SI Appendix

A conserved subcomplex within the bacterial cytokinetic ring activates cell wall synthesis by the FtsW-FtsI synthase

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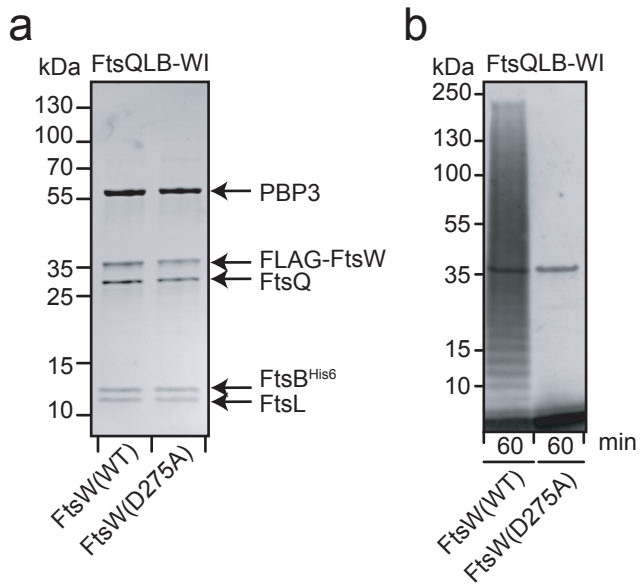

Figure S1: Comparison of FtsQLBWI complex activity with FtsQLB-W(D275A)I. a. Coomassie stained gel of the indicated complexes purified and labeled as in **Figure 1a. b.** PG polymerization activity of the indicated complexes was assayed as in **Figure 1b.**

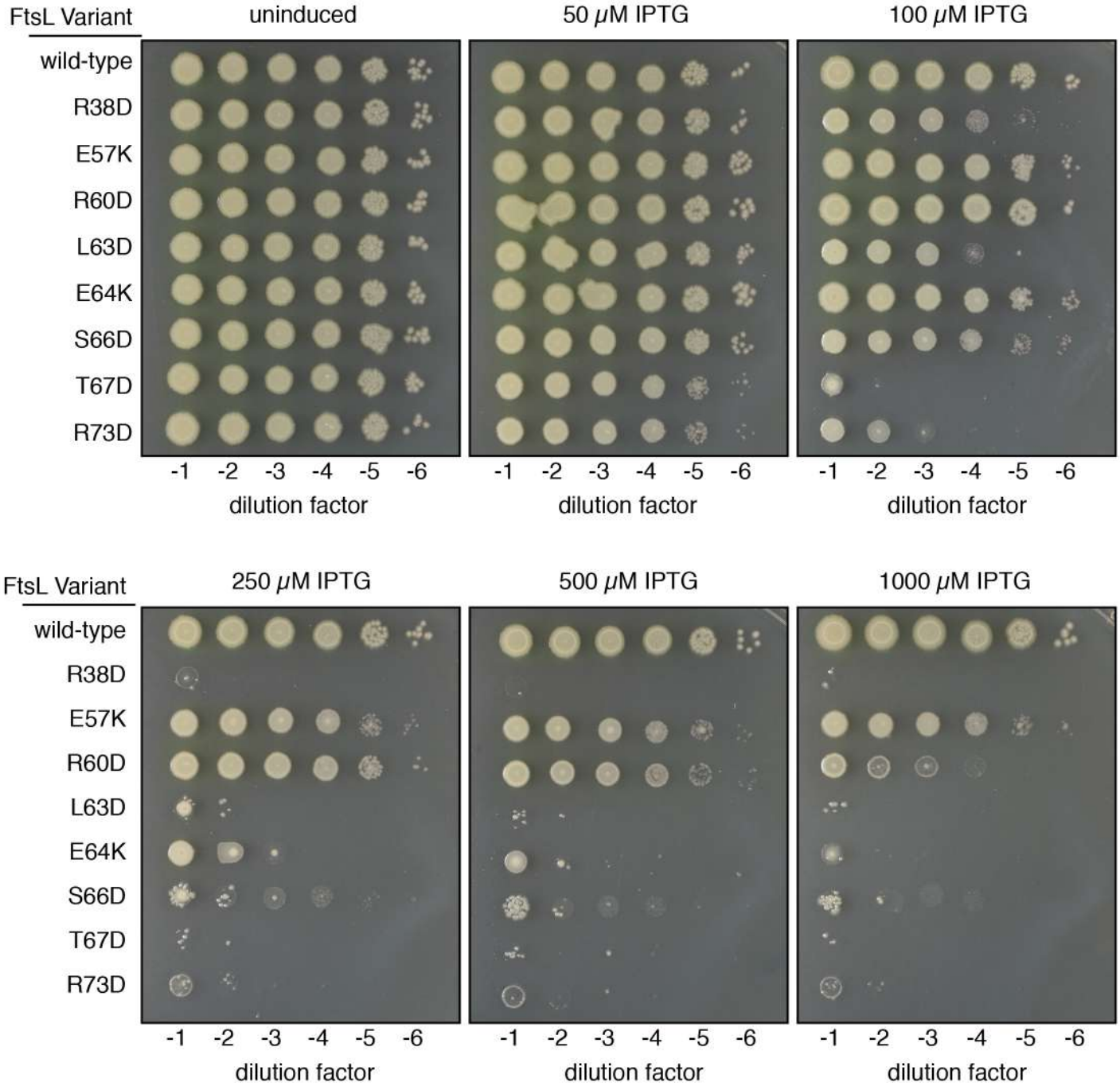

Figure S2: Effect of inducer concentration on variant toxicity. Ten-fold serial dilutions of *P. aeruginosa* PAO1 cells harboring expression plasmids producing the indicated FtsL variant were plated on LB agar with the indicated IPTG concentration to induce overproduction of the FtsL variants.

