Supplementary information

Structure and reconstitution of a hydrolase complex that may release peptidoglycan from the membrane after polymerization

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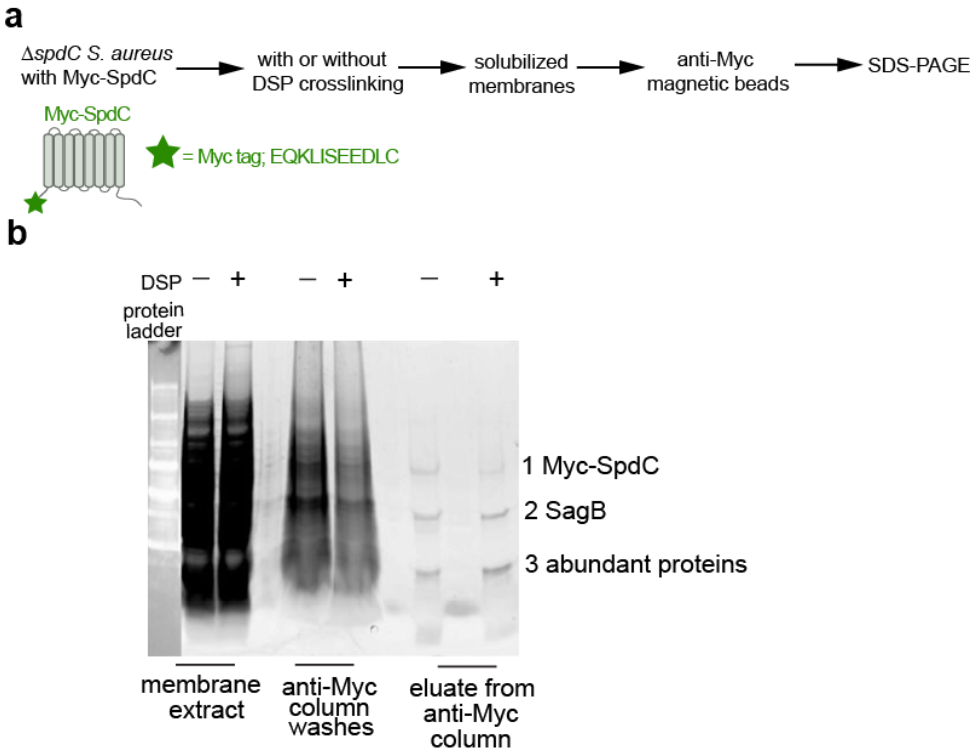
Beta lactam treatment	Gene number	Gene name	Corrected p-value	Ratio
Oxacillin	*USA300HOU_RS09425*	*sagB*	1.48E-13	0.0358
	USA300HOU_RS12635	*spdC*	5.26E-17	0.0599
Cefoxitin	*USA300HOU_RS09425*	*sagB*	1.84E-06	5.0994
	USA300HOU_RS12635	*spdC*	4.48E-11	6.2723
Mecillinam	*USA300HOU_RS09425*	*sagB*	6.16E-07	0.1675
	USA300HOU_RS12635	*spdC*	4.19E-06	0.2869

Supplementary Table 1. Treatment of a methicillin-resistant *Staphylococcus aureus* transposon library with sublethal concentrations of beta-lactams identified *sagB* and *spdC* as the only two genes with a unique cell wall phenotype. Genes significantly enriched and depleted under cefoxitin, mecillinam, or oxacillin conditions were identified using a two-sided Mann-Whitney U test corrected for multiple hypothesis testing using the Benjamini-Hochberg method.



Supplementary Figure 1. The interaction between SagB and SpdC was first observed by co-immunoprecipitation of SagB with a Myc-epitope tagged SpdC in a *S. aureus* Δ*spdC* background. Untreated cells and cells treated with a chemical crosslinker, DSP (dithiobis(succinimidyl propionate); Thermo Scientific, Catalog # 22585), were lysed and their membranes isolated. Solubilized cell membranes were applied to anti-myc magnetic beads, which were washed extensively before bound proteins were eluted and then analyzed using SDS-PAGE. Protein bands from the sample without crosslinker (1-3) were then subjected to LC-MS-MS analysis, which identified the middle band as SagB (Supplementary Table 2).

Band 1:

Unique peptides	Total peptides	Reference	Gene symbol	MW (kDa)
35	102	P02976_SPA_STAA8	*spa*	56.40
16	17	Q2FY15_Q2FY15_STAA8	*SAOUHSC_01659*	51.05
14	15	Q2FWW1_Q2FWW1_STAA8	*SAOUHSC_02161*	65.53
13	14	Q2FWF0_ATPB_STAA8	*atpD*	51.37
13	14	Q2FWH5_Y2316_STAA8	*SAOUHSC_02316*	56.91
13	13	Q2FYZ4_GLPD_STAA8	*glpD*	62.35
12	14	Q2FWE8_ATPA_STAA8	*atpA*	54.55
12	12	Q2FV16_Q2FV16_STAA8	*mqo*	55.96
11	21	Q2FVT1_LYRA_STAA8	***spdC***	46.76
10	10	Q2G0N0_EFTU_STAA8	*tuf*	43.08

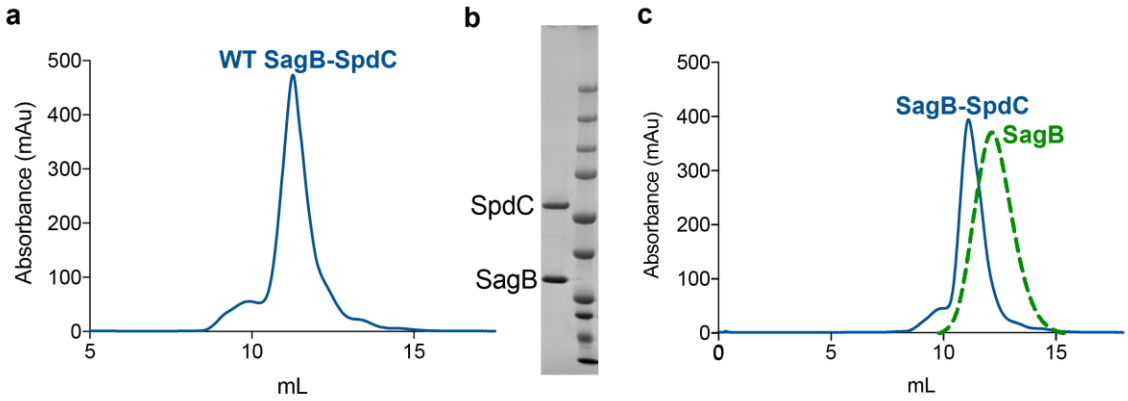
Band 2:

Unique peptides	Total peptides	Reference	Gene symbol	MW (kDa)
15	30	P60430_RL2_STAA8	*rplB*	30.14
12	16	Q2FWW9_Q2FWW9_STAA8	*SAOUHSC_02152*	32.93
12	13	Q2FXF4_Q2FXF4_STAA8	***sagB***	32.49
11	11	Q2G2S6_PRSA_STAA8	*prsA*	35.62
10	10	Q2FXZ9_Y1676_STAA8	*SAOUHSC_01676*	35.16
10	10	Q2G2D8_Q2G2D8_STAA8	*SAOUHSC_00634*	35.05
9	15	Q2FW06_RL3_STAA8	*rplC*	23.7
8	14	Q2G0B5_Q2G0B5_STAA8	*SAOUHSC_00690*	26.67
8	8	Q2FVL2_Q2FVL2_STAA8	*SAOUHSC_02699*	28.89
8	8	Q2G0N0_EFTU_STAA8	*tuf*	43.08

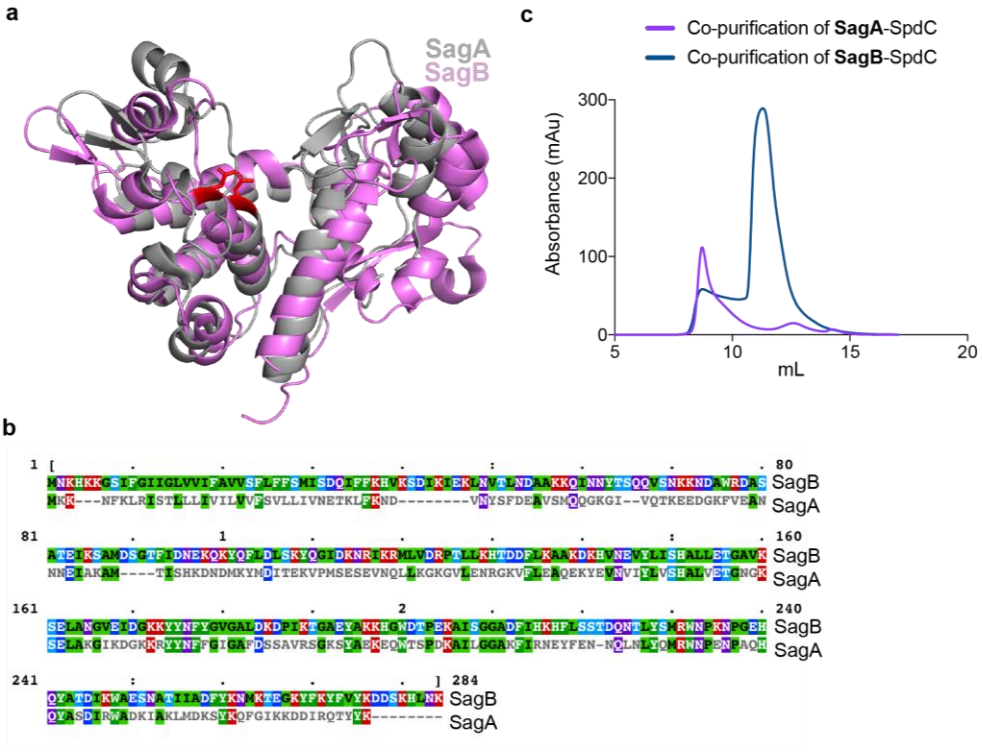
Band 3:

Unique peptides	Total peptides	Reference	Gene symbol	MW (kDa)
10	10	Q2G0F2_Q2G0F2_STAA8	*SAOUHSC_00617*	18.58
9	29	Q2FXS8_RL21_STAA8	*rplU*	11.33
9	9	Q2FW30_RS13_STAA8	*rpsM*	13.71
8	16	Q2FXQ1_RL20_STAA8	*rplT*	13.68
7	8	P0A0F8_RL15_STAA8	*rplO*	15.59
7	8	Q2G247_Y1855_STAA8	*SAOUHSC_01855*	17.99
7	7	Q2FW11_RL22_STAA8	*rplV*	12.83
7	7	Q2FVN6_Q2FVN6_STAA8	*SAOUHSC_02666*	13.33
6	7	Q2FW23_RS5_STAA8	*rpsE*	17.73
6	6	Q2FXM1_Q2FXM1_STAA8	*SAOUHSC_01814*	15.22

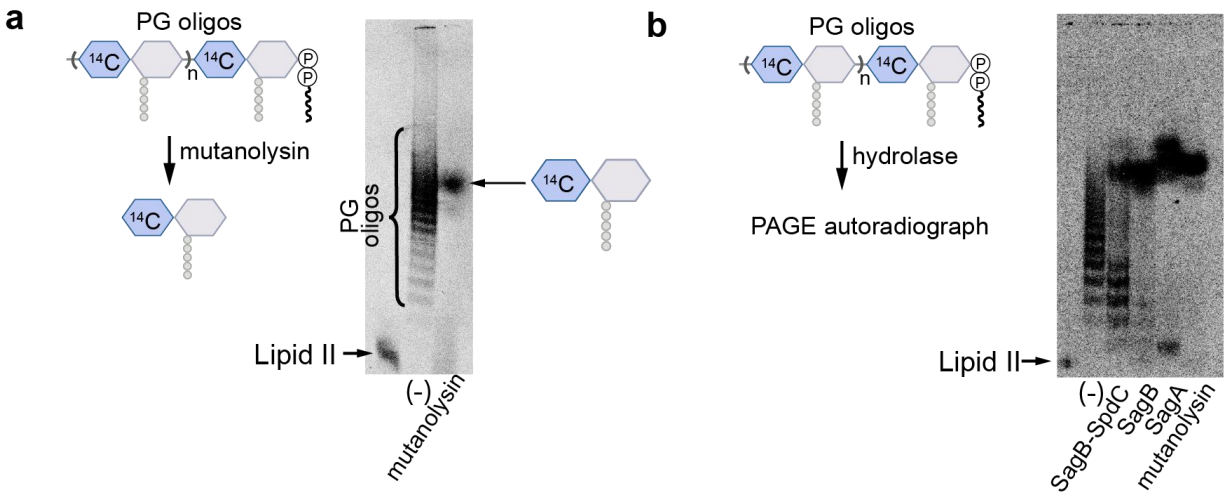
Supplementary Table 2. List of proteins identified by LC-MS-MS analysis of the sample obtained from immunoprecipitation of Myc-SpdC. Each protein band shown in Supplementary Figure 1 was analyzed separately. For each of the three bands observed in SDS-PAGE, here is the list of the top ten proteins with the highest coverage in LC-MS-MS analysis.



