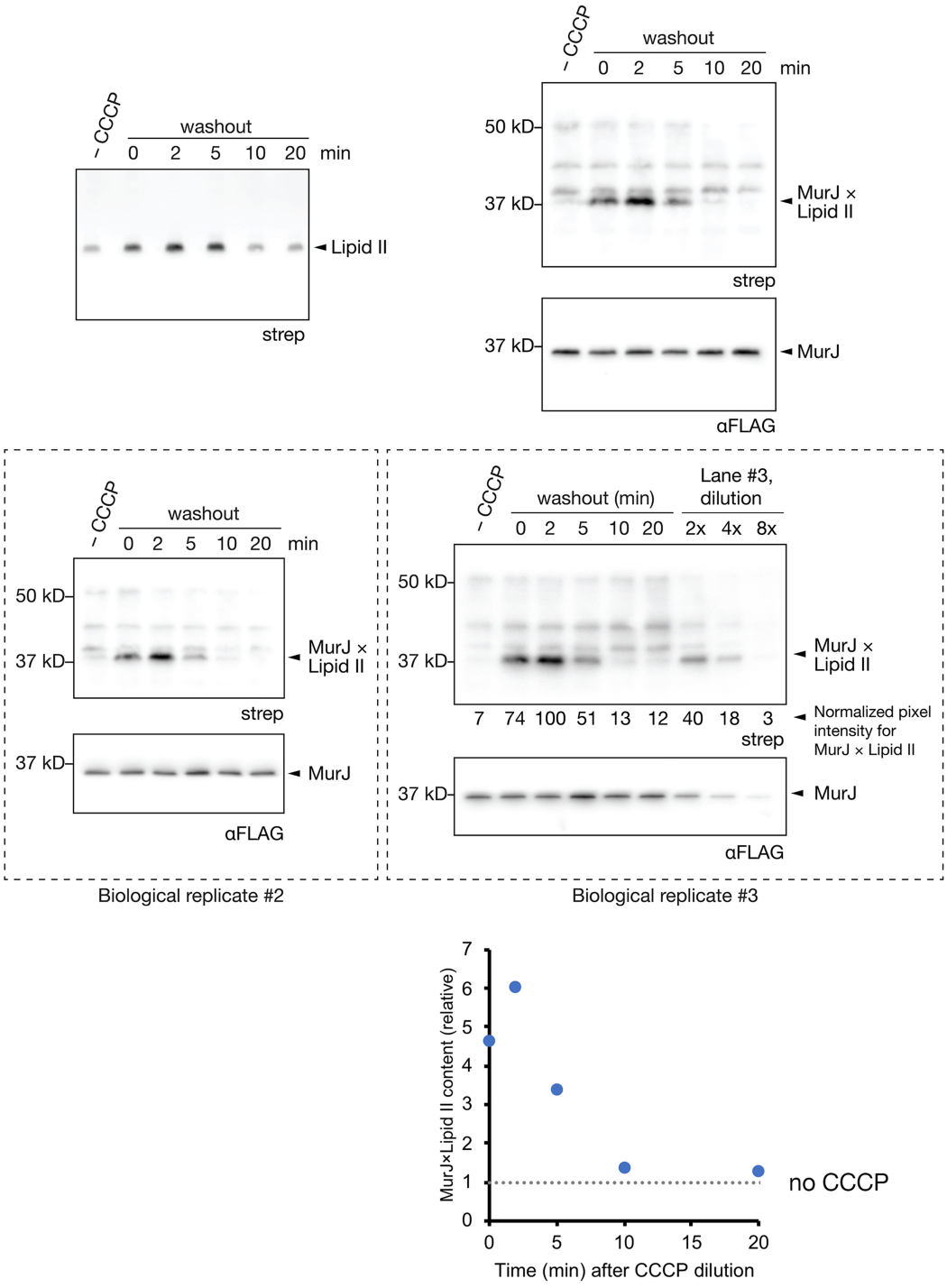
Supplementary figure S8. Uncropped western blots from **Figure 2d**.