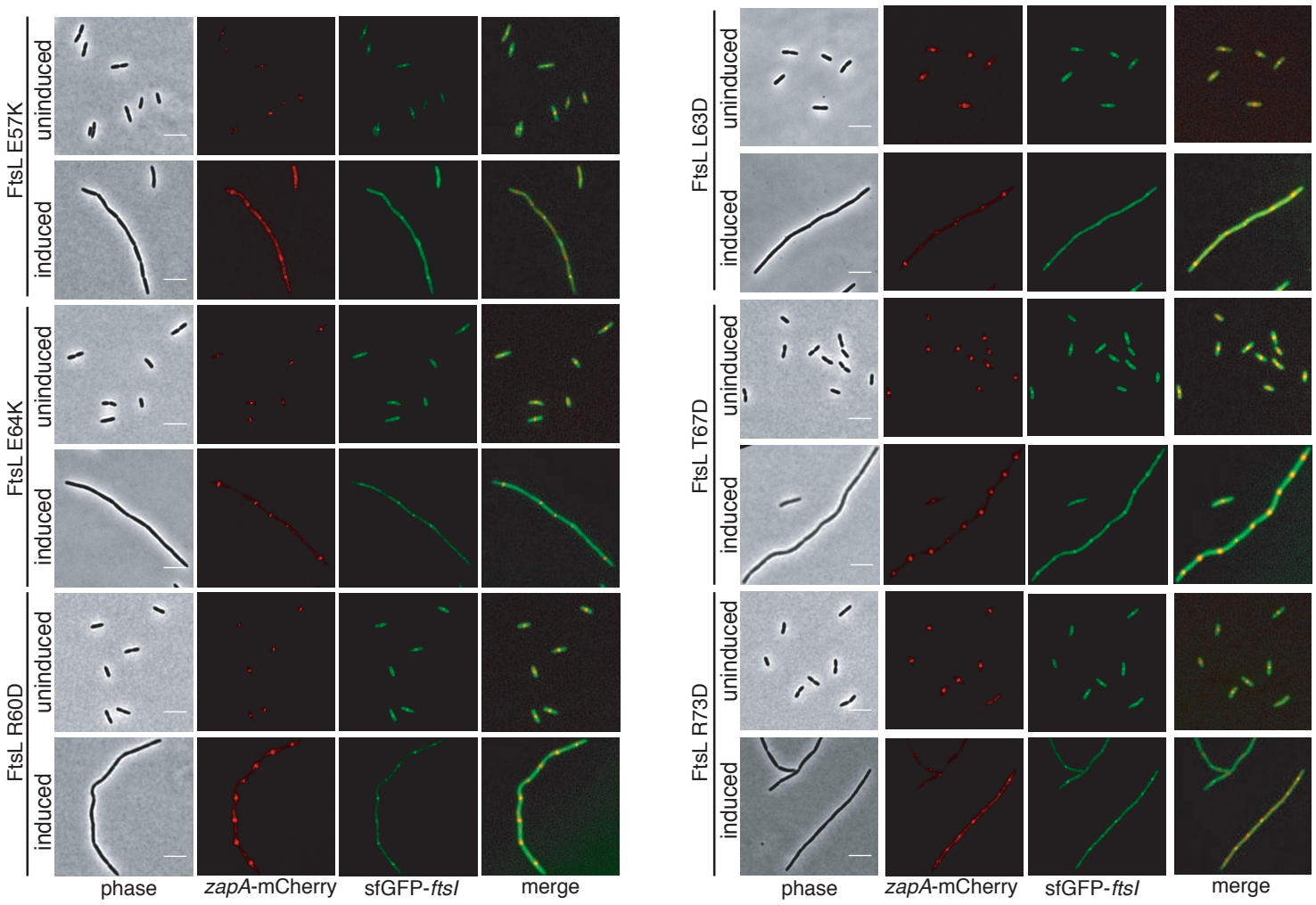

Figure S3: Cell division phenotype induced by additional ^{DN}FtsL variants. Shown are phase contrast, mCherry, GFP, and merged mCherry/GFP micrographs of *P. aeruginosa* PAO1 cells grown with or without IPTG to induce production of the indicated FtsL variants from derivatives of the pLSM11 plasmid. Scale bar = 5 μm. Representative images of two independent experiments are shown.

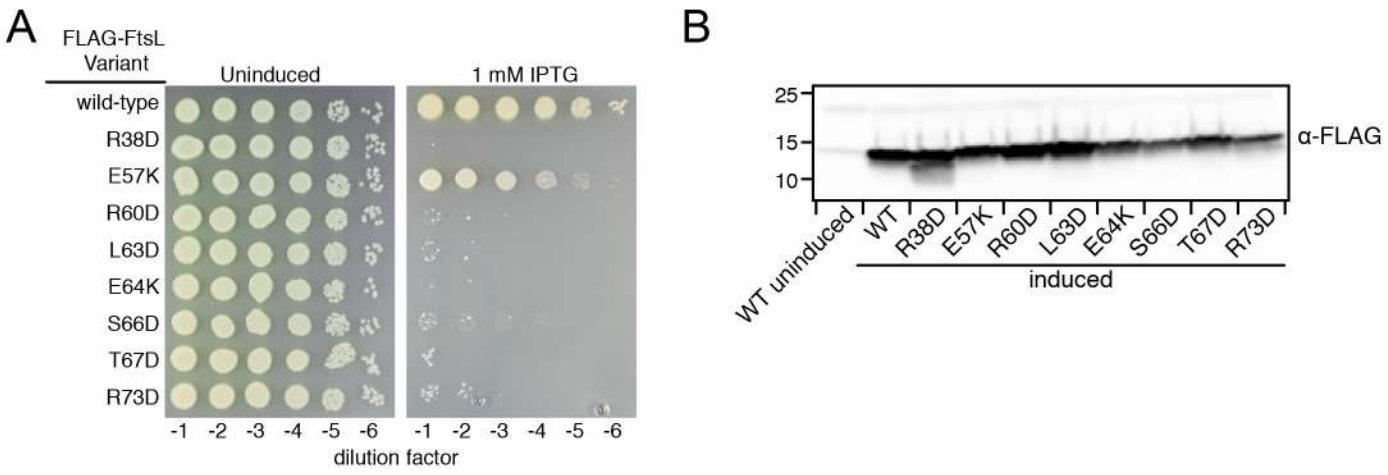

Figure S4: Activity and accumulation of FLAG-tagged ^{DN}FtsL variants **a**. Ten-fold serial dilutions of *P. aeruginosa* PAO1 cells harboring expression plasmids for production of the indicated FLAG-tagged FtsL variant. DIlutions were plated on LB agar with or without IPTG to induce the FLAG-FtsL variants as indicated. **b**. Detection of the indicated FLAG-FtsL variants from cell extracts with anti-FLAG antibodies.

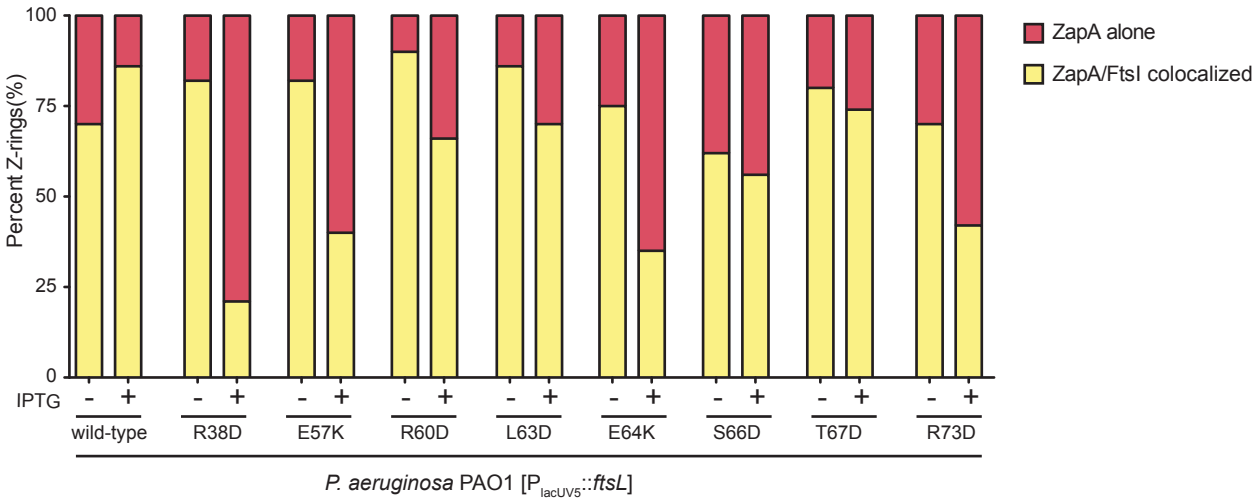

Figure S5: Quantification of ZapA and FtsI co-localization. ZapA rings were identified by manual examination of images using FIJI software, and assessed for the presence or absence of an associated FtsI ring. At least 50 rings were examined per strain, and per condition.