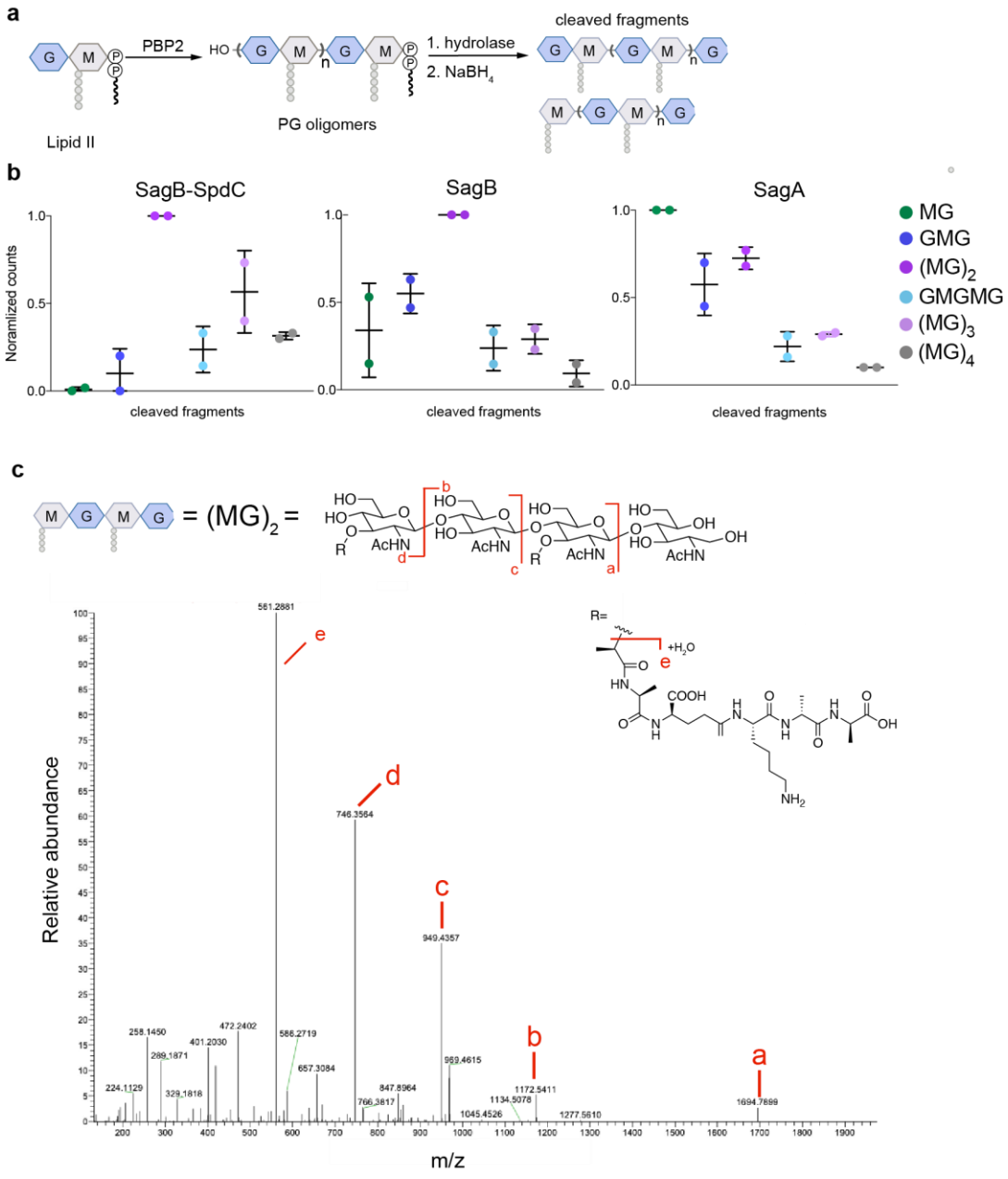
Supplementary Figure 2. Co-purification of SagB and SpdC results in a stable SagB-SpdC complex. **a,** An expression plasmid containing an N-terminal SUMO-FLAG epitope tag on SpdC and a C-terminal hexa-histidine tag on SagB was transformed into *E. coli* BL21-DE3 that also contained an arabinose-inducible Ulp1 protease plasmid. Purification steps included two affinity columns: nickel resin to pull down SagB-His_6 and an anti-FLAG M1 antibody resin to pull down an amino-FLAG-SpdC (SUMO removed by Ulp1 during co-expression). Eluate fractions were then concentrated and further purified by size exclusion chromatography with an Increase Sephadex S200 column 10/300 GL. **b,** SDS-PAGE of the dominant peak, which corresponds to the correct molecular weight of the DDM micelle, SagB, and SpdC, shows the presence of SpdC and SagB. **c,** Overlaid size exclusion chromatograms from a SagB-SpdC co-purification and a full-length SagB purification show a peak shift to a higher molecular weight in the presence of SpdC.



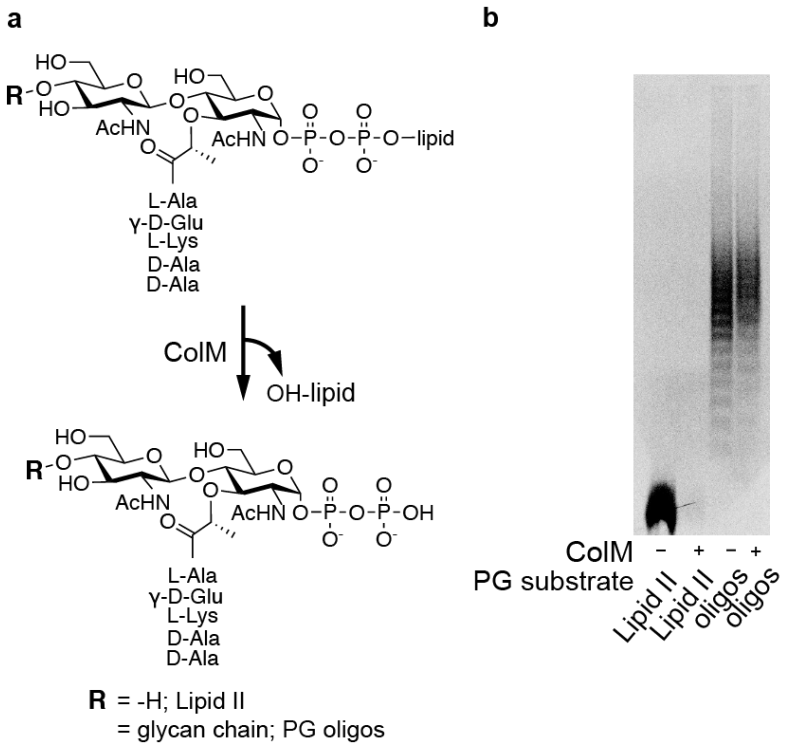
Supplementary Figure 3. SagB is related to the other *S. aureus* membrane-anchored glucosaminidase, SagA, but SagA does not form a stable complex with SpdC. a, Alignment of SagA and SagB glucosaminidase domains, colored gray and magenta, respectively (PDB# 4PIA and 6FXP)[4,5]. **b,** Primary sequence alignment of SagA (bottom) and SagB, with residues colored by type. Alignments were made using ClustalO and then coloured in MView (https://www.ebi.ac.uk/Tools/msa/clustalo/). SagA and SagB are 53% similar. **c,** Parallel attempts were made to co-purify SagB-SpdC and SagA-SpdC using FLAG-SpdC and histidine-tagged SagA or SagB. Solubilized membranes were purified on nickel resin and then anti-FLAG resin. Eluate from anti-FLAG resin was concentrated and run through a size exclusion column. A stable SagB-SpdC complex was obtained (blue trace), whereas SagA (violet trace) did not co-elute from anti-FLAG resin with FLAG-SpdC.



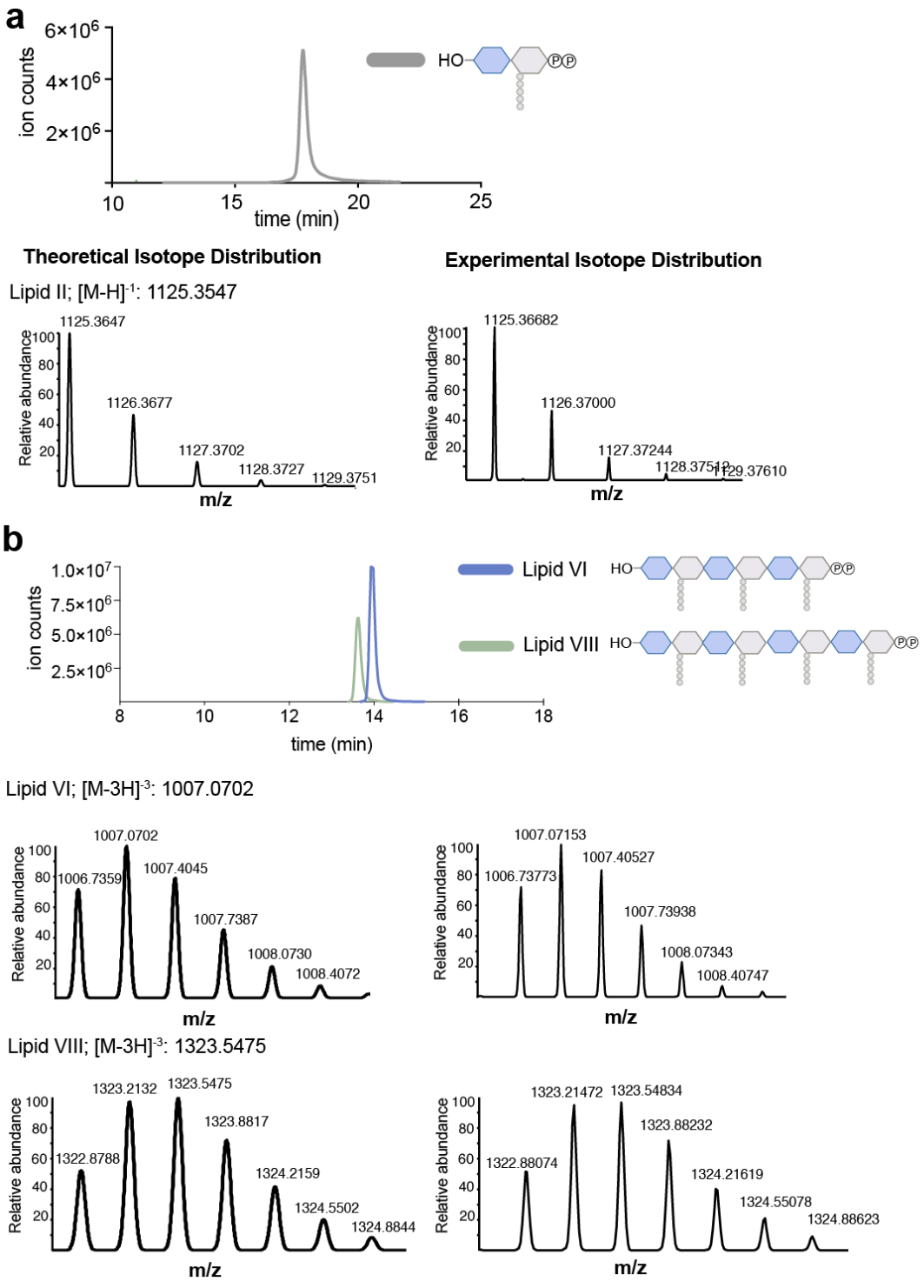
Supplementary Figure 4. Muramidase cleavage of radiolabeled peptidoglycan oligomers results in a diffuse signal at the same approximate position as cleavage products of glucosaminidases. a, Mutanolysin is a well-characterized muramidase that cleaves peptidoglycan strands into disaccharide units[1]. Radiolabeled peptidoglycan oligomers run as a ladder (lane 2), but incubation with mutanolysin (lane 3) changes the ladder to a diffuse signal that migrates towards the middle of the gel. The gel was replicated at least three times. **b,** Radiolabeled Lipid II incubated with SgtB* (SgtB^{Y181D}) resulted in a ladder of PG oligomers as visualized in the PAGE autoradiograph[2,3]. Radiolabeled product bands are similar to hydrolase reactions with PG oligomers prepared using PBP2 (see main text, Fig. 2b). PG oligomer treatment with SagB-SpdC resulted in two discrete signals on the autoradiograph, a ladder at the bottom of the gel and a signal towards the top of the gel that was also present in SagB, SagA, and mutanolysin reactions. These experiments were performed at least three times with similar results.