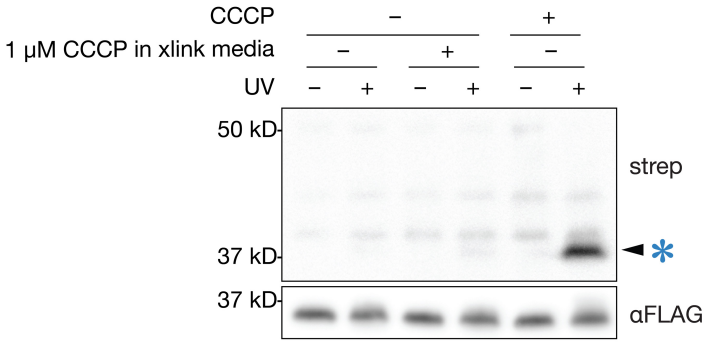
Supplementary figure S9. MurJ and MurJ×Lipid II can be resolved by SDS-PAGE. Crosslinked Flag-MurJ^{F22pBpa} sample was run on a 10% gel (4% stacking layer) and transferred to a PVDF membrane. Following on-membrane biotinylation, visualization was performed using a mixture of the IR-dye-tagged M2®-700 (2 μg/mL) and Strep-800 (1 μg/mL).



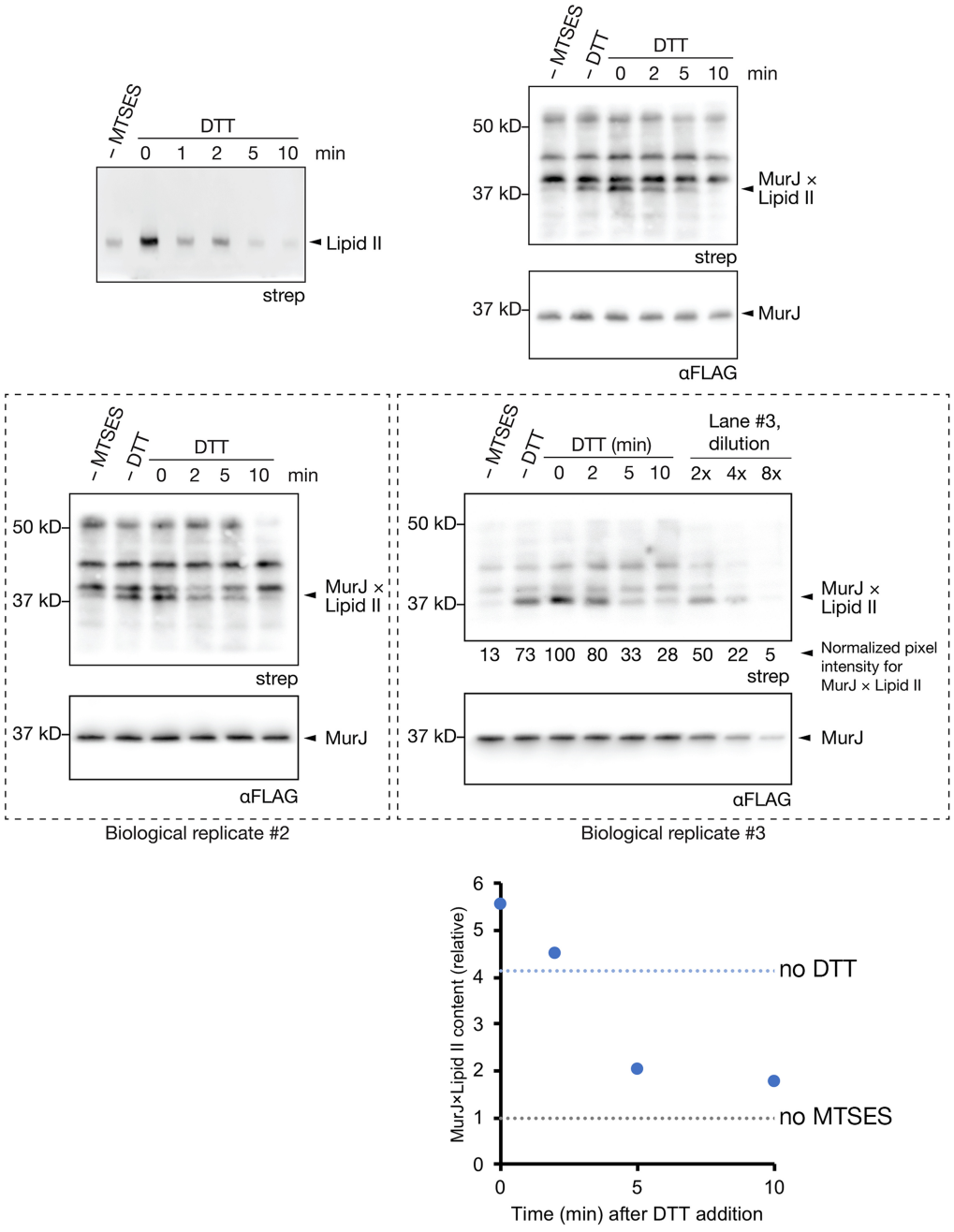
Supplementary figure S10. Uncropped western blots from **Figure 3b**, including biological replicates and quantification of MurJ×Lipid II content for the third replicate.