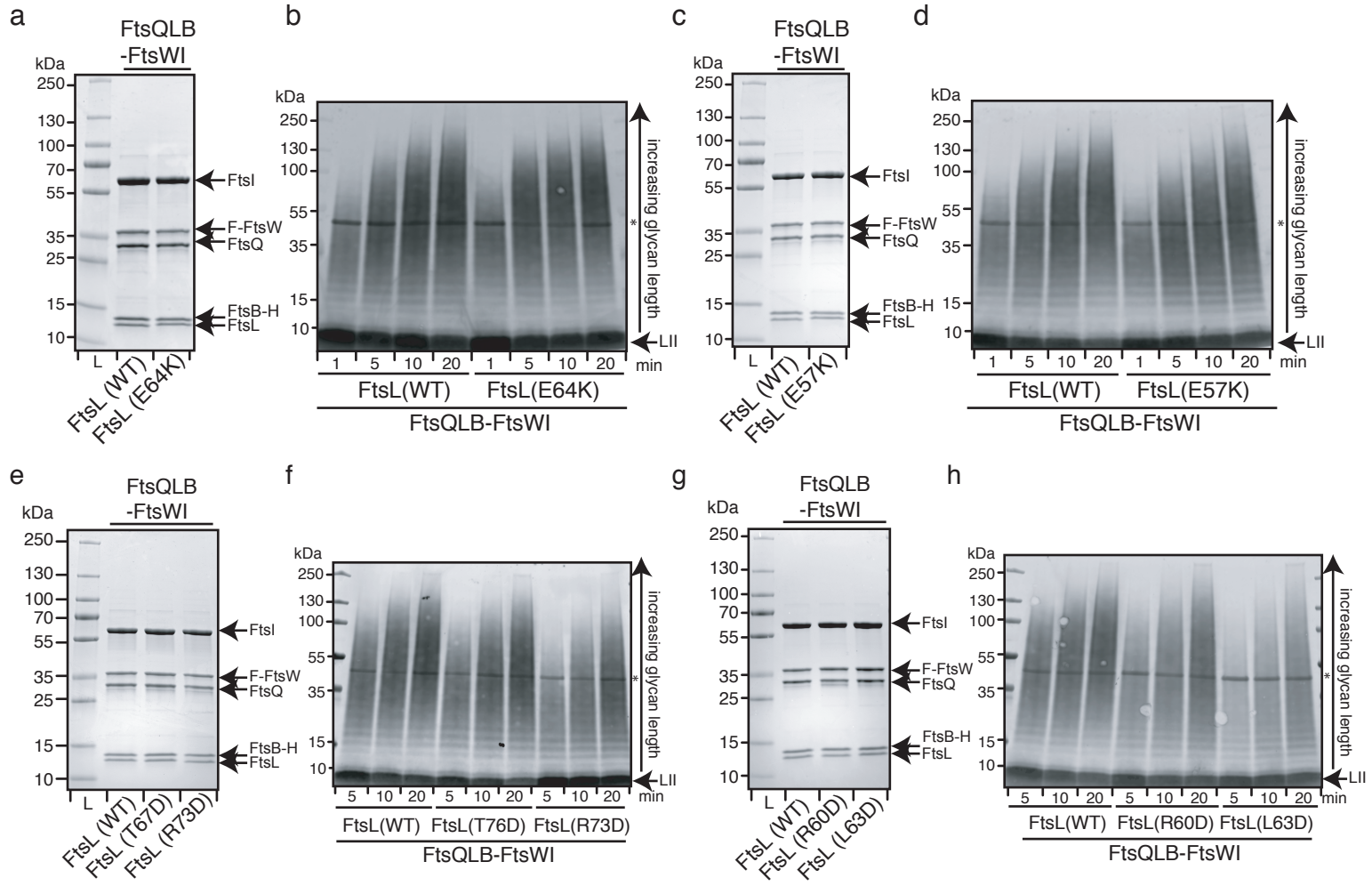

Figure S6: A subset of ^{DN}FtsL variants retain FtsWI activation activity. a, c, e, and g. Coomassie stained gel of the indicated complexes purified and labeled as in **Figure 1a.** **b, d, f, h.** PG polymerization activity of the indicated complexes was assayed as in **Figure 1b**. Representative images of three independent experiments for each ^{DN}FtsL variant are shown.

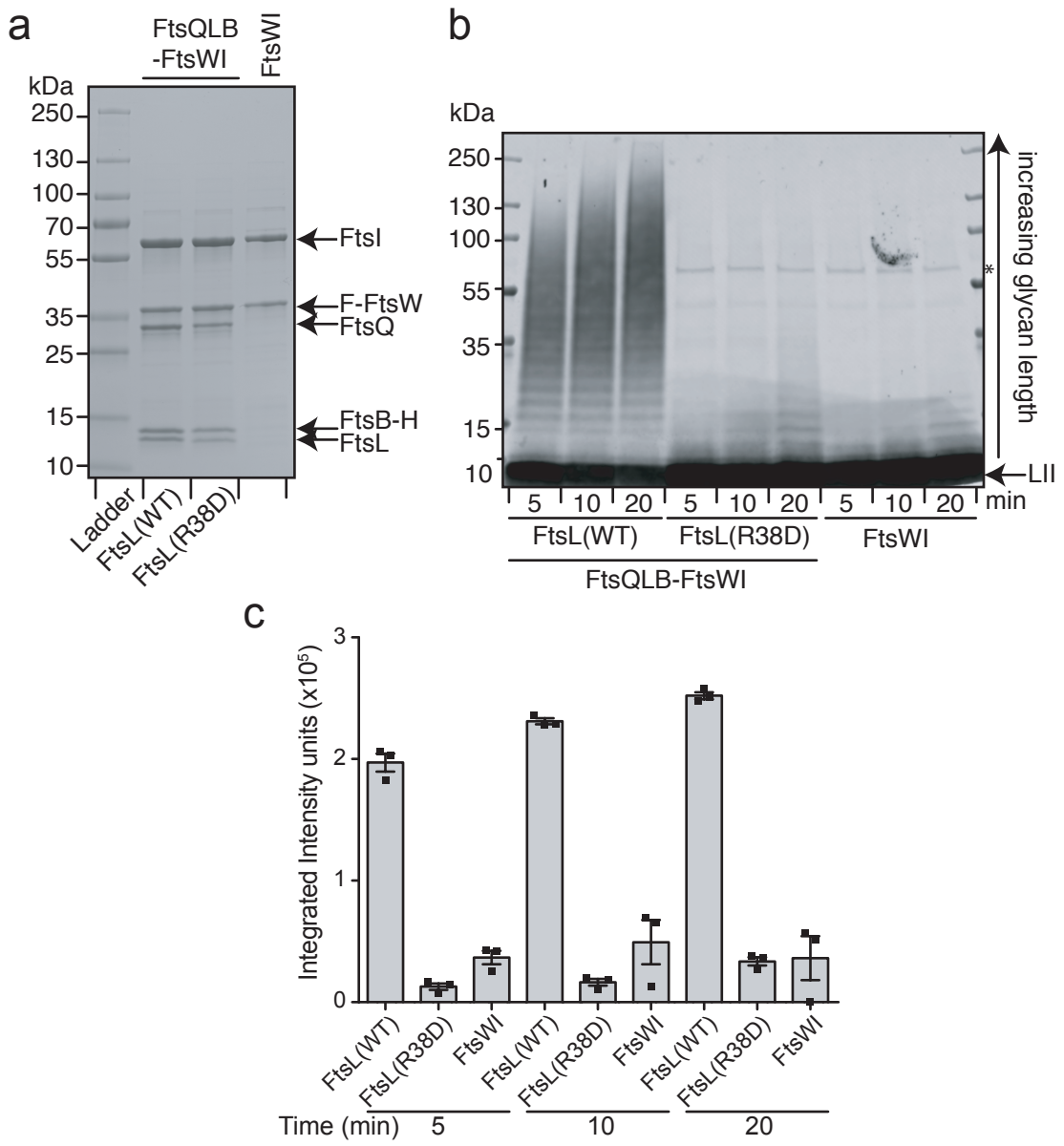

Figure S7: Comparison of FtsQL(R38D)B-WI complex activity with FtsWI alone. a. Coomassie stained gel of the indicated complexes purified and labeled as in **Figure 1a. b.** PG polymerization activity of the indicated complexes was assayed as in **Figure 1b.** For both panels, representative images of three independent experiments are shown. **c.** The accumulation of glycan fragments from three independent replicates of polymerization time-courses were quantified using densitometry. Error bars represent standard error of the mean.

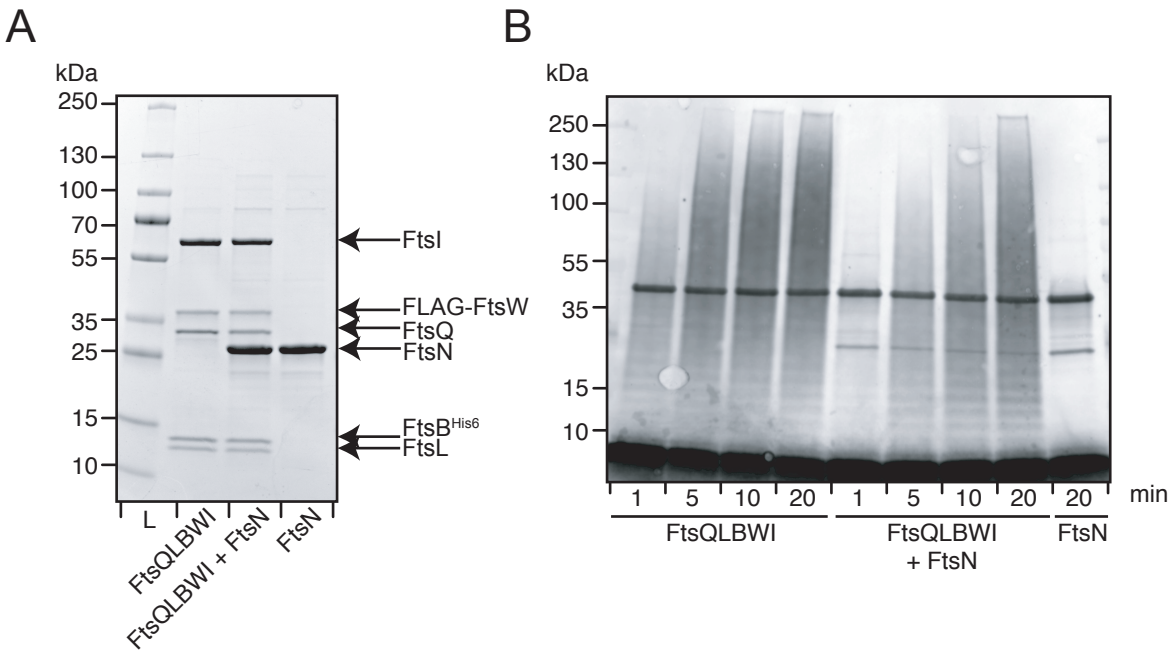

Figure S8: The periplasmic domain of FtsN does not enhance the polymerase activity of FtsW. a. Coomassie stained gel of the indicated proteins purified and labeled as in Figure 1a. FtsQLBWI was mixed with buffer or with excess FtsN(46-231) and equivalent volumes of each sample were loaded on the gel. **b**. PG polymerization activity of the indicated proteins was assayed as in Figure 1b.