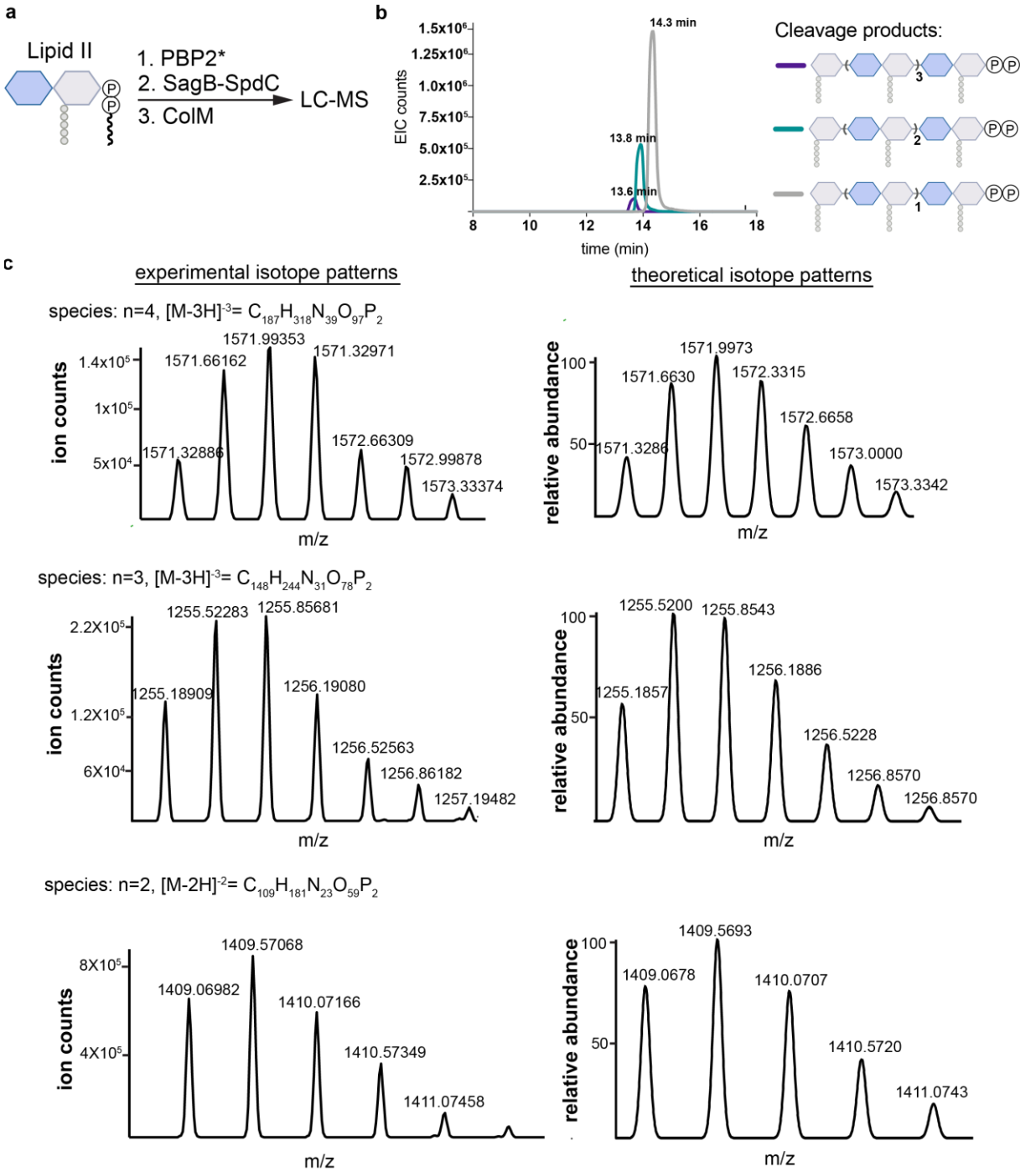
Supplementary Figure 5. SagB, SagA, and the SagB-SpdC complex cleave peptidoglycan strands into oligosaccharide fragments, but the complex favors longer products. **a**, This scheme shows the isolation of cleaved peptidoglycan fragments for LC-MS analysis. Peptidoglycan oligomers were incubated with a glucosaminidase (SagA, SagB, or SagB-SpdC). After the cleavage reaction, the reaction mixture was treated with sodium borohydride to reduce any lipid-free fragments[1]. **b**, Molecular ions corresponding to the expected muropeptides were extracted. Integrated peak areas for the muropeptides were normalized relative to one. The bars represent the averages of two biological experiments and the error bars represent standard deviation from the mean. These products presumably correspond to the diffuse signal and slower migrating products visualized in the PAGE autoradiograph experiments (see main text Fig. 2). These structures are consistent with glucosaminidase cleavage and also confirm the endolytic cleavage of nascent peptidoglycan oligomers. **c,** Targeted MS-MS of the tetrasaccharide cleavage product $(MG)_2$ from the SagB-SpdC reaction confirms that a GlcNAc is present on the reducing end. The $[M]^{+1}$ was targeted for fragmentation.



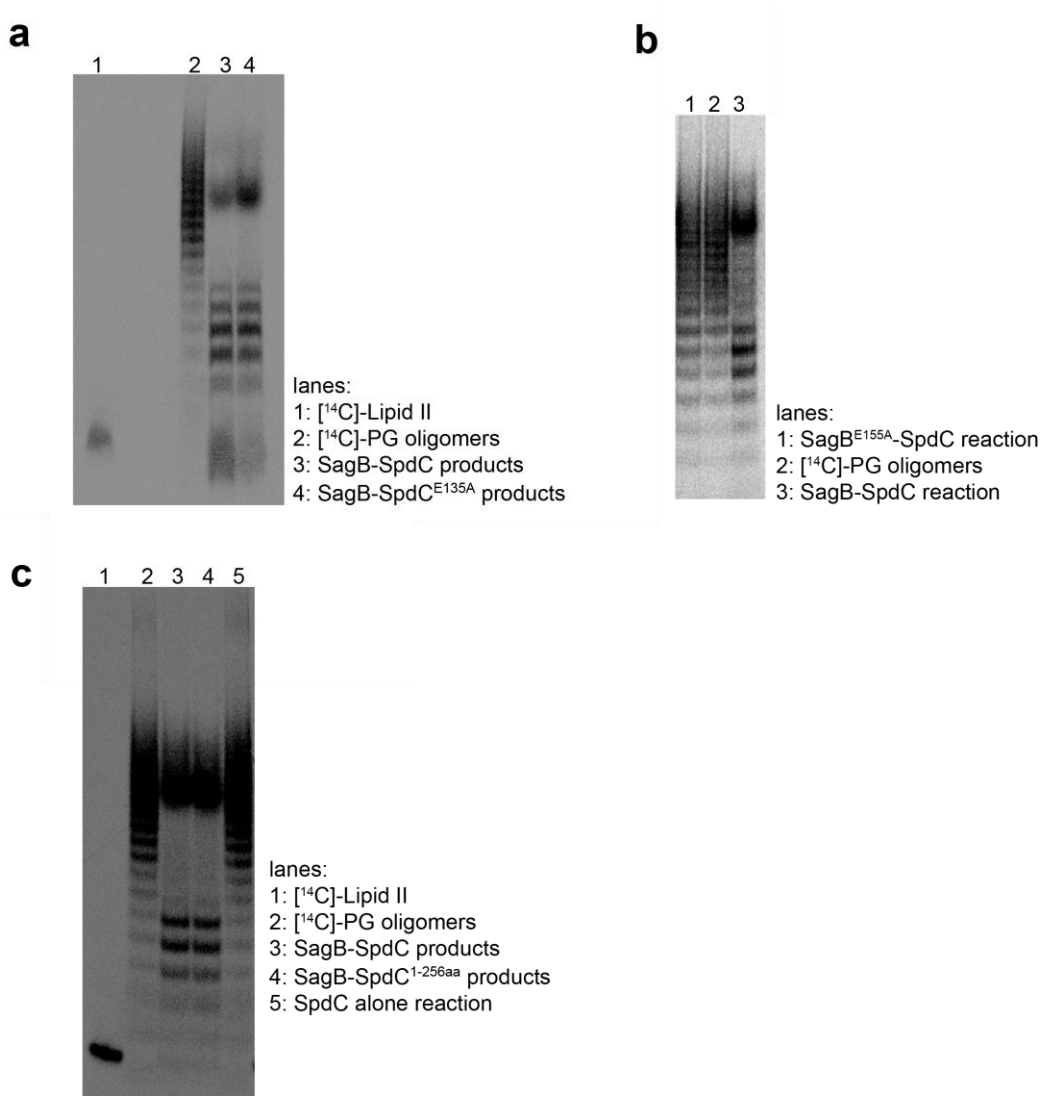
Supplementary Figure 6. Colicin M (ColM) cleaves the lipid anchor from Lipid II and PG oligomers. Radiobeled Lipid II runs as a single band at the bottom of PAGE autoradiograph; the signal disappears upon treatment with ColM presumably due to its inability to stay within the gel matrix. Radiolabeled PG oligomers run as a discrete ladder. When incubated with ColM, the oligomers also react but the products migrate similarly to the longer oligos (compare lane 3 to lane 4). Mass spectrometry confirmed the reactions (Supplementary Figure 7). The experiment was replicated at least two times.