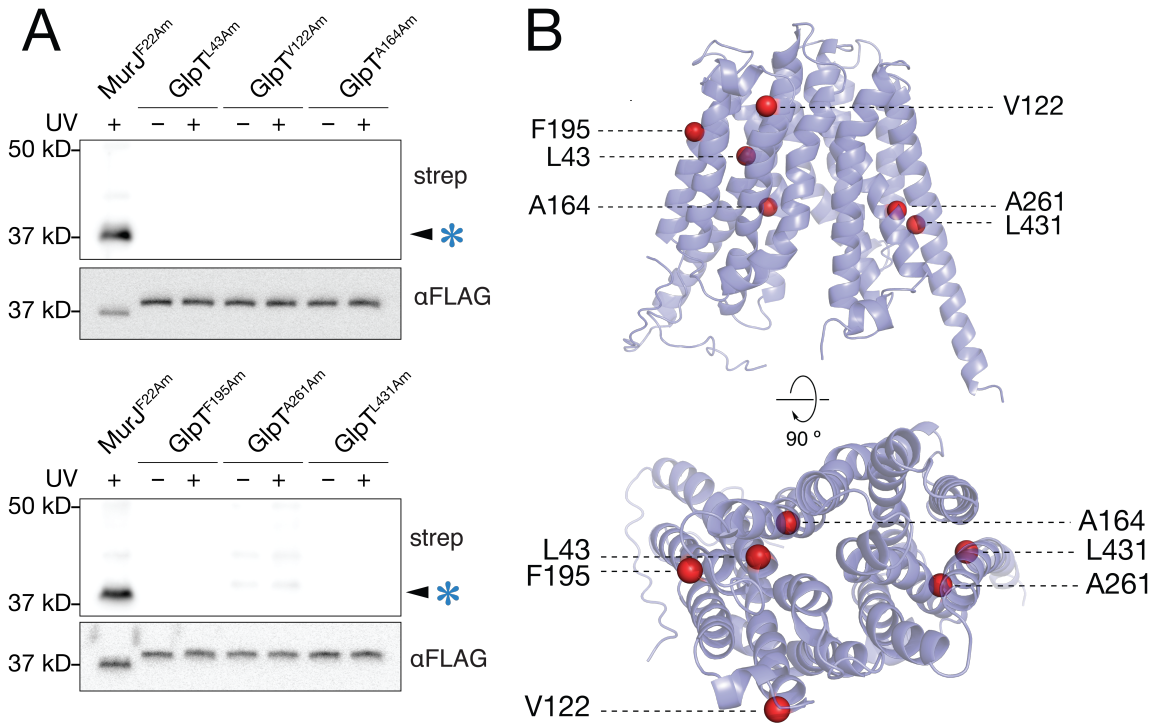
Supplementary figure S11. Treatment with 1 μM CCCP does not accumulate MurJ×Lipid II. Cultures of cells expressing MurJ^{F22pBpa} were treated with DMSO or 100 μM CCCP and subsequently diluted 100× into fresh media containing no CCCP or 1 μM CCCP, irradiated, and assessed for MurJ×Lipid II formation by on-membrane biotinylation.