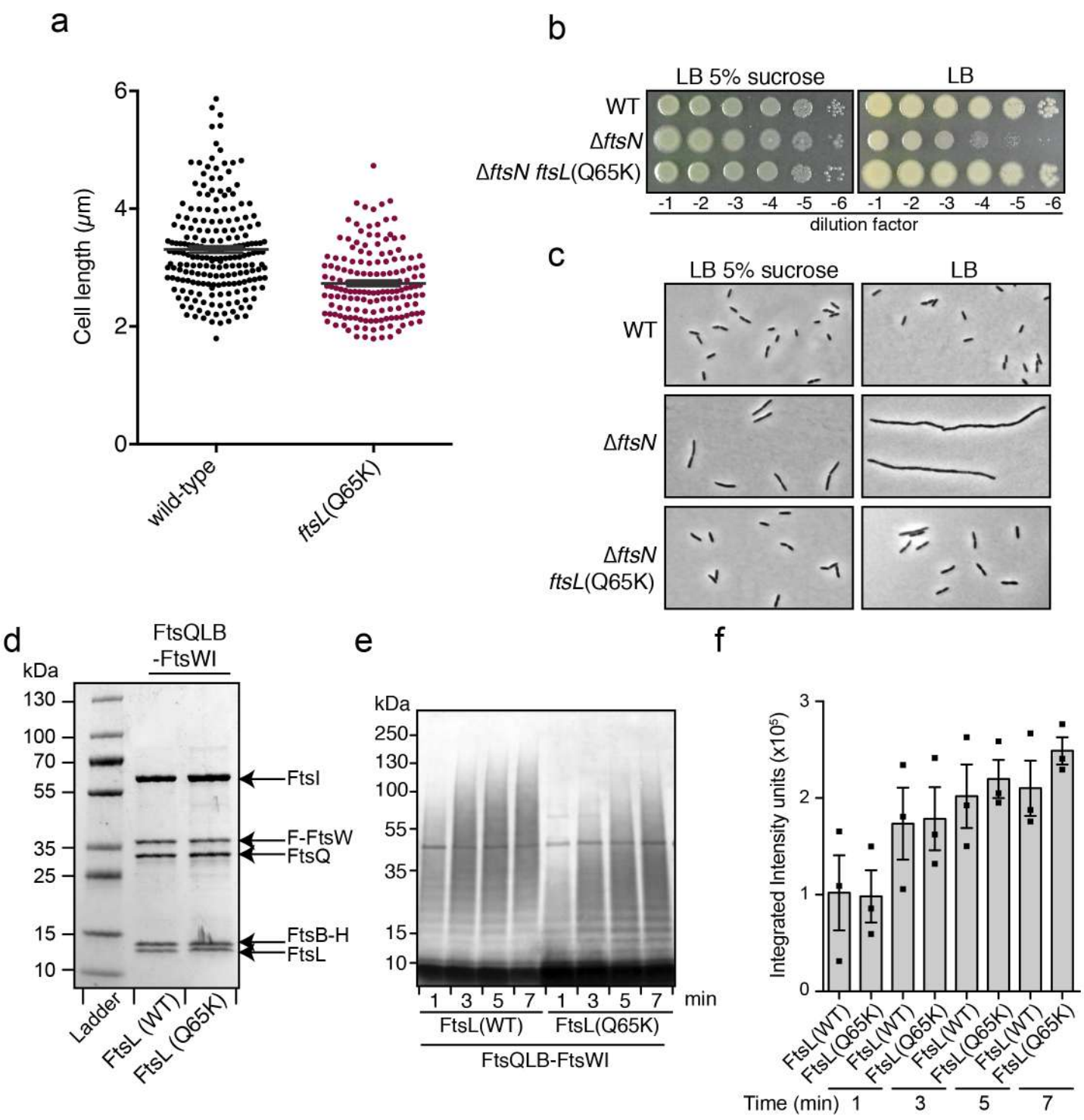

Figure S9: A ^{SF}FtsL variant does not further activate FtsQLB-WI complexes. a. Overnight cultures of wild-type *P. aeruginosa* and *P. aeruginosa ftsL*(Q65K) were backdiluted to OD_{600} = 0.05 and allowed to grow for 4h at 42°C in M9 media supplemented with glucose. Cells were fixed, immobilized, and imaged using phase-contrast microscopy. At least 150 cells from micrographs of two independent experiments were analyzed using Oufti. Error bars represent standard error of the mean **b.** Ten-fold serial dilutions of *P. aeruginosa* PAO1 cells with the indicated chromosomal mutations. Dilutions were plated on LB agar with or without 5% sucrose as indicated. **c.** Phase contrast micrographs of the indicated strains grown in LB with or without 5% sucrose as indicated. **d.** Coomassie stained gel of the indicated complexes purified and labeled as in **Figure 1a. e.** PG polymerization activity of the indicated complexes was assayed as in **Figure 1b. f.** The accumulation of glycan fragments from three independent replicates of polymerization time-courses were quantified using densitometry. Error bars represent standard error of the mean.

Table S1: FtsL mutations generated

Mutation*	Dominant-Negative Phenotype Observed
R3A	No
R8A	No
Y35H	No
W36H	No
N37D	No
R38A	No
R38D	**Yes**
Q39E	No
L40D	No
L41D	No
N42D	No
S43D	No
L44D	No
Y45H	No
S46D	No
E47A	No
E47K	No
L48D	No
S49D	No
V50D	No
R51A	No
R51D	No
D52A	No
D52K	No
K53A	No
K53D	No
A54L	No
Q55E	No
A56L	No
A56K	No
E57A	No
E57K	**Yes**
W58H	No
G59L	No
R60A	No
R60D	**Yes**
L61D	No
I62D	No
L63D	**Yes**
E64A	No
E64K	**Yes**
Q65E	No
S66D	**Yes**
T67D	**Yes**
W68H	No
T69D	No
A70L	No
H71A	No

S72D	No
R73A	No
R73D	**Yes**
I74D	No
E75A	No
E75K	No
S76D	No
L77D	No
A78L	No
V79D	No
E80A	No
E80K	No
Q81E	No
L82D	No
R83A	No
R83D	No
M84K	No
R85A	No
R85D	No
V86D	No
P87G	No
D88A	No
D88K	No
P89G	No
A90L	No
E91A	No
E91K	No
V92D	No
R93A	No
R93D	No
M94K	No
V95D	No
A96L	No
P97G	No

*Plasmids for production of *ftsL* variants were generated by GenScript using site directed mutagenesis with pLSM11 as the template. All plasmids were sequence verified.

**Dominant negative activity was assessed by streaking transformants on LB Gent 30 agar with or without inducer (1 mM IPTG) added to the medium. Strains that exhibited a significant defect in growth on media containing inducer were further characterized.

Table S2: FtsB mutations

Mutation*	Dominant-Negative Phenotype Observed
G27L	No
D28K	No
G29L	No
S30D	No
L31D	No
A32L	No
Q33E	No
V34D	No
R35D	No
D36K	No
L37D	No
Q38E	No
K39E	No
Q40E	No
I41D	No
A42L	No
D43K	No
Q44E	No
H45A	No
G46L	No
E47K	No
N48D	No
E49K	No
R50D	No
L51D	No
L52D	No
E53K	No
R54D	No
N55D	No
R56D	No
I57D	No
L58D	No
E59K	No
A60L	No
E61K	No
V62D	No
A63L	No
E64K	No
L65D	No
K66E	No
K67E	No
G68L	No
T69D	No

E70K	No
T71D	No
V72D	No
E73K	No
E74K	No
R75D	No
A76L	No
R77D	No
H78A	No
E79K	No
L80D	No
G81L	No
M82K	No
V83D	No
K84D	No
D85K	No
G86L	No
E87K	No
T88D	No
L89D	No
Y90H	No
Q91E	No
L92D	No
A93L	No
K94D	No

*Plasmids of *ftsB* were generated by GenScript using site directed mutagenesis with pLSM61 as a template. All plasmids were sequence verified.
**Dominant negative activity was assessed by streaking transformants on LB G30 agar with or without inducer (1 mM IPTG) added to the medium.