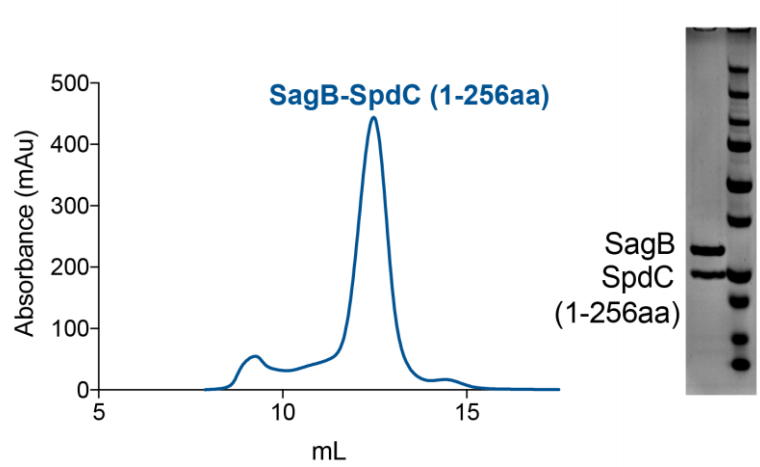
Supplementary Figure 7: ColM cleaves both Lipid II and linear peptidoglycan strands.



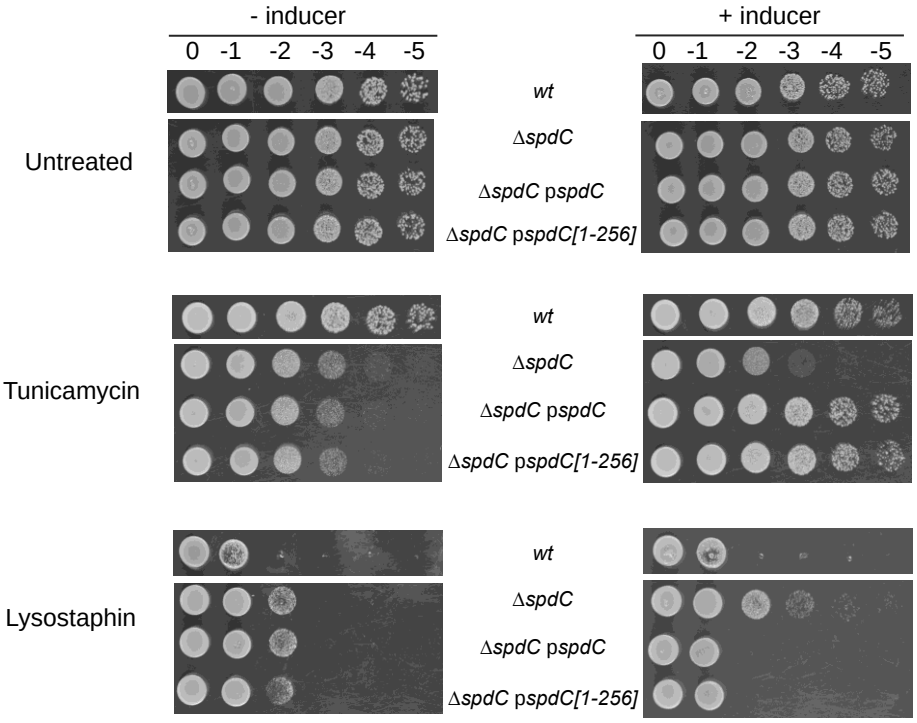
Supplementary Figure 8. LC-MS analysis of SagB-SpdC reactions incubated with colicin M (colM) confirms the identity of short, lipid-linked cleavage products. Pre-assembled peptidoglycan oligomers were incubated with SagB-SpdC, and then treated with ColM. Chromatogram traces and isotope patterns for three cleaved peptidoglycan oligomers containing a diphosphate indicate that ColM cleaved the lipid anchor. The odd number of sugars is consistent with glucosaminidase activity.



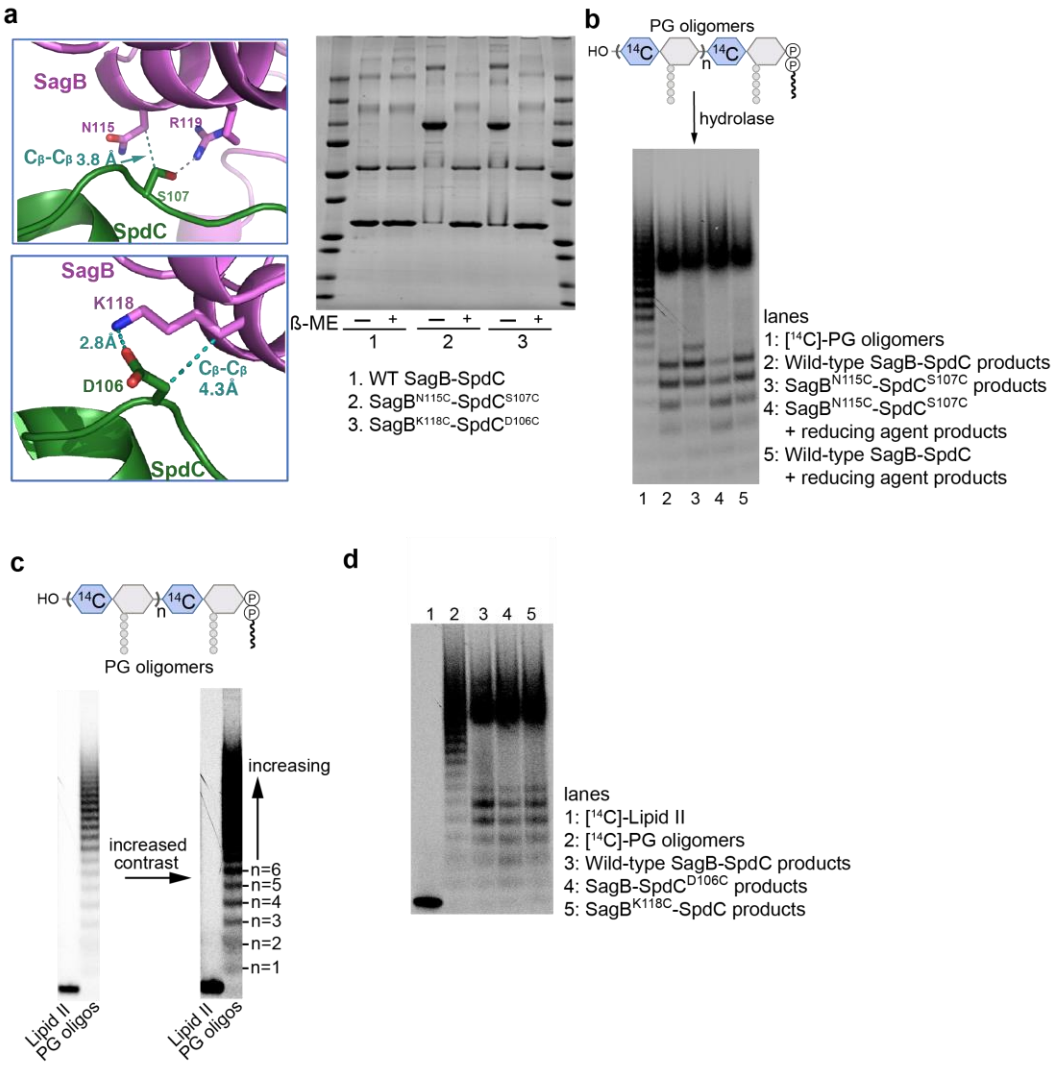
Supplementary Figure 9. The cleavage products of SagB-SpdC require the catalytic glutamate in SagB, but not the cytoplasmic C-terminal part or the CAAX proteolytic glutamate of SpdC. a, Activity of wild-type SagB-SpdC in the presence of radiolabeled peptidoglycan oligomers was tested alongside SagB-SpdC^{E135A}, in which the proposed CAAX proteolytic active site glutamate[9] is mutated to an alanine. Similar cleavage products are observed, indicating that the conserved SpdC residue is not important for SagB-SpdC glucosaminidase activity. **b,** In the presence of radiolabeled peptidoglycan oligomers, the activity of wild-type SagB-SpdC was compared to the activity of a variant predicted to be catalytically-inactive[10], SagB^{E155A}-SpdC. The similarity between the SagB^{E155A}-SpdC reaction (lane 1) and control peptidoglycan oligomers (lane 2) indicates that E155 is critical for cleavage. **c,** Radiolabeled peptidoglycan oligomers (lane 2) were incubated with wild-type SagB-SpdC (lane 3), truncated SagB-SpdC$^{1-256}$, which lacks the C-terminal, cytoplasmic region of SpdC (lane 4), and SpdC alone (lane 5). Purification of SagB-SpdC$^{1-256}$ is shown in Supplementary Fig. 10. SpdC alone does not affect the peptidoglycan oligomers, and the cytoplasmic region of SpdC is not required for the activity of the complex. The gels were replicated at least three times.



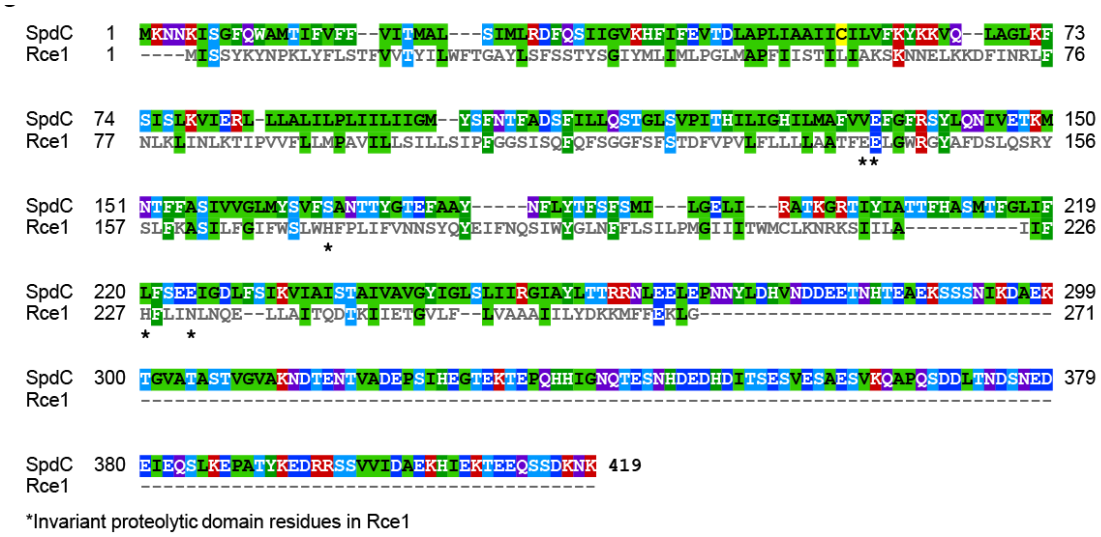
Supplementary Figure 10. SagB and a truncated SpdC variant lacking its C-terminal, cytoplasmic region (SpdC$^{1-256}$) co-purify as a stable complex. Expression and purification conditions used to obtain SagB-SpdC$^{1-256}$ complex were similar to those used in purification of the wild-type SagB-SpdC complex. A stable 1:1 protein complex was obtained after tandem affinity purifications and indicates that the C-terminal cytoplasmic region of SpdC is not required for complexation. *In vitro* and *in vivo* activity of this complex is also similar to that of wild-type SagB-SpdC (see Supplementary Figure 9).