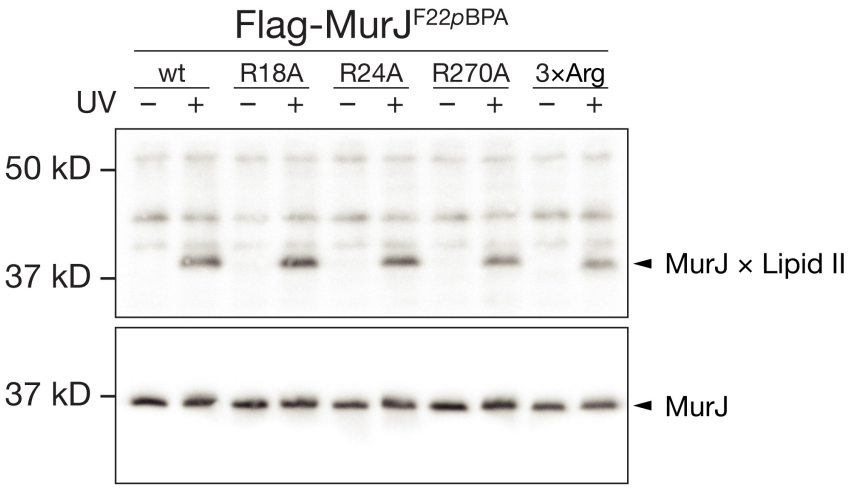
Supplementary figure S12. Uncropped western blots from **Figure 3d** including biological replicates and quantification of MurJ×Lipid II content for the third replicate.



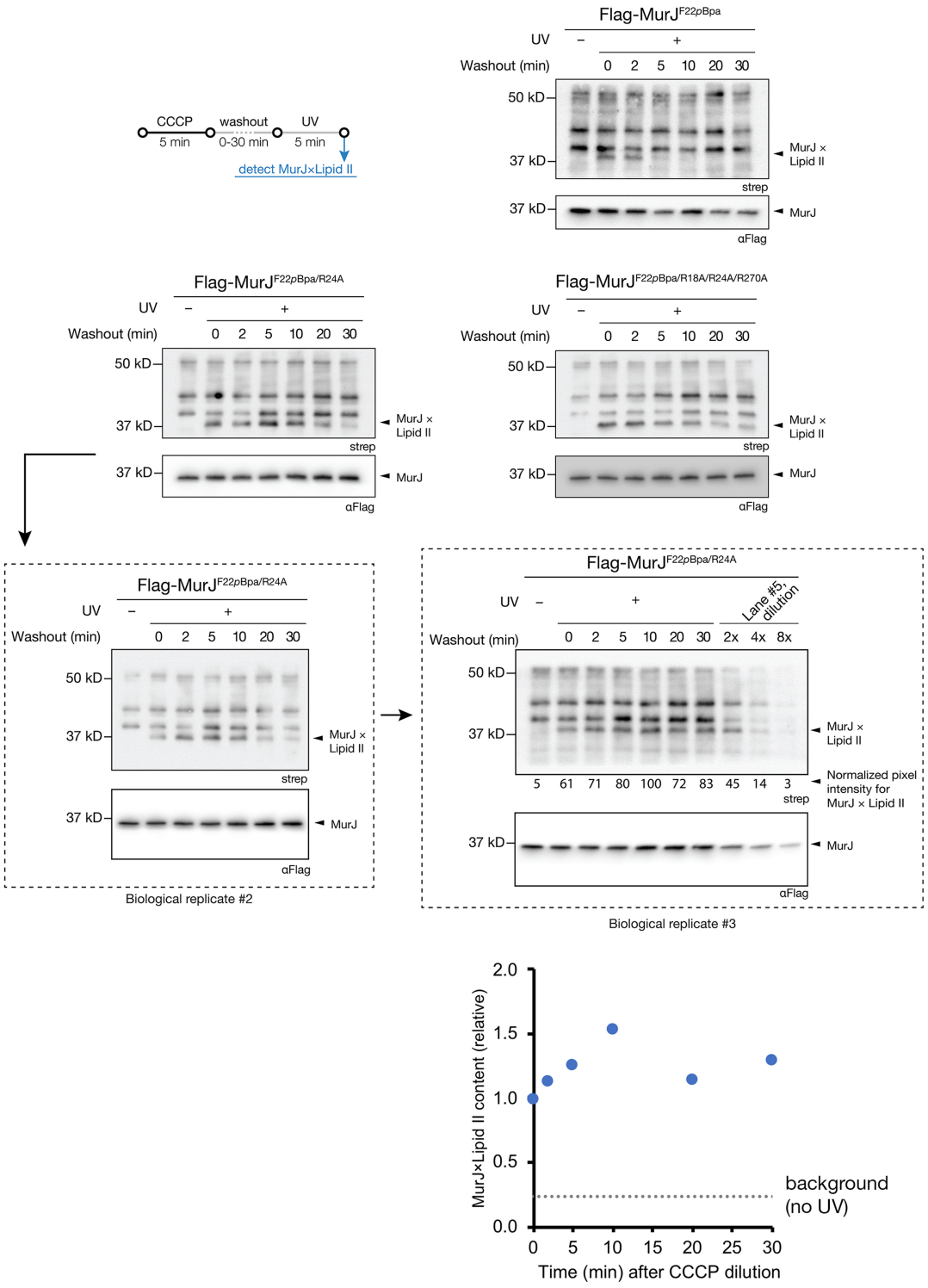
Supplementary figure S13. The diffuse membrane protein GlpT does not crosslink to Lipid II. (a) Strains expressing *p*Bpa-containing GlpT were crosslinked alongside MurJ^{F22pBpa} and assessed for MurJ×Lipid II formation by on-membrane biotinylation. (b) Location of the 6 sites of *p*Bpa incorporation in GlpT as viewed from the membrane (top) and from the periplasm (bottom).