Table S3. Oligonucleotide primers used in this study

Primer	Sequence (5'-3')*
oLSM38	TAATGAGCTCACGGGAGGAAAGATGAGCCGTCTCTTCGTCAAGCG
oLSM39	TAATTCTAGATCATGGCGCCACCATCCTGAC
oLSM40	GATGACGGAGCAGTACGCCATTCATGGATGACCCCCCAGGGCC
oLSM41	AGGCCCTGGGGGGTCATCCATGAATGGCGTACTGCTCCGTCATC
oLSM42	TTAAGCATTATGCGGCCGCAAGCTTTCACTGCACGGCGCTGGC
oLSM43	CCGCGACGGTGGCCACGGCCAGCGCCGTGCAGTGAAAGCTTGCGGCCGCATAATGCTTAA
oLSM44	GGGGCTACGTAACCTCAAATATGTATATCTCCTTCTTATACTTAACTAATATACTAAGAT
oLSM45	TAAGAAGGAGATATACATATTTGAGGTTACGTAGCCCCTACTG
oLSM46	GCTCGAGTGCGGCCGCAAGCTTCTTGGCGAGCTGGTAGAGGGT
oLSM47	GAAACCCTCTACCAGCTCGCCAAGAAGCTTGCGGCCGCACTCGA
oLSM48	AGGAGATATACATATATGAGGTTACGTAGC
oLSM49	GCTACGTAACCTCATATATGTATATCTCCT
oLSM56	TCAGGATGGTGGCGCCATGAAAGCTTGCGGCCGCATAATGCTTAA
oLSM57	GCGCTTGACGAAGAGACGGCTCATGGATGACCCCCCAGGGCC
oLSM58	CAAGGCCCTGGGGGGTCATCCATGAGCCGTCTCTTCGTCAAGCG
oLSM59	GCATTATGCGGCCGCAAGCTTTCATGGCGCCACCATCCTGAC
oLSM62	TAGGGGCTACGTAACCTCATATATGTATATCTCCTTCTTATACTTAACTAATATACTAAG
oLSM63	AAGAAGGAGATATACATATATGAGGTTACGTAGCCCCTACTG
oLSM64	CATTATGCGGCCGCAAGCTTTCAGTGGTGGTGGTGGTGGTG
oLSM65	TCGAGCACCACCACCACCACCACTGAAAGCTTGCGGCCGCATAATGCTTAA
oLSM66	TTGACGAAGAGACGGCTCATATATGTATATCTCCTTCTTATACTTAACTAATATACTAAG
oLSM67	AAGAAGGAGATATACATATATGAGCCGTCTCTTCGTCAAGCG
oLSM68	TCTTTACCAGACTCGAGTCATGGCGCCACCATCCTGAC
oLSM69	GGATGGTGGCGCCATGACTCGAGTCTGGTAAAGAAACCGCT
oLSM99	GCAGCAGCCTAGGTTAATCAGCCACGCCCTCCTTTTGCG
oLSM100	GCAAAAGGAGGGCGTGGCTGATTAACCTAGGCTGCTGCCACC
oLSM106	ATGCGTGGTTCTCACCACCACC
oLSM109	GGTGGTGGTGAGAACCACGCATGGTATATCTCCTTATTAAAGTTAAACAAAATTATTTCT
oLSM110	CAGAGAACAGATTGGTGGTTCCATGAATGGCGTACTGCTCC
oLSM111	AGTACGCCATTCATGGAACCACCAATCTGTTCTCTGTGAGC
oLSM124	TAATTCTAGAACGGGAGGAAAGATGAATGGCGTACTGCTCCGTCATC
oLSM114	CTAAAATTGTGAGTGCTCACTGCACGGCGCTGGC
oLSM115	CGTGCAGTGAGCACTCACAATTTTAGGCACCCCA
oLSM116	CACGGGAGGAAAGATGAGGTTACGTAGCCCCTACTG
oLSM117	ACGTAACCTCATCTTTCCTCCCGTGAGCTCGAATT
oLSM118	CAGCTCGCCAAGTGAGCACTCACAATTTTAGGCACCCCA
oLSM119	TGTGAGTGCTCACTTGGCGAGCTGGTAGAGGG
oLSM120	ACGGGAGGAAAGATGAGCCGTCTCTTCGTCAAGCG
oLSM121	GAGACGGCTCATCTTTCCTCCCGTGAGCTCGAATT
oLSM125	TAATAAGCTTTCATGGCGCCACCATCCTGAC
oLSM128	GCACCACTACGTGCTCGAGAAATG
oLSM129	CTGCGCAACTGTTGGGAAGG
oLSM169	CAGATTGGTGGTTCCATGAGCCGTCTCTTCGTCAA
oLSM170	CGGCTCATGGAACCACCAATCTGTTCTCTGTGAGC
oLSM186	TAATGAATTCACGGGAGGAAAGATGAGGTTACGTAGCCCCTACTG
oLSM187	ATTATCTAGATCACTTGGCGAGCTGGTAGAGG
oLSM188	ATGTAAAGCAAGCTTGACTCTCCCACTTCTTCCCACTC
oLSM189	GTTCTTCACCTTTAGATTTCATGGCGCCACCATCCTGAC
oLSM190	GCGCCATGAAATCTAAAGGTGAAGAACTGTTCACCGGT
oLSM191	CAGACCAGCCGGACCCTCGAGTTTGTAGAGCTCATCCATGCCGTGC
oLSM192	ACTCGAGGGTCCGGCTGGTCTGAAACTGAATTATTTCCAGGGCGCCCT
oLSM193	ATTAAGGTACCGAATTCTCAGCCACGCCCTCCTTTTGC

oLSM194	CGTGGCTGAGAATTCGGTACCTTAATTAATTTCCACGG
oLSM195	GTGGGAGAGTCAAGCTTGCTTTACATTTATGCTTCCGGCT
oLSM196	AAGGCGCAGGCCAAATGGGGCCGGTTG
oLSM197	CAACCGGCCCCATTTGGCCTGCGCCTT
oLSM198	CGGTTGATTCTCAAACAGAGCACCTGG
oLSM199	CCAGGTGCTCTGTTTGAGAATCAACCG
oLSM200	ACCTACTGGAACGATCAACTGCTCAAC
oLSM201	GTTGAGCAGTTGATCGTTCCAGTAGGT
oLSM202	ATTCTCGAGCAGGATACCTGGACCGCC
oLSM203	GGCGGTCCAGGTATCCTGCTCGAGAAT
oLSM204	GCCGAGTGGGGCGATTTGATTCTCGAG
oLSM205	CTCGAGAATCAAATCGCCCCACTCGGC
oLSM206	GGCCGGTTGATTGATGAGCAGAGCACC
oLSM207	GGTGCTCTGCTCATCAATCAACCGGCC
oLSM208	CTCGAGCAGAGCGATTGGACCGCCCAT
oLSM209	ATGGGCGGTCCAATCGCTCTGCTCGAG
oLSM210	ACCGCCCATAGCGATATCGAAAGCCTG
oLSM211	CAGGCTTTCGATATCGCTATGGGCGGT
oLSM212	CAGACCAGCCGGACCCTCGAGGGCTTCGCCGGCATCGGCCGGATTC
oLSM213	GAATTCCAATTCCCAAGCGAGAGCC
oLSM214	CTTCGCCGGCATCGTTATTAGGATCCGCCAGCACCTTTG
oLSM215	CTCGAGGGTCCGGCTGGTCTGTCCAAG
oLSM216	AAGCTTCGACCCGCTGGAATACCATG
oLSM217	CGGATCCTAATAACGATGCCGGCGAAGCCTGACC
oLSM237	CCTGGGGGGTCATCCCTCGAACCCGGGCGCAAG
oLSM88	TTCTTTACCAGACTCGAGTCAGCGGGCCTTGCGTTGCT
oLSM89	GCAACGCAAGGCCCGCTGACTCGAGTCTGGTAAAGAAACCGCT
oLSM238	CTTGCGCCCGGGTTCGAGGGATGACCCCCCAGGGCC

*Restriction site underlined

Table S4. Bacterial strains used in this study

Strain	Description*	Reference
E. coli		
DH5α	Host strain for plasmid cloning	Invitrogen
BL21(DE3)	Expression strain for protein production	[1]
CAM333	C43(DE3) Δ*ponB*, Δ*pbpC*, Δ*mtgA*	[2]
LSM9	C43(DE3) Δ*pbpC*, Δ*mtgA fhuA*-	This study
P. aeruginosa		
PAO1	Wild-type	S. Lory
LSM10	PAO1 sfGFP-*ftsI zapA*-mCherry	This study
GT225	PAO1 Δ*ftsN*	This study
GT244	PAO1 *ftsL*(Q65K)	This study
GT234	PAO1 Δ*ftsN ftsL*(Q65K)	This study