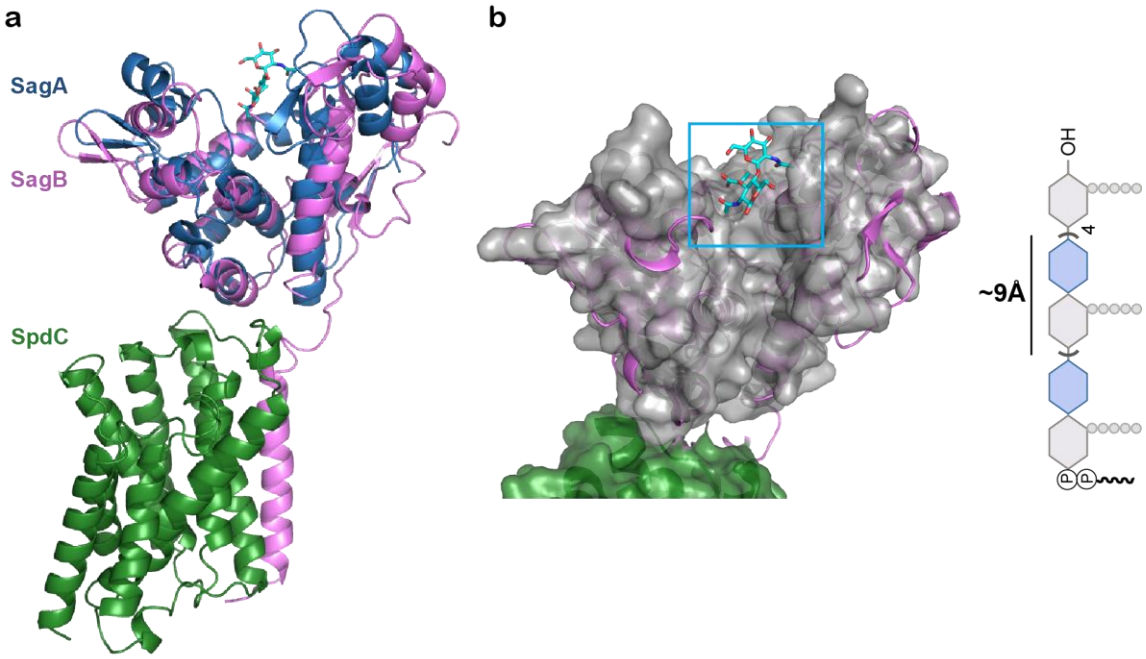
Supplementary Figure 11. The cell wall phenotypes of SpdC are unaffected by its cytoplasmic truncation. Spot dilution series performed as described in Extended Data Figure 3.



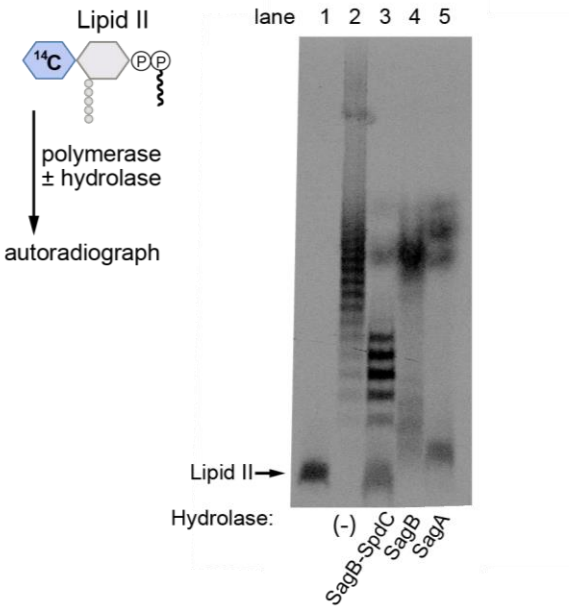
Supplementary Figure 12. Disulfide-stabilized SagB-SpdC complexes cleave nascent peptidoglycan to a longer distribution of lipid-linked oligomers than the wild-type complex. a, Close-up view of two interactions at the extracellular interface between SagB (violet) and SpdC (forest green). Sidechains of the relevant residues are shown as sticks. SpdC serine 107 hydrogen bonds to SagB arginine 119, however, its C_β is closest to the C_β of SagB asparagine 115. Wild-type SagB-SpdC, $SagB^{N115C}$-$SpdC^{S107C}$, and $SagB^{K118C}$-$SpdC^{D106C}$ were purified and then samples were mixed with 2x SDS-PAGE loading buffer with or without β-mercaptoethanol (β-ME), run on an acrylamide gel, and stained with coomassie. **b,** To test the effects of the cysteine substitutions and disulfide formation, radiolabeled PG oligomers (lane 1) were incubated with wild-type SagB-SpdC or $SagB^{N115C}$-$SpdC^{S107C}$ in the presence or absence of reducing agent. **c,** To assess the length (in sugar or disaccharide units) of lipid-linked, linear peptidoglycan fragments, we compare samples to Lipid II and the ladder of PG oligomers produced from it by PBP2. Each "step" in the ladder corresponds to the addition of a MurNAc-GlcNAc disaccharide. **d,** Single cysteine mutations in SagB-SpdC do not alter the distribution of cleavage products. Gels in **a-d** were repeated at least three times.



Supplementary Figure 13. Sequence alignment of SpdC and Rce1 shows partial conservation of catalytic residues. Sequence alignment of SpdC and Rce1 generated using Clustal Omega and colored in MView. Stars under the alignment mark positions of the catalytic residues in Rce1.



Supplementary Figure 14. Alignment of SagB-SpdC with SagA crystal structures shows that the reducing end of a substrate glycan strand would be adjacent to the SagB-SpdC interface. a, A crystal structure of SagA (blue) in complex with GlcNAc-MurNAc fragment (sticks) (PDB #4PI7) was aligned to SagB-SpdC (violet and forest green) using the glucosaminidase domain of SagB as the target. The orientation of the disaccharide fragment indicates that the reducing end faces SpdC and that the non-reducing end of the peptidoglycan substrate faces the top of the active site cleft. **b,** The same alignment shown in **a**, with SagA shown as a gray surface. The length of a disaccharide unit in the linear glycan chain is approximately 9 angstroms.



Supplementary Figure 15. SagB-SpdC cleaves peptidoglycan oligomers with an active polymerase present. Synthetic, [^{14}C]-Lipid II was incubated with PBP2 and a hydrolase (SagB-SpdC, SagB, or SagA). Similar cleavage products are observed in the presence of pre-assembled peptidoglycan oligomers (see main text Fig. 2b).

Data Collection	**SagB-SpdC**
Space Group	C2
Unit Cell	
Dimensions (a, b, c), Å	156.7, 54.8, 96.4
Angles (α, β, γ), °	90.0, 107.2, 90.0
Resolution range, Å	46.98 - 2.60 (2.71 - 2.60)
Completeness, %	99.3 (97.2)
R_{merge}	0.16 (1.86)
Mean I/σ(I)	4.7 (0.8)
Multiplicity	5.8 (5.1)
$CC_{1/2}$	0.994 (0.530)
Refinement	
No. unique reflections	24207
Rwork, % / Rfree, %	25.3 / 27.9
Average B ($Å^2$) protein	98.7
Average B ($Å^2$) ligands	121.8
Average B ($Å^2$) waters	87.7
Ramachandran plot	
Favored/disallowed (%)	96.82 / 0.00
rmsd from ideal geometry	
Bond lengths, Å	0.0025
Bond angles, °	0.49
PDB code	6U0O

Supplementary Table 3. Data collection and refinement statistics for SagB-SpdC$^{1-256}$.

Primer Name	Sequence (3'-5')
SM1	GATCTGATTGGATCCAATATTGAGCATACGCAATATC
SM2	CTAGCAAGCGCTTTGTATATGTAACCTCCATTAG
SM3	CTAATGGAGGTTACATATACAAAGCGCTTGCTAG
SM4	GATCTGATTGTCGACCATGACGCTGGGAATTGG
SM43	GATCTGATTGGATCCCATAATGACATCATGTTCATGTAC
SM44	CCTATCACCTCAAATGGTTCGCTATATGTAACCTCCATTAGG
SM45	CCTAATGGAGGTTACATATAGCGAACCATTTGAGGTGATAGG
SM46	GAGGTACTAGCAAGCGCTTTGTCCTAGGTACTAAAACAATTCATC
SM47	GATGAATTGTTTTAGTACCTAGGACAAAGCGCTTGCTAGTACCTC
SM48	GATCTGATTGTCGACGCAGAGCGCATCGGTCTTTTCG
SM124	GATTGGTACCGTTACCTAATGGAGG
SM125	TTAAGCTCAGCTTATTAATGGTGATGGTGATGGTGTTTGTTTTTATC TGAAGATTGTTC
SM130	CTGATGGCGTTCGTAGTAGCATTCGGATTCCGTTCATAC
SM131	GTATGAACGGAATCCGAATGCTACTACGAACGCCATCAG
SM132	GTAGTAGAATTCGGATTCGCATCATACTTACAAAATATTG
SM133	CAATATTTTGTAAGTATGATGCGAATCCGAATTCTACTAC
SM134	TATATTGCAACGACATTCGCAGCTTCAATGACATTCGGA
SM135	TCCGAATGTCATTGAAGCTGCGAATGTCGTTGCAATATA
SM165	GATTGGTACCAATGGAGGTTACATATAATGGAACAAAAACTTATTTC TGAAGAAGATCTGAAGAACAATAAAATTTCTGGTTTTCAATG
SM166	GATTGCTCAGCTTATTATTTGTTTTTATCTGAAGATTGTTCTTC
oJP21	CGGCGCTCAGCTCAGTGGTGGTGGTGGTGGT
oJP22	TGATGGTACCAACAATGACCTAAGAGGTGTGGATATGAATAAACAC AAGAAAGGTTCTATTTTTGG
oJP25	TGATGGTACCAATGGAGGTTACATATAA
oJP26	CGGCGCTCAGCTTATTATTTGTTTTTAT
oJP30	TGATGGTACCAATGGAGGTTACATATAATGGAGCAAAAACTTATTTC TGAAGAAGATC
oJP31	CGGCGCTCAGCTTATTATTTGTTTTTATCTGAAGATTGTTCCTCAG
F_pKTarO	GCTTATCAAAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATC
R_pkTarO	CAACAATGACCTAAGAGGTGTGGATGGATCCCCGGGTACCG

F_1kb+_sagB	AAGATATATGTTCAATAAAATAACTTAGTTGGGAATGGGCTTCATTTCAG
R_1kb+_sagB	GATTACGCCAAGCTTGCATGCCTGCAGGTCGACTTTGATAAGC
F_1kb(-)_sagB	CGGTACCCGGGGATCCATCCACACCTCTTAGGTCATTGTTG
R_1kb(-)_sagB	ATTGCGAGATTTGGGTTGTTGAGCCATAGTCTTTCTCTTTGATTTAAAAG
F_tetR_sagB	TTAAATCAAAGAGAAAGACTATGGCTCAACAACCCAAATCTCGCAATTTG
R_tetR_sagB	AAGCCCATTCCCAACTAAGTTATTTTATTGAACATATATCTTACTTTATC
oJP32	CCGGGGATCCCGAAGGTCATGTTCATTAAAGTATGTTG
oJP33	AACTCAAATTGCGAGATTTGGGTTGATCCACACCTCTTAGGTCATTGTTG
oJP35	GCAGGTCGACCATCAATAATGAATAATATGCCTACAGTTTTAATA
oJP49	TTTGATAAGCTACGAGTTGTTTTT
oJP50	ATCCACACCTCTTAGGTCATTGT
oJP51	CTAAGAGGTGTGGATCAACCCAAATCTCGCAAT
oJP52	TCGTAGCTTATCAAACTAAGTTATTTTATTGAACAT
oJP53	GGATCCCCGGGTACCGAG
oJP54	GGTACCCGGGGATCCCGAAGGTCATGTTCATTAAAGTATG
oJP79	TAGAGAATAGGAACTTCATCCACACCTCTTAGGTCATTGTTG
oJP80	GAAAGTATAGGAACTTCTTTGATAAGCTACGAGTTGTTTTTATGACTC
oJP81	CGGCGCTCAGCTTATGCAATACCTCGGATAATTAAGCTTAAAC
oTD73	TATTCTCTAGAAAGTATAGGAACTTCGCGAACCATTTGAGGTGATAGGTAAGATTAT
oTD74	TACTTTCTAGAGAATAGGAACTTCTCCTAGGTACTAAAACAATTCATCCAGTAA
oTD145	CAACCCAAATCTCGCAATTTGAGTTG
F_SpdC	CATCCACTGAATATATGAAGAACAATAAAATTTCTGGTTTTCAATG
R_SpdC	GCTCGAATTCGGATCCTTATTTGTTTTTATCTGAAGATTGTTCTT
F_DUET_FLAG_SpdC	CAGATAAAAACAAATAAGGATCCGAATTCGAGCTCGG
R_DUET_FLAG_SpdC	GAAATTTTATTGTTCTTCATATATTCAGTGGATGACCCCCCAG
F_SagB	GAGATATACATATGAATAAACACAAGAAAGGTTCTATTTTTGG