Supplementary figure S14. Uncropped blots from **Figure 4c**.



Supplementary figure S15. Uncropped blots from **Figure 4d** including biological replicates and quantification of MurJ×Lipid II content for the third replicate.



Supplementary table S1. Strains used in this study.

Strain	Genotype	Source
NR4128	MG1655 *ΔmurJ::FRT ΔmalE::(araC PARA uppS FRT) ΔlacIZYA::FRT* (pRC7KanMurJ)	This study
NR4158	MG1655 *ΔmurJ::FRT ΔmalE::(araC PARA uppS FRT) ΔlacIZYA::FRT* (pRC7KanMurJ; pSUP-BpaRS-6TRN)	This study
NR4292	NR4158 (pET23/42FLAGMurJΔCys/F22Am)*	This study
NR5684	NR4158 (pET23/42FLAGMurJΔCys/F22Am/A29C)*	This study
AM39	NR4158 (pET23/42FLAGMurJΔCys/R18A/F22Am)	This study
AM40	NR4158 (pET23/42FLAGMurJΔCys/F22Am/R24A)	This study
AM41	NR4158 (pET23/42FLAGMurJΔCys/F22Am/R270A)	This study
AM42	NR4158 (pET23/42FLAGMurJΔCys/R18A/F22Am/R24A/R270A)	This study
AM43	NR4158 (pET23/42FLAGMurJΔCys/F22Am)	This study
AM68	NR4158 (pET23/42FLAGGlpT/L43Am)	This study
AM70	NR4158 (pET23/42FLAGGlpT/V122Am)	This study
AM73	NR4158 (pET23/42FLAGGlpT/A164Am)	This study
AM35	NR4158 (pET23/42FLAGGlpT/F195Am)	This study
AM74	NR4158 (pET23/42FLAGGlpT/A261Am)	This study
AM79	NR4158 (pET23/42FLAGGlpT/L431Am)	This study

* Plasmid pRC7KanMurJ was lost from this strain as described in the section **Construction and growth of Amber library**

Supplementary table S2. Plasmids used in this study.

Plasmid ID	Description	Source
–	pET23/42FLAGMurJΔCys/F22Am	This study
–	pET23/42FLAGMurJΔCys/F22Am/A29C	This study
pAM052	pET23/42FLAGMurJΔCys/R18A/F22Am	This study
pAM053	pET23/42FLAGMurJΔCys/F22Am/R24A	This study
pAM054	pET23/42FLAGMurJΔCys/F22Am/R270A	This study
pAM056	pET23/42FLAGMurJΔCys/R18A/F22Am/R24A/R270A	This study
pAM087	pET23/42FLAGGlpT/L43Am	This study
pAM089	pET23/42FLAGGlpT/V122Am	This study
pAM092	pET23/42FLAGGlpT/A164Am	This study
pAM048	pET23/42FLAGGlpT/F195Am	This study
pAM093	pET23/42FLAGGlpT/A261Am	This study
pAM098	pET23/42FLAGGlpT/L431Am	This study
–	pSUP-BpaRS-6TRN	Ref. 1
–	pRC7KanMurJ	Ref. 2