Table S5. Plasmids used in this study

Plasmid	Description*	Reference
pETDuet-1	IPTG-inducible protein expression vector containing two multiple cloning sites; pBR322-derived ColE1 replicon; $Carb^R$	Novagen
pETDuet-FLAG	Modified pETDuet-1 containing a N-terminal His_6-SUMO-FLAG tag at the first insertion site and C-terminal His_6 tag at the second site; $Carb^R$	[3]
pCOLADuet-1	IPTG-inducible protein expression vector containing two multiple cloning sites; COLA replicon, Kan^R	Novagen
pAM174	Encodes arabinose-inducible Ulp1[L403-K621] protease; Cm^R	[2]
pPBP4	*S. aureus* His_6-PBP4 expression vector; Kan^R	[4]
pPSV38	*P. aeruginosa* expression vector containing IPTG-inducible P_{lacUV5} promoter; $Gent^R$	[5]
pLSM3	His_6-SUMO-FLAG-*Pa*FtsW & *Pa*PBP3-His_6 expression vector; $Carb^R$	[3]
pLSM10	*His_6-SUMO-FLAG-Pa-ftsQ* & *Pa-ftsB*-His_6, expression vector; $Carb^R$	This study
pLSM11	*P. aeruginosa* P_{lacUV5}-*Pa-ftsL* expression vector (pPSV38); $Gent^R$	This study
pLSM13	*His_6-SUMO-FLAG-Pa-ftsQ* & *Pa-ftsB*-His_6 *Pa-ftsB* has TTG mutated to ATG, expression vector; $Carb^R$	This study
pLSM14	*His_6-SUMO-FLAG-Pa-ftsL* & *Pa-ftsB*-His_6; *Pa-ftsB* has TTG mutated to ATG, expression vector; $Carb^R$	This study
pLSM15	*His_6-SUMO-FLAG-Pa-ftsQ*, *Pa-ftsB*-His_6, & *Pa-ftsL*, *Pa-ftsB* has TTG mutated to ATG, expression vector; $Carb^R$	This study
pLSM33	*His_6-SUMO-FLAG-Pa-ftsW* & *Pa-ftsI*, expression vector; Kan^R	This study
pLSM34	*His_6-SUMO-Pa-ftsQ*, *Pa-ftsB*-His_6 & *Pa-ftsL*. expression vector; $Carb^R$	This study
pLSM51	*His_6-SUMO-Pa-ftsL* & *Pa-ftsB*-His_6; *Pa-ftsB* has TTG mutated to ATG, expression vector; $Carb^R$	This study
pLSM61	*P. aeruginosa* P_{lacUV5}-*Pa-ftsB* expression vector (pPSV38); $Gent^R$	This study
pLSM63	pEXG2 containing the full-length *ftsI* sequence, fused at the N-terminus to sfGFP, for allelic exchange into *P. aeruginosa*	This study
pAAY24	pEXG2 containing the full-length *zapA* sequence, fused at the C-terminus to mCherry, for allelic exchange into *P. aeruginosa*	This study
pLSM75	pLSM34 with *ftsL* containing the mutation E57K	This study
pLSM76	pLSM34 with *ftsL* containing the mutation E64K	This study
pLSM77	pLSM34 with *ftsL* containing the mutation R38D	This study
pLSM78	pLSM34 with *ftsL* containing the mutation S66D	This study
pLSM87	pLSM34 with *ftsL* containing the mutation R60D	This study
pLSM88	pLSM34 with *ftsL* containing the mutation L63D	This study
pLSM89	pLSM34 with *ftsL* containing the mutation T67D	This study
pLSM90	pLSM34 with *ftsL* containing the mutation R73D	This study
pGT204	pEXG2 containing *ftsL* Q65K for allelic exchange into *P. aeruginosa*	This study
pGT206	pEXG2 containing Δ*ftsN* for allelic exchange into *P. aeruginosa*	This study
pLSM54	*His_6-SUMO-Pa-ftsQ*, *Pa-ftsB^{ATG}*-His_6, & *Pa-ftsL^{Q65K}* expression vector, $Carb^R$	This study
pLSM72	*His_6-SUMO-FLAG-Pa-ftsN$^{46-231}$* expression vector, $Carb^R$	This study

*Abbreviations: $Carb^R$, ampicillin/carbenicillin resistance; Cm^R, chloramphenicol resistance; $Gent^R$, gentamycin resistance; Kan^R, kanamycin resistance

MATERIALS AND METHODS

Plasmid construction

pLSM10 [P_{T7}::*H-SUMO-FLAG-Pa-ftsQ*(*PA4409*) and P_{T7}::*Pa-ftsB*(*PA3634*)-H] is a pET-Duet derivative. Full-length *ftsQ* and *ftsB* were amplified from *P. aeruginosa* PAO1 gDNA using the primer pairs oLSM41 and oLSM42, and oLSM45 and oLSM46, respectively. The promoter region found between both genes was amplified from pLSM3 using oLSM43 and oLSM44. The backbone of pLSM3 was amplified using oLSM40 and oLSM47. Overlap extension PCR was used to stitch together *ftsQ*, the promoter region, and *ftsB*. This fragment and the backbone were then joined using Gibson assembly.

pLSM11 [P_{lacUV5}::*Pa-ftsL*(*PA4419*)] is a pPSV38 derivative. The *PA4419* [M1-P97] gene encoding full-length *ftsL* was amplified from PAO1 genomic DNA using primers oLSM38 and oLSM39. After digestion with SacI and XbaI, the PCR product was ligated into pPSV38 to generate pLSM11, for overexpressing *Pa-ftsL* in PAO1.

pLSM13 [P_{T7}::*H-SUMO-FLAG-Pa-ftsQ* and P_{T7}::*Pa-ftsB*^{ATG}-H] is a pET-Duet derivative. Using pLSM10 as a template, the start codon of *ftsB* was mutated from TTG to ATG using site directed mutagenesis (QuikChange Lightning, Agilent) with primers oLSM48 and oLSM49.

pLSM14 [P_{T7}::*H-SUMO-FLAG-Pa-ftsL* and P_{T7}::*Pa-ftsB*^{ATG}-H] is a pET-Duet derivative. Using pLSM13 as a template, the backbone of the plasmid was amplified using oLSM56 and oLSM57, designed to exclude *ftsQ*. *Pa-ftsL* [M1-P97] was amplified from genomic DNA using oLSM58 and oLSM59. The fragments were ligated using Gibson assembly.

pLSM15 [P_{T7}::*H-SUMO-FLAG-Pa-ftsQ* P_{T7}::*Pa-ftsB*^{ATG}-H P_{T7}::*Pa-ftsL*] is a pET-Duet derivative. Using pLSM13 as a template, insertion of *ftsL* was accomplished by amplifying full length *ftsL* [M1-P97] from PAO1 gDNA using primers oLSM67 and oLSM68. The T7 promoter for *ftsL* was amplified using oLSM65 and oLSM66 from pLSM3. *ftsB* was amplified from pLSM13 using oLSM63 and oLSM64. The pET-Duet backbone of pLSM3 was amplified using oLSM62 and oLSM69. *ftsB*, promoter region, and *ftsL* were spliced together using overlap extension PCR. This fragment was joined to the backbone using Gibson assembly.

pLSM34 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*^{ATG}-H P_{T7}::*Pa-ftsL*] is a pET-Duet derivative. As pLSM15 generates a FLAG-tagged *ftsQ*, and pLSM33 also generates a FLAG-tagged *ftsW*, we removed the FLAG tag from *ftsQ*. To generate this plasmid, *ftsQ* (from M1) through to *ftsL* (P97) was amplified using the primer pair oLSM110 and oLSM68 from pLSM15, and oLSM111 and oLSM69 to amplify the pET-Duet backbone. The pieces were assembled using Gibson assembly to generate a plasmid encoding His-SUMO-FLAG-*ftsQ*, *ftsB*-His6 and *ftsL* each under the control of a separate T7 promoter.

pLSM33 [P_{T7}::*H-SUMO-FLAG-Pa-ftsW*(*PA4413*) and P_{T7}::*Pa-ftsI*(*PA4418*)] is a pCOLADuet derivative. The *P. aeruginosa* PAO1 genes *ftsW* [M1-R399] and *ftsI* [M1-G579], were sub-cloned into pCOLADuet-1 (Novagen) for expression from pLSM3. *FtsW* and *ftsI* were amplified from pLSM3 using the primer pair oLSM106 and oLSM99. The backbone of pCOLADuet was amplified using oLSM100 and oLSM109, and the fragments were joined using isothermal assembly to make pLSM33. pLSM33 encodes His-SUMO-FLAG-*ftsW* and *ftsI* each under control of a pT7 promoter.