R_SagB	GCAAGCTTCTTATTCAAATGTTTACTGTCATCTTTATACAC
F_DUET_SagB	ACAGTAAACATTTGAATAAGAAGCTTGCGGCCG
R_DUET_SagB	TCTTGTGTTTATTCATATGTATATCTCCTTCTTATACTTAACTAATATAC
R_Trunc_SpdC	CCGAGCTCGAATTCGGATCCTTATGCAATACCTCGGAT
F_DUET_trunc_SpdC	ATCCGAGGTATTGCATAAGGATCCGAATTCGAGCTCGG
F_SagB_alone	TTAAGAAGGAGATATACCATGAATAAACACAAGAAAGGTTCTATTTTTGG
R_SagA_alone	GCAAGCTTCTTATTCAAATGTTTACTGTCATCTTTATACAC
F_plasmid_SagA	ACAGTAAACATTTGAATAAGAAGCTTGCGGCCG
R_plasmid_SagA	AGAACCTTTCTTGTGTTTATTCATGGTATATCTCCTTCTTAAAGTTAAAC
F_soluble_SagB	ATGGCTAGCCAGATATTTTTCAAACATGTTAAATCCG
R_soluble_SagB	CATTTGCTGTCCACCAGTCATTTACTTATTCAAATGTTTACTGTCATC
F_plasmid_soluble_SagB	GATGACAGTAAACATTTGAATAAGTAAATGACTGGTGGACAGCAAATG
R_plasmid_soluble_SagB	GCTAGCCATATGGCTGCCG
F_SagB_NRIKRMLVD_to_SEVNQLLKG	CAAGGGATTGATAAA TCTGAAGTTAACCAATTGCTAAAAGGT AGACCAACGTTATTGAAACATACGGATG
R_SagB_NRIKRMLVD_to_SEVNQLLKG	GTTGGTCTACCTTTTAGCAATTGGTTAACTTCAGATTTATCAATCCC TTGATACTTTGATAAATCTAAAA
F_SagB_interface_mut	TCTAGAATTAATCAAATGTTAGACGGTAGACCAACGGAATTGAAACA TACGGATGATTTCTTAAAAGCTG
R_SagB_interface_mut	TTC CGT TGG TCT ACC GTC TAA CAT TTG ATT AAT TCTAGATTT ATC AAT CCC TTG ATA CTT TGA TAA ATC
F_SpdC_D106R	GGTATGTACAGCTTTAATACATTTGCAAGAAGCTTTATTTTATTACAA TCAACAGGC
R_SpdC_D106R	GCC TGT TGA TTG TAA TAA AAT AAA GCT TCT TGC AAA TGT ATT AAA GCT GTA CAT ACC
F_SagB_K118D	GTATCAAGGGATTGATAAAAATAGAATTGATCGTATGTTAGTAGATA GACCAACG
R_SagB_K118D	CGTTGGTCTATCTACTAACATACGATCAATTCTATTTTTATCAATCCT TGATAC
R_pETduet_s2_SagB1-5	TTTCTTGTGTTTATTCATATGTATATCTCCTTCTTATACTTAAC
F_pETduet_SagA5	GTTAAGTATAAGAAGGAGATATACATATGAATAAACAC AAGAAA AATTTCAAGTTACGCATTTCAACGC
F_SagA_TM_with_SagB_overlap	CGGATTTAACATGTTTGAACAATTTAGTTTCATTCACGATGAGTAAT ACAGC

R_SagA_TM_with_SagB_overlap	CGGATTTAACATGTTTGAACAATTTAGTTTCATTCACGATGAGTAATACAGC
F_SagB_TM_then_SagB_F36	CTCATTTTTATTTTTCTCAATGATATCCGATACTAAATTGTTTAAAAATGATGTGAATTACTC
R_SagB_TM_then_SagB_F36	GAGTAATTCACATCATTTTTAAACAATTTAGTATCGGATATCATTGAGAAAAATAAAAATGAG
R_SpdC_D106C	GCCTGTTGATTGTAATAAAATAAAGCTGCATGCAAATGTATTAAAGCTGTACATACC
F_SpdC_S107C	GTACAGCTTTAATACATTTGCAGATTGCTTTATTTTATTACAATCAACAGGC
R_SpdC_S107C	GCCTGTTGATTGTAATAAAATAAAGCAATCTGCAAATGTATTAAAGCTGTAC
F_SagB_N115C	TTATCAAAGTATCAAGGGATTGATAAATGTAGAATTAAACGTATGTTAGTAGATAGAC
R_SagB_N115C	GTCTATCTACTAACATACGTTTAATTCTACATTTATCAATCCCTTGATACTTTGATAA
F_SagB_K118C	GTATCAAGGGATTGATAAAAATAGAATTTGCCGTATGTTAGTAGATAGACCAACG
R_SagB_K118C	CGTTGGTCTATCTACTAACATACGGCAAATTCTATTTTTATCAATCCC TTGATAC

Supplementary Table 4. Primers used in this study

Strain	**Genotype**	**Source**
RN4220 *S. aureus*	Wild-type	[12]
TD011	RN4220 *S. aureus* (pTP44)	[13]
HG003 *S. aureus*	Wild-type	[14]
TM283	*S. aureus* USA300 cured of pUSA300HOUMR; Tn library host	[15]
SHM002	HG003 Δ*spdC*	This study
SHM056	HG003 Δ*spdC*::*kan*^{R}	This study
SHM226	HG003 Δ*spdC*::*kan*^{R}, pSM_*spdC*_Myc	This study
JP012	HG003 *sagB::Tn-erm*^{R}	This study
JP051	HG003 *sagB::Tn-erm*^{R} (pJP15)	This study
JP053	HG003 *sagB::Tn-erm*^{R} (pJP19)	This study
JP054	HG003 Δ*spdC::kan*^{R} (pJP17)	This study
JP061	HG003 Δ*spdC*::*kan*^{R} (pSM_*spdC*_E135A)	This study
JP062	HG003 Δ*spdC*::*kan*^{R} (pSM_*spdC*_R139A)	This study
JP063	HG003 Δ*spdC*::*kan*^{R} (pSM_*spdC*_H210A)	This study
JP064	HG003 Δ*spdC*::*kan*^{R} (pJP22)	This study
JP065	HG003 Δ*spdC*::*kan*^{R} *sagB::Tn-erm*^{R}	This study
JP128	HG003 Δ*spdC::kan*^{R} (pJP42)	This study
JP132	HG003 Δ*sagB::kan*^{R}	This study
BL21(DE3) *E. coli*	*F- ompT hsdSB*(rB- mB-) *gal dcm* (DE3)	Novagen
NovaBlue(DE3) *E. coli*	*endA1 hsdR17* (rk12- mk12+) *supE44thi-1 recA1 gyrA96 relA1 lac* (DE3) *F' proAB lacI*^{Q}*ZΔM15::Tn10*(Tet^R)*gyrA96*(nal^R) *thi-1 recA1 relA1 lac glnV44 F'*[*::Tn10 proAB*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] *hsdR17*(*rK–mK+*)	Novagen
Plasmid	**Description**	**Source**

pTarKO	*cam*^{R} vector for making gene deletions in *S. aureus*	[16]
pKFC	*cam*^{R} vector for making gene deletions in *S. aureus*	[17]
pKFC_*spdC_kan*	pKFC vector to make Δ*spdC*::*kan*^{R} marked deletion in *S. aureus*	This study
pKFC_*spdC*	pKFC vector to make Δ*spdC* unmarked deletion in *S. aureus*	This study
pTP63	atc-inducible, integrative *cam*^{R} expression vector for *S. aureus*	[13]
pSM_*spdC_myc*	pTP63-cMyc-*spdC* containing native *spdC* ribosome-binding site	This study
pSM_*spdC_his*	pTP63-*spdC*-His_6 containing native *spdC* ribosome-binding site	This study
pSM_*spdC*_E135A	pTP63-*spdC E135A*-His_6 containing native *spdC* ribosome-binding site	This study
pSM_*spdC* _R139A	pTP63-*spdC R139A*-His_6 containing native *spdC* ribosome-binding site	This study
pSM_*spdC* _H210A	pTP63-*spdC H210A*-His_6 containing native *spdC* ribosome-binding site	This study
pJP15	pTP63-*sagB*-His_6 containing native *sagB* ribosome-binding site and 7 amino acid linker	This study
pJP17	pTP63-cMyc-*spdC* containing native *spdC* ribosome-binding site	This study
pJP19	pTP63-*sagB* E155A-His_6 containing native *sagB* ribosome-binding site and 7 amino acid linker	This study
pJP22	pTP63-cMyc-*spdC* E135A/R139A/H210A containing native *spdC* ribosome-binding site	This study
pJP42	pTP63-cMyc-*spdC[aa1-256]* containing native *spdC* ribosome-binding site	This study
pJP47	pTarKO vector to make Δ*sagB::kan*^{R} marked deletion in *S. aureus*	This study
pETDuet-FLAG	*amp*^{R} vector for dual protein expression	[18]
pAM174	*cam*^{R} vector for Ulp1 protease expression	[19]
pET24b(+)-*sgtBY181D*	*amp*^{R}-marked *S. aureus* $SgtB^{WT}$-His_6 expression vector	[3]
pMW1010	pET28b(+)-His6-*EF_3129* [T36-P429] expression vector for *E. faecalis* PBPX, *kan*^{R}	[6]
p*pbp2*^{S398G}	pET_42a containing a PBP2 construct with a S398G, TP inactive mutation	[8]
p*spdC_sagB*	pDUET containing a SUMO-FLAG-SpdC and a SagB-His_6	This study
p*spdC_sagB with TM of SagA*	pDUET containing a SUMO-FLAG-SpdC and a SagB-His_6 with the TM of SagA	This study
p*spdC_sagA with TM of SagB*	pDUET containing a SUMO-FLAG-SpdC and a SagA-His_6 with the TM of SagB	This study
pspdC alone	pDUET containing only SUMO-FLAG-SpdC	This study
p*spdC*^{E135A}*_sagB*	pDUET containing a SUMO-FLAG-SpdC with E135A mutation and a SagB-His_6	This study
p*spdC_sagB*^{E155A}	pDUET containing a SUMO-FLAG-SpdC and a SagB-His_6 with a E155A mutation	This study

pDUET_*spdC^{D106R}_sagB*	pDUET containing a SUMO-FLAG-SpdC with a D106R mutation and a SagB-His_6	This study
pDUET_*spdC_sagB* *interface* mutant*	pDUET containing a SUMO-FLAG-SpdC and a SagB-His_6 the following mutations: N115S, K118N, R119Q, V122D, D123G, L127E	This study
p*spdC^{S107C}_sagB^{N115C}*	pDUET containing a cysteine pair SUMO-FLAG-SpdC (S107C) and a SagB-His_6 with a N115C	This study
p*spdC^{D106C}_sagB^{K118C}*	pDUET containing a cysteine pair SUMO-FLAG-SpdC (D106C) and a SagB-His_6 with a K118C	This study
p*sagB*	pDUET containing a full-length SagB-His_6	This study
p*sagB$^{(33\text{-}end)}$*	pET_28(b-) containing a soluble, SagB construct lacking the transmembrane domain (33-end aa)	This study
p*spdC*	pDUET containing a SpdC with an amino terminal sumo and flag epitope fusion	This study
p*spdC^{truncated}_sagB*	pDUET containing a truncated SpdC lacking the cytoplasmic carboxy terminus (1-253 aa) with an amino terminal Sumo and flag epitope fusion and a SagB-His_6	This study
p*spdC_sagA*	pDUET containing a SpdC with an amino terminal sumo and flag epitope fusion and a SagA-His_6	This study

Supplementary Table 5. Strains and plasmids used in this study

References:

1. Lebar, M. D. *et al.* Forming cross-linked peptidoglycan from synthetic gram-negative Lipid II. *J. Am. Chem. Soc.* **135**, 4632-4635, doi:10.1021/ja312510m (2013).