Materials

Biotin-D-lysine (BDL) and PBP4[Y21-Q383] were prepared as previously described.[3] PBP5[D30-N385] was prepared as previously described[4] Sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) was purchased from Toronto Research Chemicals. 4-Benzoyl-L-phenylalanine (*p*Bpa) was obtained from Bachem. 6-Aza-2-thiothymine was purchased from Alfa Aesar. M2®-HRP was fluorescently tagged using AzureSpectra Antibody Labeling Kit 700 (AC2188). IRDye 800CW Streptavidin was purchased from LI-COR. Streptavidin-HRP was purchased from KPL. LB Miller media and agar were purchased from Becton Dickinson. All other chemicals were purchased from Sigma-Aldrich. Glucose (0.2%) M63 minimal broth and agar were prepared as described previously.[5] All liquid cultures were grown under aeration at 37 °C unless otherwise noted. Growth was monitored by measuring the optical density at 600 nm (OD_{600}). Western blotting was performed on a 0.2 μM Immun-Blot® PVDF membrane (Bio-Rad). HRP-conjugated antibodies were visualized using Amersham ECL Prime Western Blotting Detection Reagent. UV irradiation was carried out via a Blak-Ray® B-100AP High-Intensity UV Lamp, 365 nm (UVP, Upland, CA). Structures were visualized in PyMOL.[6]

Construction and growth of Amber library

The MurJ-Amber library was constructed by site-directed mutagenesis to introduce the TAG codon into the coding region of plasmid pET23/42FLAGMurJΔCys (Ref. 7) as a template. Competent cells of strain NR4128 (MG1655 *ΔmurJ::FRT ΔmalE::(araC PARA Upps FRT) ΔlacIZYA::FRT* (pRC7KanMurJ)) were transformed with the pET23/42FLAGMurJΔCys/Am library and plated on LB agar supplemented with carbenicillin (50 μg/mL) as a selection marker. The plasmid pRC7KanMurJ (Ref. 2) has a partitioning defect (which causes it to be easily lost in the presence of a functional *murJ* allele) and encodes a wild-type *murJ* allele in addition to the enzyme β-galactosidase. Colonies grown on agar supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) will therefore appear blue unless another functional allele of murJ is present. Transformants of NR4128 with pET23/42FLAGMurJΔCys/Am were thus grown in liquid LB media supplemented with carbenicillin (50 μg/mL), made competent by resuspension in TSS buffer,[8] transformed with the plasmid pSup-BpaRS-6TRN (Ref. 1), which encodes the tRNA and corresponding aminoacyl-tRNA synthetase for *p*Bpa, and plated on LB supplemented with carbenicillin (50 μg/mL) and chloramphenicol (30 μg/mL) to retain the pET23/42FLAGMurJΔCys and pSup-BpaRS-6TRN plasmids, respectively, and X-gal (150 μg/mL). White colonies, indicating complementation by the $MurJ^{Am}$ variant, were purified on LB agar supplemented with carbenicillin (50 μg/mL), chloramphenicol (30 μg/mL), *p*Bpa (480 μM), and X-gal (150 μg/mL). *p*Bpa-dependent expression was confirmed by assessing Flag-MurJ levels in cultures grown in the presence or absence of *p*Bpa (480 μM) via western blot (M2® αFlag). In the case of R→A mutant pET23/42 vectors, which encode nonfunctional MurJ, a similar strain construction protocol to the above was carried out, except that no pRC7KanMurJ loss is possible, requiring a merodiploid strain. As such, X-gal was omitted, and transformation of pET23/42FLAGMurJΔCys(R→A) into NR4128 was followed by transformation with pSup-BpaRS-6TRN. The same selection markers were used as described above, taking care to add kanamycin for AM43 to retain

pRC7KanMurJ. For GlpT, the homogeneously diffuse inner-membrane glycerol-3-phosphate transporter,[9-10] a pET23/42FLAGGlpT vector and merodiploid Amber library was similarly constructed by transformation into NR4128.