pLSM51 [P_{T7}::*H-SUMO-Pa-ftsL* and pT7::*ftsB*-H] is a pET-Duet derivative. Plasmid containing His-Sumo-*Pa-ftsL* and *Pa-ftsB*-His6 each under the control of a pT7 promoter. Using pLSM14 as a template, *ftsL* and *ftsB* were amplified using oLSM169 and oLSM46 and the pET-Duet plasmid backbone was amplified using oLSM170 and oLSM47, together these PCR products exclude the FLAG tag sequence. The fragments were ligated using Gibson assembly.

pLSM61 [P_{lacUV5}-*Pa-ftsB*] is a pPSV38 derivative**.** The *PA3634* [M1-K94] gene encoding *ftsB* was amplified from pLSM13 using primers oLSM186 and oLSM187. After digestion with EcoRI and XbaI, the PCR products were ligated into pPSV38 to generate pLSM61, for overexpressing *Pa-ftsB* in PAO1.

pLSM63 [sfGFP-*ftsI*] is a pEXG2 derivative. To generate an allelic exchange plasmid containing sfGFP-*ftsI*, the homologous region upstream of *ftsI* (2047 bp) including the promoter for the operon, was amplified from PAO1 gDNA using oLSM188 and oLSM189. The region encoding sfGFP was amplified using primers oLSM190 and oLSM191, such that the first two residues of *ftsI* (MK) were N-terminal to the sfGFP sequence. The *ftsI* gene was amplified from PAO1 gDNA using oLSM192 and oLSM193. Primers oLSM191 and oLSM192 encode the LEGPAGL linker sequence between sfGFP and *ftsI*. The pEXG2 backbone was amplified using oLSM194 and oLSM195. Each of the four amplified sequences were ligated using Gibson assembly.

pAAY24 [*zapA*(*PA5227*)-mCherry] is a pEXG2 derivative. To generate an allelic exchange plasmid containing *zapA*-mCherry, the homologous region upstream of *zapA* (226 bp) and the full-length *zapA* was amplified from gDNA using oLSM212 and oLSM213. Using oLSM214 and oLSM215 the mCherry sequence was amplified and fused to a LEGPAGL linker. Using oLSM216 and oLSM217, 526 bp of the sequence downstream of *zapA* was amplified. These pieces were stitched together using overlap extension PCR to generate a single insert. Following digestion with EcoRI and HindIII, the insert was ligated into pEXG2 and sequence verified prior to allelic exchange.

pLSM75 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and P_{T7}::*Pa-ftsL E57K]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM196 and oLSM197.

pLSM76 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and P_{T7}::*Pa-ftsL E64K]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM198 and oLSM199.

pLSM77 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and P_{T7}::*Pa-ftsL R38D]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM200 and oLSM201.

pLSM78 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and P_{T7}::*Pa-ftsL S66D]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM202 and oLSM203.

pLSM87 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and P_{T7}::*Pa-ftsL R60D]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM204 and oLSM205.

pLSM88 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and P_{T7}::*Pa-ftsL L63D]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM206 and oLSM207.

pLSM89 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and *Pa-ftsL T67D]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM208 and oLSM209.

pLSM90 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*-H and *Pa-ftsL R73D]** is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM210 and oLSM211.

pGT204 [*ftsL* Q65K] is a pEXG2 derivative**.** To generate an allelic exchange plasmid containing $ftsL^{Q65K}$, sequence surrounding *ftsL* (1720 bp upstream and 1711 bp downstream), which includes the promoter for the operon, was amplified from PAO1 gDNA using oLSM212 and oLSM213. After digestion with HindIII and XbaI, the insert was ligated into pEXG2. The assembled plasmid was sequenced, and then subjected to site-directed mutagenesis (QuikChange Lightning, Agilent) using primers oLSM74 and oLSM75 to introduce the Q65K mutation. The plasmid was then sequenced prior to proceeding with allelic exchange.

pGT206 [Δ*ftsN* (*PA5052*)] is a pEXG2 derivative**.** To generate an allelic exchange plasmid with the deleted *ftsN* gene, 511 bp of the sequence upstream and sequence encoding the first six residues of *ftsN* was amplified using oLSM214 and oLSM215. 518 bp of the downstream sequence, including the last ten residues and the stop codon of *ftsN* were amplified using oLSM216 and oLSM217. PCR products were joined using overlap extension PCR using the primers oLSM214 and oLSM217. Following digestion with HindIII and EcoRI, insert was ligated into pEXG2 and sequence verified prior to allelic exchange.

pLSM54 [P_{T7}::*H-SUMO-Pa-ftsQ* P_{T7}::*Pa-ftsB*^{ATG}-H P_{T7}::*Pa-ftsL*^{Q65K}] is a pET-Duet derivative. Site directed mutagenesis (QuikChange Lightning, Agilent) of pLSM34 was performed using oLSM74 and oLSM75.

pLSM72 [pT7::*H-SUMO-FLAG-Pa-ftsN*$^{46-231}$] is a pET-Duet derivative. Sequence for *ftsN* was amplified from PAO1 gDNA using oLSM237 and oLSM88. The backbone of pLSM3 was amplified using oLSM89 and oLSM238. The resulting PCR products were then joined using Gibson assembly.

Allelic replacement in P. aeruginosa PAO1

The fusion alleles encoded by pLSM63 and pAAY24 were introduced into *P. aeruginosa* via mating with donor strain *E. coli* SM10. Merodiploids were selected on VBMM (Vogel-Bonner minimal medium) containing 30 μg/mL Gent. SacB-mediated counterselection was carried out by selecting for double cross-over mutations on LB 5% (w/v) sucrose. Strains containing gene fusions were identified by PCR with primers targeting the flanking regions of the fusion.

Deletion of *ftsN* was accomplished by introducing pGT206 into *P. aeruginosa* via conjugation with donor strain *E. coli* SM10, and following the selection steps as described above. Following counterselection, strains containing the desired deletion were identified via PCR with primers flanking *ftsN*. The strain was maintained on LB 5% sucrose.

To generate *ftsL**(Q65K) Δ*ftsN*, pGT204 was first introduced into *P. aeruginosa* via mating using *E. coli* SM10. Merodiploids were selected on VBMM containing 30 μg/mL Gent. SacB-mediated counterselection was carried out by selecting for double cross-over mutations on LB 5% (w/v) sucrose. The *ftsL** region was amplified by PCR with primers flanking *ftsL**, and the PCR product was sent for sequence analysis. Strains containing the desired mutation were subjected to a second round of allelic exchange to introduce the *ftsN* deletion allele as described above.

Electroporation of P. aeruginosa

P. aeruginosa strains were made competent using previously described methods [6]. Plasmids encoding FtsL and FtsB variants were received from GenScript as 4 μg of lyophilized powder. Plasmids were resuspended in 40 μL of MilliQ water to a final concentration of 100 ng/μL. For electroporation, 1 μL of plasmid DNA was added to 40 μL of competent *P. aeruginosa* cells. Transformation was achieved using standard protocols and transformants were selected for using 30 μg/mL Gent.

P. aeruginosa viability assays

Overnight cultures of LSM10 derivatives, containing vectors producing the indicated alleles of *ftsL* from an IPTG-inducible (P_{lacUV5}) plasmid were normalized to an OD_{600} of 2.4 before being serially diluted. Aliquots (5 μL) of the dilutions were spotted onto LB Gent agar with or without 1 mM IPTG. Plates were incubated at 37°C for 24 h (LB) at which point the plates were imaged.

Construction of E. coli protein overexpression strain

LSM9 was generated by moving a Δ*fhuA::kan* allele from a BL21(DE3) background (gift from Ry Young) into CAM333 by P1-transduction to confer resistance against T1 phage.