2. Schaefer, K., Matano, L. M., Qiao, Y., Kahne, D. & Walker, S. In vitro reconstitution demonstrates the cell wall ligase activity of LCP proteins. *Nat. Chem. Biol.* **13**, 396-401, doi:10.1038/nchembio.2302 (2017).

3. Rebets, Y. *et al.* Moenomycin resistance mutations in Staphylococcus aureus reduce peptidoglycan chain length and cause aberrant cell division. *ACS Chem. Biol.* **9**, 459-467, doi:10.1021/cb4006744 (2014).

4. Mihelic, M. *et al.* The mechanism behind the selection of two different cleavage sites in NAG-NAM polymers. *IUCrJ* **4**, 185-198, doi:10.1107/S2052252517000367 (2017).

5. Pintar, S., Borisek, J., Usenik, A., Perdih, A. & Turk, D. Domain sliding of two Staphylococcus aureus N-acetylglucosaminidases enables their substrate-binding prior to its catalysis. *Commun. Biol.* **3**, 178, doi:10.1038/s42003-020-0911-7 (2020).

6. Welsh, M. A. *et al.* Identification of a Functionally Unique Family of Penicillin-Binding Proteins. *J. Am. Chem. Soc.* **139**, 17727-17730, doi:10.1021/jacs.7b10170 (2017).

7. Qiao, Y. *et al.* Detection of lipid-linked peptidoglycan precursors by exploiting an unexpected transpeptidase reaction. *J. Am. Chem. Soc.* **136**, 14678-14681, doi:10.1021/ja508147s (2014).

9. Qiao, Y. *et al.* Lipid II overproduction allows direct assay of transpeptidase inhibition by beta-lactams. *Nat. Chem. Biol.* **13**, 793-798, doi:10.1038/nchembio.2388 (2017).

9. Grundling, A., Missiakas, D. M. & Schneewind, O. Staphylococcus aureus mutants with increased lysostaphin resistance. *J. Bacteriol.* **188**, 6286-6297, doi:10.1128/JB.00457-06 (2006).

10. Alcorlo, M., Martinez-Caballero, S., Molina, R. & Hermoso, J. A. Carbohydrate recognition and lysis by bacterial peptidoglycan hydrolases. *Curr Opin Struct Biol* **44**, 87-100, doi:10.1016/j.sbi.2017.01.001 (2017).

11. Do, T. *et al.* Staphylococcus aureus cell growth and division are regulated by an amidase that trims peptides from uncrosslinked peptidoglycan. *Nat. Microbiol.* **5**, 291-303, doi:10.1038/s41564-019-0632-1 (2020).

12. Nair, D. *et al.* Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. *J. Bacteriol.* **193**, 2332-2335, doi:10.1128/JB.00027-11 (2011).

13. Pang, T., Wang, X., Lim, H. C., Bernhardt, T. G. & Rudner, D. Z. The nucleoid occlusion factor Noc controls DNA replication initiation in Staphylococcus aureus. *PLoS Genet.* **13**, e1006908, doi:10.1371/journal.pgen.1006908 (2017).

14. Herbert, S. *et al.* Repair of global regulators in Staphylococcus aureus 8325 and comparative analysis with other clinical isolates. *Infect Immun* **78**, 2877-2889, doi:10.1128/IAI.00088-10 (2010).

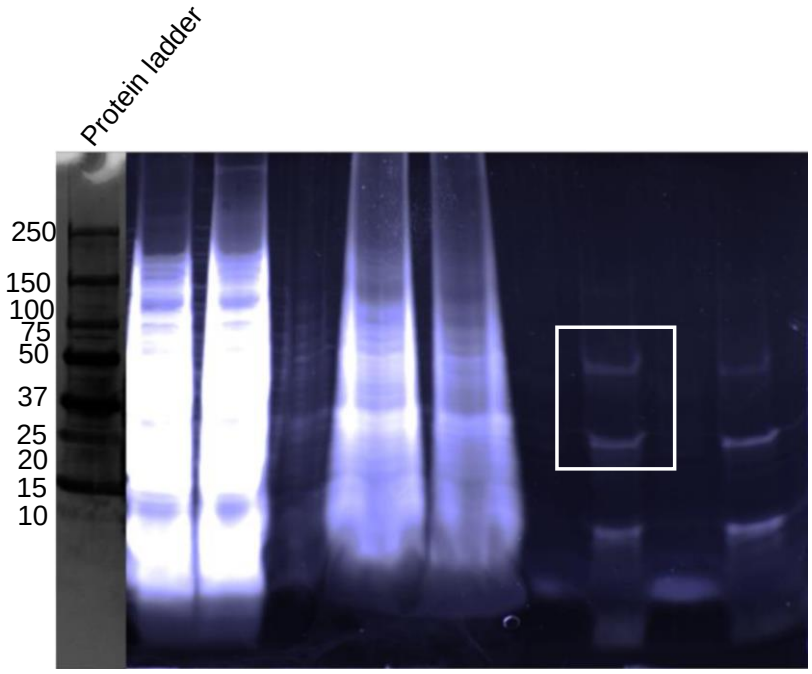
15. Coe KA, Lee W, Stone MC, Komazin-Meredith G, Meredith TC, Grad YH, et al. Multi-strain Tn-Seq reveals common daptomycin resistance determinants in *Staphylococcus aureus*. *PLoS Pathog.* **15**, e1007862. doi:10.1371/journal.ppat.1007862 (2019)

16. Lee, W. *et al.* Antibiotic Combinations That Enable One-Step, Targeted Mutagenesis of Chromosomal Genes. *ACS Infect. Dis.* **4**, 1007-1018, doi:10.1021/acsinfecdis.8b00017 (2018).

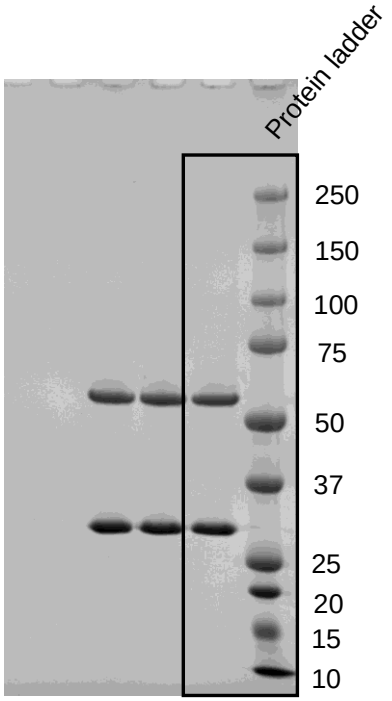
17. Kato, F. & Sugai, M. A simple method of markerless gene deletion in Staphylococcus aureus. *J. Microbiol. Methods* **87**, 76-81, doi:10.1016/j.mimet.2011.07.010 (2011).

18. Taguchi, A. *et al.* FtsW is a peptidoglycan polymerase that is functional only in complex with its cognate penicillin-binding protein. *Nat. Microbiol.* **4**, 587-594, doi:10.1038/s41564-018-0345-x (2019).

19. Meeske, A. J. *et al.* SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* **537**, 634-638, doi:10.1038/nature19331 (2016).



Unmodified gel shown in Supplementary Figure 1.



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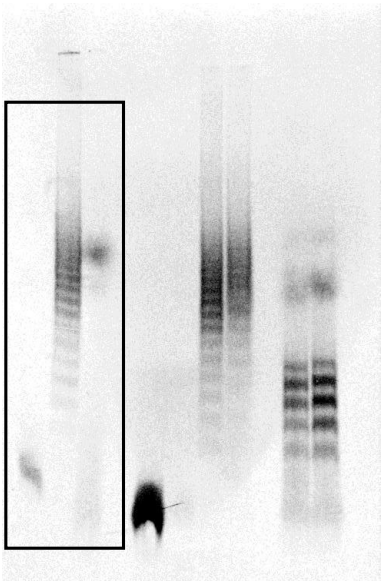

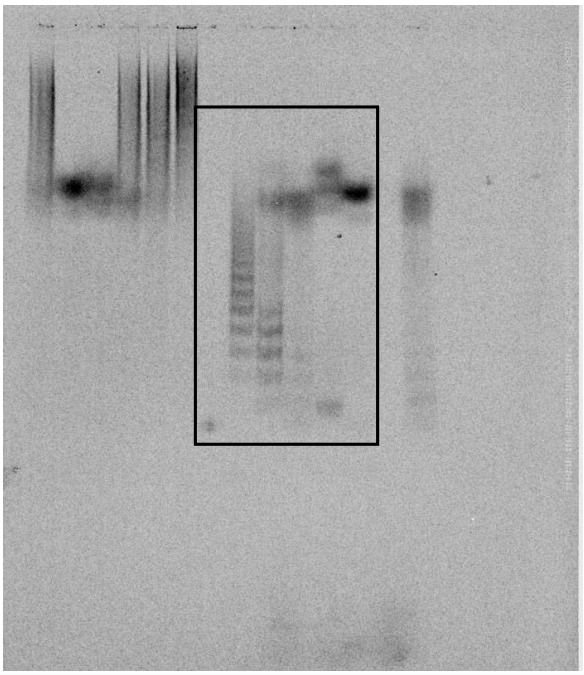

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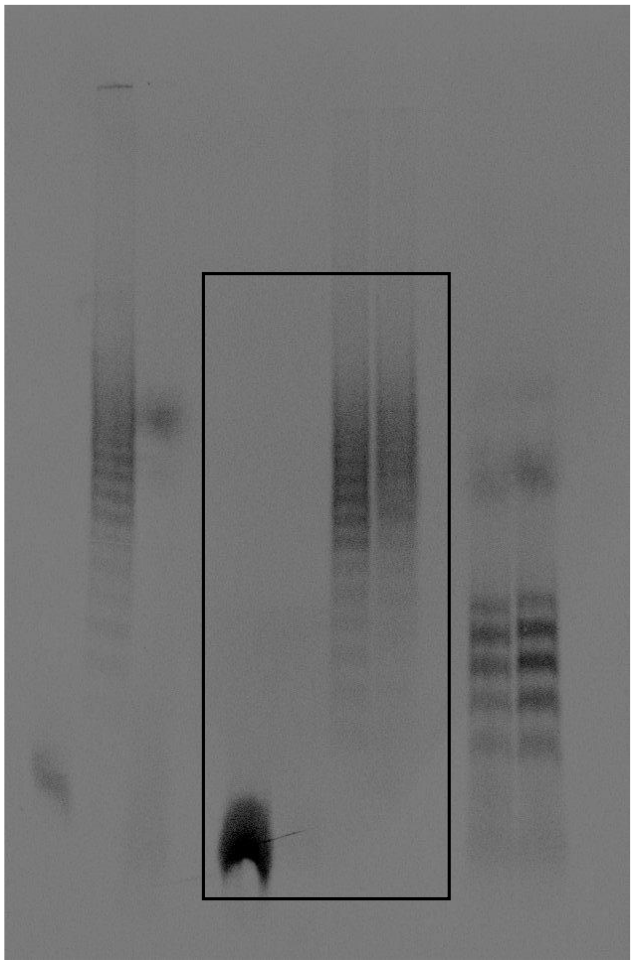