Extraction and detection of Lipid II from cultures

Lipid II extraction protocol was adapted from previously published protocols.[3, 11] Aliquots (1 mL) of cultures were pipetted into 3.5 mL of a mixture of methanol and chloroform (2:1) in borosilicate glass culture tubes (16 × 100 mm) and shaken for 2 min at 1000 rpm, 25 °C in a ThermoMixer C© (Eppendorf) to form a single phase. Cell debris was pelleted by centrifugation for 2 min at 2000 × *g*. To the supernatant, 2 mL chloroform was added, followed by acidification with 0.1 N HCl to pH 1, as determined by pH indicator strips. Samples were shaken and centrifuged as above to form a two-phase system. As much of the aqueous upper layer was removed without disturbing the interface between the aqueous and organic phases, and 1 mL methanol was subsequently added to form a single liquid phase upon shaking. Samples were transferred to 1.5-mL conical microcentrifuge tubes by glass pipette, then dried by nitrogen stream at 40 °C. Dried samples were dissolved in 150 µL of a mixture of methanol and chloroform (2:1) by shaking for 2 minutes at 2000 rpm, 25 °C, then centrifuged at 21,000 × *g* for 1 minute and dried by nitrogen stream at 40 °C. This was repeated with 40 µL organic mixture, then crude lipid extracts were dissolved in 10 µL DMSO by shaking 10 min at 500, 1000, and 2000 rpm, 50 °C. Extracts were stored at -20 °C.

Crude Lipid II extracts (2 µL) were added to 12.5 mM HEPES-NaOH (pH 7.5), 100 µM $MnCl_2$, 250 µM Tween 80, 3 mM BDL, 8 µM PBP4[Y21-Q383] (10 µL total volume), then incubated 1 h at 25 °C to biotinylate Lipid II. Reactions were quenched by the addition of 10 µL of 2× SDS sample buffer and shaking. SDS samples (3 µL) were loaded onto a 14% polyacrylamide gel (pH 8.8) and run for 40 min at a constant 30 mA current using a running buffer composed of 25 mM Tris, 192 mM glycine, 0.1% SDS. Samples were transferred onto PVDF membranes at 10 V for 1 h, using a transfer buffer composed of 25 mM Tris, 192 mM glycine. Membranes were blocked 20 min in StartingBlock™ Buffer (Thermo #375378) then incubated 1 hr in a 1:10,000 dilution of streptavidin-HRP in TBS + 0.02% (w/v) Tween 20 followed by washing in the same buffer (8 × 10 min). Washed membranes were treated with ECL Reagent and visualized using an Azure c400 imaging system (Azure Biosystems, Inc., Dublin, CA).

Capture of MurJ×Lipid II by photocrosslinking

30 mL cultures of Amber library strains were grown to mid-log phase (OD_{600} ≈ 0.3) in M63 minimal medium supplemented with 30 µg/mL chloramphenicol, 50 µg/mL carbenicillin (+ 50 µg/mL kanamycin for AM39-43), 480 µM *p*Bpa, and then pelleted (4,000 × *g*, 6 min). The pellet was resuspended in 900 µL of the same media at 37 °C and incubated for 5 min at 37 °C with shaking (550 rpm, ThermoMixer C®). CCCP was added to 100 µM final (from a 10 mM stock in DMSO) and the cultures were incubated for another 5 min at 37 °C with

shaking and then shifted to 16 °C for an additional 10 min. 450 µL aliquots of CCCP-treated cultures were diluted into 45 mL of the same media (pre-cooled to 16 °C) and irradiated with UV light for 5 min at room temperature with gentle stirring. Prior to UV irradiation, a 1 mL aliquot of cells was taken for Lipid II level quantification (see **Extraction and detection of Lipid II from cultures**). Non-photocrosslinked controls were covered with aluminum foil during this step. Irradiated samples and controls were then collected (8,000 × *g*, 6 min, 4 °C) and pellets frozen at -80 °C. For inhibition with MTSES, the procedure was performed as described, with the exception that OD 1 cultures were treated with 400 µM MTSES for 5 min at 37 °C before dilution into media supplemented with 10 mM DTT. For pre-treatment with fosfomycin, OD 0.3 cultures were resuspended to OD 1 in M63 media supplemented with 0.23 M sucrose (osmoprotectant). Following 45 min treatment with 100 µg/mL fosfomycin, cells were treated with CCCP and cross-linked as above.