Purification of P. aeruginosa FLAG-FtsW-FtsI

For expression of *P. aeruginosa* FtsW-FtsI, *E. coli* expression strain LSM9 containing pAM174 and the expression plasmid (pLSM33) was grown in 1 L TB supplemented with 2 mM $MgCl_2$, ampicillin, and chloramphenicol at 37 ºC with shaking until OD_{600} was 0.7. The culture was cooled to 20 ºC before inducing protein expression with 1 mM IPTG and 0.1% arabinose. Cells were harvested 18 h post-induction by centrifugation (4,200 x g, 15 min, 4 ºC). To purify FLAG-FtsW and FtsI, the cells were resuspended in lysis buffer B (50 mM HEPES pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5 M DTT) and lysed by passage through a cell disruptor (Constant Systems) at 25,000 psi twice. Membranes were collected by ultracentrifugation (100,000 x g, 1 h, 4 ºC). The membrane pellets were resuspended in solubilization buffer B (20 mM HEPES pH 7.0, 0.5 M NaCl, 20% glycerol, and 1% DDM (Thermo Fisher)) and rotated end over end for 1 h at 4 ºC before ultracentrifugation (100,000 x g, 1 h, 4 ºC). The supernatant was supplemented with 2 mM $CaCl_2$ and loaded onto a homemade M1 α-Flag antibody resin. The resin was washed with 25 column volumes (CVs) of wash buffer C (20 mM HEPES pH 7.0, 0.5 M NaCl, 20% glycerol, 2 mM $CaCl_2$, 0.1% DDM) and the bound protein was eluted from the column with five CVs of elution buffer (20 mM HEPES pH 7.0, 0.5 M NaCl, 20% glycerol, 0.1% DDM, 5 mM EDTA pH 8.0, and 0.2 mg/mL FLAG peptide). Fractions containing the target protein were

concentrated and the protein concentration was measured via Bradford assay. Proteins were then aliquoted and stored at -80 ºC.

Co-expression and purification of P. aeruginosa FtsW-FtsI, FtsL-FtsB

E. coli expression strain LSM9 containing pAM174 and the expression plasmids pLSM33 and pLSM51 were grown in 1 L TB supplemented with 2 mM $MgCl_2$, ampicillin, kanamycin and chloramphenicol at 37 ºC with shaking until OD_{600} was 0.7. The expression and purification protocol is identical to that described for FtsW-FtsI above.

Co-expression and purification of P. aeruginosa FtsW-FtsI, FtsQ-FtsB-FtsL

For expression of *P. aeruginosa* FtsW-FtsI and FtsQ-$FtsB_{His6}$-FtsL, *E. coli* expression strain LSM9 containing pAM174 and the expression plasmids pLSM33 and pLSM34 were grown in 2 L TB supplemented with 2 mM $MgCl_2$, ampicillin, kanamycin, and chloramphenicol at 37 ºC with shaking until OD_{600} was 0.7. The culture was cooled to 20 ºC before inducing protein expression with 1 mM IPTG and 0.1% arabinose. Cells were harvested 18 h post-induction by centrifugation (4,200 x g, 15 min, 4 ºC). Protein complexes of FLAG-FtsW, FtsI, FtsQ, $FtsB_{His6}$ and FtsL were purified as described above. Expression and purification of *P. aeruginosa* FLAG-FtsW-FtsI and all FtsQ-$FtsB_{His6}$-^{DN}FtsL variants follow the same protocol described above except instead of pLSM34, the expression plasmid for the corresponding ^{DN}FtsL (pLSM75, 76, 77, 78, 87, 88, 89, 90) variant was co-transformed along with pLSM33.

PG glycosyltransferase assays

The protocol for detecting Lipid II/peptidoglycan was adapted from previously published methods[4,7,8]. Prior to performing the assay, protein concentrations were measured using the Bradford assay in order to normalize samples. Briefly, a standard curve was generated by using bovine serum albumin (BSA). Absorbance was read at 595 nm. Target protein concentration in μM was calculated by interpolation from a standard curve using molecular weights for FtsW-FtsI (108642 g/mol), FtsW-FtsI-FtsB-FtsL (132138 g/mol), or FtsW-FtsI-FtsQ-FtsB-FtsL (164371g/mol) assuming 1:1, 1:1:1:1 or 1:1:1:1:1 protein complex formation. From these calculations, stocks with a normalized concentration (11 μM) of protein complexes were prepared, aliquoted and frozen at -80ºC. Normalization was assessed by running equivalent volumes of an aliquot for each preparation on an SDS-PAGE gel and staining with Coomassie Brilliant Blue.

For the assays, the indicated protein complex (0.5 μM) was added to a 1x reaction buffer (125 mM HEPES pH 7.5, 20 mM $MnCl_2$, 2.5 mM Tween 80, 200 μM cephalexin, 30% DMSO) containing *E. faecalis* Lipid II (10 μM) in a total volume of 10 μL. The samples were incubated at room temperature for various time points as indicated. Following the incubation, reactions were heat-quenched at 95°C for 2 min. After cooling, 2 μL biotin-D-lysine (BDL, 20 mM) and 1 μL *S. aureus* PBP4 (50 μM) were added to the reaction mixture and the samples were incubated for 1 h. Reactions was quenched by the addition of 13 μL of 2x Laemmli sample buffer and samples were loaded onto a 4-20% polyacrylamide gel. The peptidoglycan product was then transferred onto a PVDF membrane and the membrane was fixed by incubating in 0.4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Subsequently, the blot was blocked using SuperBlock blocking buffer. The biotin-labeled products were detected by incubation with IRDye 800CW Streptavidin (1:5,000 dilution). The membrane was then washed four times with TBS with 0.5% Tween-20 (TBST), followed by one wash with PBS prior to imaging using an Odyssey CLx imaging system (LI-COR Biosciences).

Densitometry analysis
As a measure of glycan polymerization, we measured the signal of individual lanes on each GT assay blot. An area of equal size that encompassed signal for the entire height of each lane, but excluding lipid II, was selected. From each integrated intensity value we subtracted a constant background value that corresponds to a D275A reaction. Intensity measurements were collected from three separate blots. Analysis was performed using FIJI software.

FLAG-FtsL plasmid construction
Plasmids encoding FLAG-FtsL and the indicated variants were synthesized by GenScript. Plasmids are a derivative of pLSM11 containing an extra 30 base pairs following the start codon to encode the FLAG peptide and a linker (DYKDDDDKGS). Lyophilized plasmids received from GenScript were resuspended in 40 µL water and electroporated into LSM10 as described above.

Western blot sample preparation and analysis
For analysis of protein levels from strains containing FLAG-FtsL variants, an overnight culture of each of the strains was allowed to grow in LB at 37°C containing 30 µg/mL gentamicin. The following day, the cultures were diluted to 0.01 and allowed to grow at 37 °C in M9-Glu containing 30 µg/ml Gent. After 2h, 1 mM IPTG was added and the cultures were allowed to grow for another 2.5h. Cultures were normalized to an OD_{600} = 0.5 and cells were collected by centrifugation at 5,000 × g for 2 min. The cell pellet was resuspended in 50 µL of 2× Laemmli buffer, boiled for 15 min at 95 °C, then centrifuged for 10 min at 21,000 x g. Samples were analyzed by SDS-PAGE followed by Western blot. For Western blot analysis, a 0.2 µm polyvinylidene difluoride (PVDF) membrane was wetted in methanol and soaked for 5 min in Trans-Blot Turbo Transfer Buffer (BioRad). Protein was transferred from the SDS-PAGE gel to the PVDF membrane using the Trans-Blot Turbo Transfer System. The membrane was blocked in 5% (w/v) skim milk powder in Tris-Buffered saline (10 mM Tris-HCl pH 7.5, 150 mM NaCl) containing 0.5% (v/v) Tween-20 (TBS-T) for 45 min at room temperature with gentle agitation. The membrane was washed in TBS-T prior to incubation with α-FLAG antibody (1:1000 dilution, F7425, Sigma-Aldrich) in TBS-T with 1% (w/v) skim milk powder at room temperature for 1.5 h. The membrane was washed three times in TBS-T for 10 min each before incubation for 1 h with secondary antibody (anti-rabbit IgG HRP, 1:1000 dilution, Rockland 18-8816-33) in TBS-T with 1% (w/v) skim milk powder. The membrane was then washed four times with TBS-T for 10 min each before developing using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific cat#34577) and imaged using the c600 Azure Biosystems platform.

FtsN expression and purification
Expression of FLAG-$FtsN^{46-231}$ was identical to that of FtsWI as described above. Cells of FLAG-$FtsN^{46-231}$ from 1L of culture were resuspended in lysis buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 20 mM $MgCl_2$, 0.5 mM DTT) and lysed by passage through a cell disruptor (Constant Systems) at 25,000 psi three times. Cell debris was pelleted by ultracentrifugation (100,000 x g, 1 h, 4 ºC). The supernatant was supplemented with 2 mM $CaCl_2$ and loaded onto a homemade M1 α-Flag antibody resin. The resin was washed with 25 CVs of wash buffer (20 mM HEPES pH 7.0, 0.5 M NaCl, 20% glycerol, 2 mM $CaCl_2$) and the bound protein was eluted from the column with five CVs of elution buffer (20 mM HEPES pH 7.0, 0.5 M NaCl, 20% glycerol, 5 mM EDTA pH 8.0, and 0.2 mg/mL FLAG

peptide). Fractions containing the target protein were concentrated and the protein concentration was measured via Bradford assay.

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