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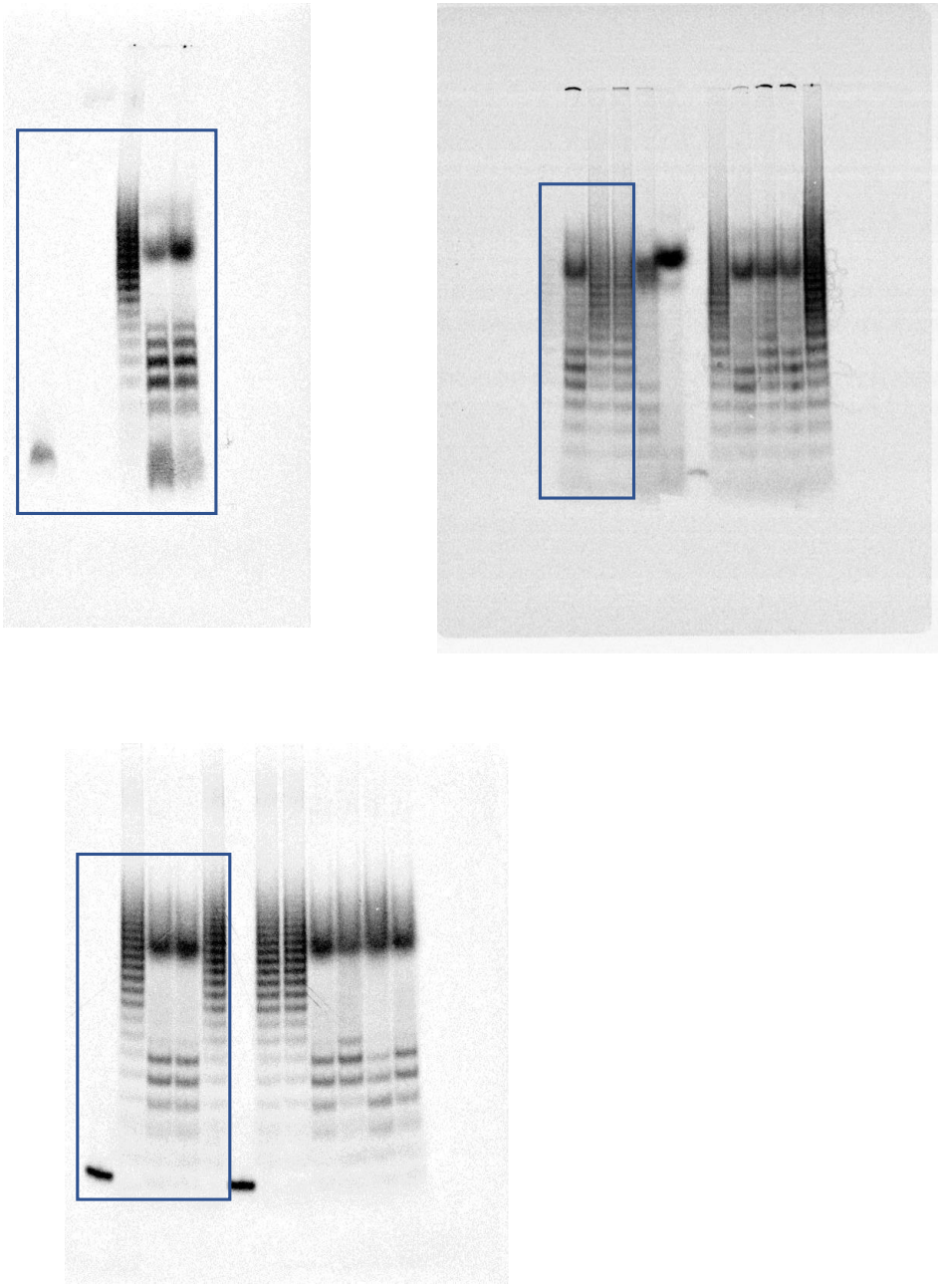

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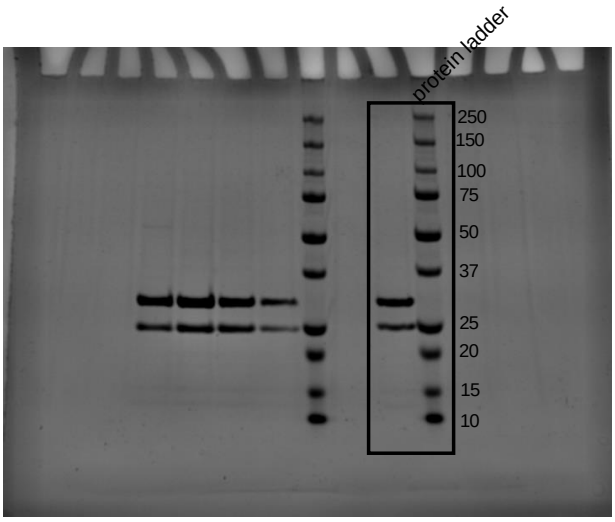

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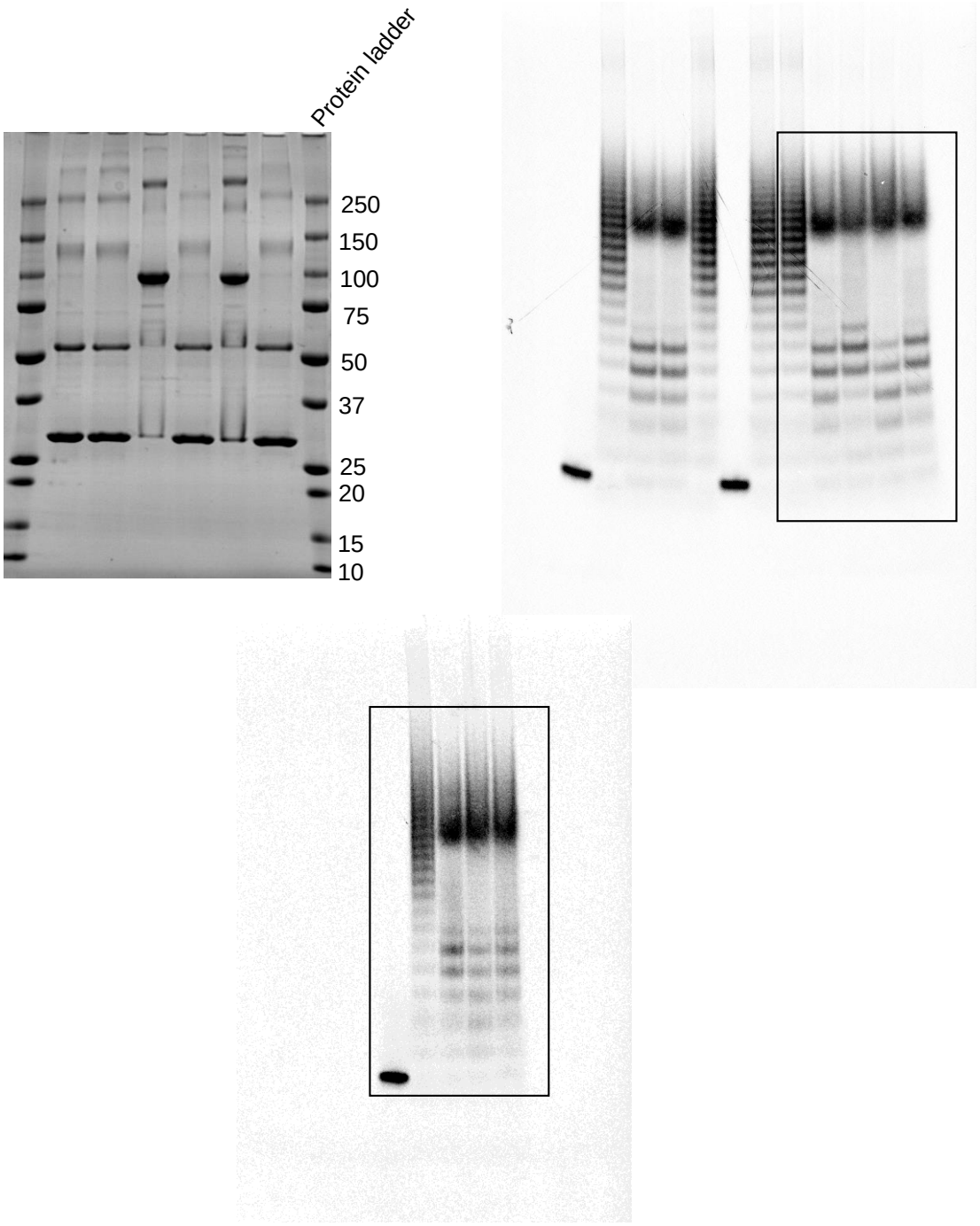
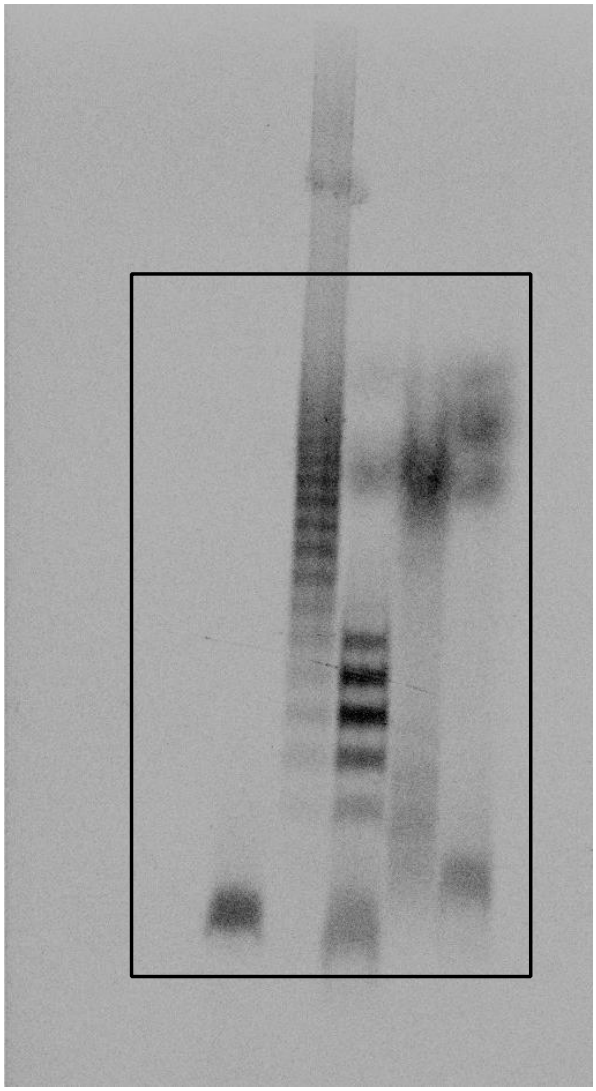


Unmodified gels and autoradiographs shown in Supplementary Figure 12.



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