On-membrane labeling and detection of MurJ×Lipid II

Cell pellets were thawed on ice, resuspended in 9 mL of 4 °C lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5 mM PMSF, 5 mM DTT), and lysed by sonication (2 min in 10-sec pulses). The lysates were pelleted for 10 min at 8,000 × *g*, 4 °C and the supernatants further pelleted for 1.5 h at 100,000 × *g*. The resulting pellets were resuspended in 100 µL of 1× SDS buffer. For αFlag blots, 2 µL of resuspended membranes were run on a 4-14% gradient gel for 45 min at 200 V, and transferred to PVDF membrane (25 V, 20 min). The membrane was blocked for 20 min (StartingBlock™ PBS buffer, ThermoFisher), washed 1 × 1 min with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5), and incubated with antibody for 1 h (10,000-fold dilution of Monoclonal ANTI-FLAG M2®-HRP, Sigma). Following 8 × 10 min washes with TBST, the membrane was visualized by chemiluminescence. For on-membrane BDL exchange reactions, 10 µL of resuspended membranes were run on a 10% gel (4% stacking layer) for 2.5 h at 120 V, and transferred to PVDF membrane (25 V, 20 min). The membrane was blocked for 20 min (StartingBlock™ PBS buffer, Thermo Fisher), washed 1 × 1 min with TBST, and incubated in BDL exchange mixture (3 mM BDL, 8 µM *S. aureus* PBP4[Y21-Q383], in 12.5 mM HEPES pH 7.5, 100 µM $MnCl_2$, 250 µM Tween 80). The membrane was then washed with TBST for 10 × 10 min and incubated for 1 h with streptavidin-HRP (10,000-fold dilution of streptavidin-HRP from KPL). Following 8 × 10 min washes, the membrane was visualized by chemiluminescence. Western blot quantification was performed using ImageJ.[12] A twofold dilution series of the strongest intensity band was prepared and run alongside samples to be quantified. A standard curve was prepared from this dilution series, fit to a linear trendline (R^2 values of 0.9965, 0.9972, and 0.9989 for Figures S10, S12, and S15, respectively), and used to convert band intensity to relative MurJ×Lipid II amount for each sample. For PBP5 treatment, membranes were transferred and blocked as described above, then incubated with a 10 µM solution of PBP5[D30-N385] in TBST for 30-75 min. The membrane was then washed with TBST for 2 × 1 min and treated with the BDL/PBP4 mixture as detailed previously.

Purification of Flag-MurJ

Cell pellets were thawed on ice, resuspended in 9 mL of 4 °C lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5 mM PMSF, 5 mM DTT), and lysed by sonication (2 min in 10-sec pulses). The lysates were pelleted for 10 min at 8,000 × *g*, 4 °C and the supernatants further pelleted for 1.5 h at 100,000 × *g*. The resulting pellets were resuspended in 50 µL of 50 mM HEPES, 250 mM NaCl, 1% DDM, and incubated for 3 h at 500 rpm, 4 °C. Meanwhile, the M2® column (250 µL of settled resin), stored in 1× HBS (20 mM HEPES pH 7.5, 250 mM NaCl) + 0.02% NaN_3, was cleaned with 500 µL of 100 mM sodium citrate pH 3. The low pH was then quenched *via* addition of 1 mL of 1× HBS, and the column equilibrated with 1 mL wash buffer (20 mM HEPES, 500 mM NaCl, 0.1% DDM). 450 µL of wash buffer were added to the sample, which was then added to the column. The flow-through was re-applied twice. After 2 × 500 µL washes with wash buffer, the Flag-labeled MurJ was eluted with 5 × 100 µL of elution buffer (20 mM HEPES, 500 mM NaCl, 0.1% DDM, 0.2 mg/mL Flag peptide). The eluate was concentrated as appropriate using a 50 kDa MWCO spin filter (5,000 × *g*) and added to SDS-PAGE loading buffer in preparation for Western blotting.

SUPPORTING INFORMATION REFERENCES

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