SUPPLEMENTAL INFORMATION FOR:

Cofactor bypass variants reveal a conformational control mechanism governing cell wall polymerase activity

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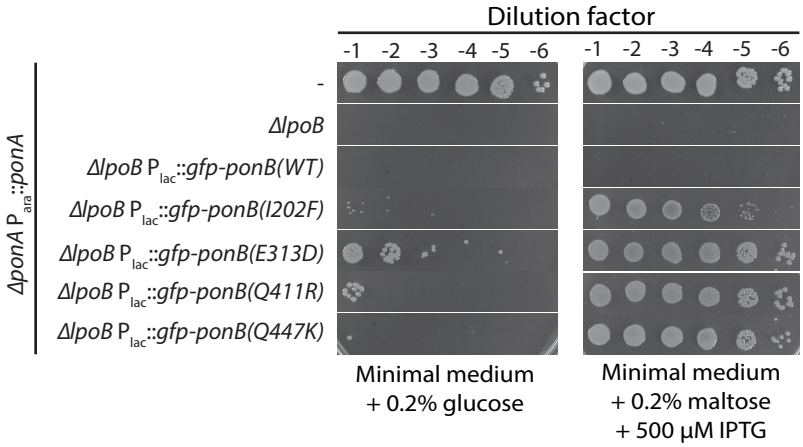

Figure S1

Figure S1. Suppression of the PBP1a- LpoB- synthetic lethal phenotype by ectopically produced GFP-PBP1b* fusions. Cells of TU121(attλTB309) [ΔponA (Para::ponA)], MM33(attλTB309) [ΔponA ΔlpoB (Para::ponA)], and MM33(attλTB309) derivatives harboring the indicated chromosomally integrated expression constructs: (attHKMM6) [Plac::gfp-ponB(WT)], (attHKMM79) [Plac::gfp-ponB(Q411R)], (attHKMM80) [Plac::gfp-ponB(I202F)], (attHKMM81) [Plac::gfp-ponB(E313D)], (attHKMM89) [Plac::gfp-ponB(Q447K)], grown and plated on the indicated media as described in Figure 2.

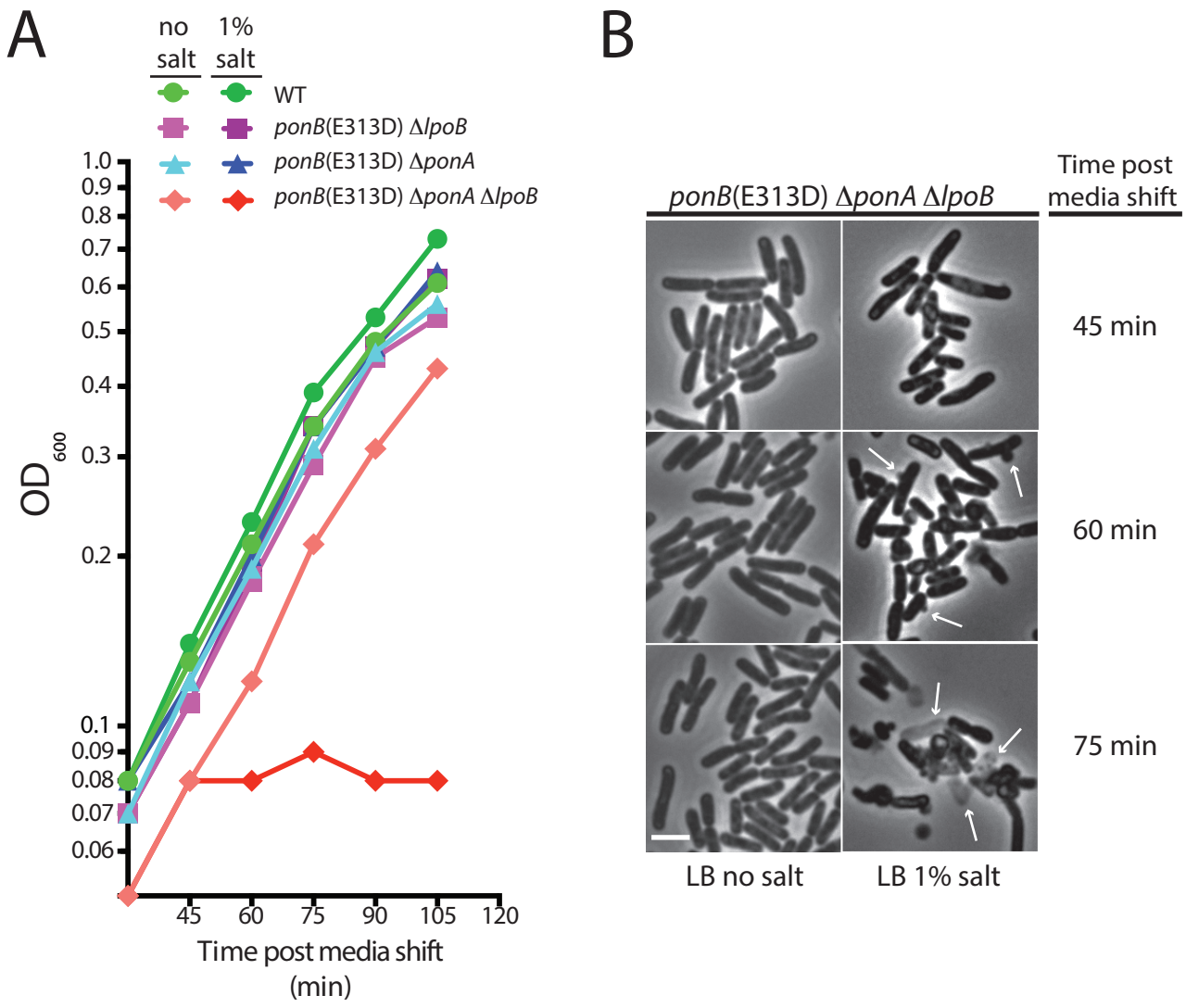

Figure S2

Figure S2. Conditional growth of cells producing PBP1b(E313D) in the absence of PBP1a and LpoB. (A) Cells of MG1655 [WT], MM49 [ponB(E313D) ΔlpoB], MM120 [ponB(E313D) ΔponA], and MM119 [ponB(E313D) ΔponA ΔlpoB] were grown overnight in LB without added salt (LB0N) at 30oC. The resulting cultures were diluted 1:100 in the same medium, grown to midlog at 37oC, and diluted to an OD600 of 0.025 in either LB0N or LB with 1% NaCl as indicated. Growth at 37oC was followed by regular OD600 measurements. Growth of strains harboring a single mutation (ponB(E313D) or ΔponA or ΔlpoB) was similar to WT in both conditions, but the growth curves were omitted from the panel so that the salt-dependant phenotype of MM119 could be more easily observed. (B) Cells of MM119 [ponB(E313D) ΔponA ΔlpoB] were grown similarly to those in (A). Cells were removed at the indicated time points, fixed, and visualized using phase-contrast optics. Bar equals 4 microns. Arrows point to membrane blebs and lysed cell ghosts observed in MM119 [ponB(E313D) ΔponA ΔlpoB] cells grown in LB with 1% NaCl.

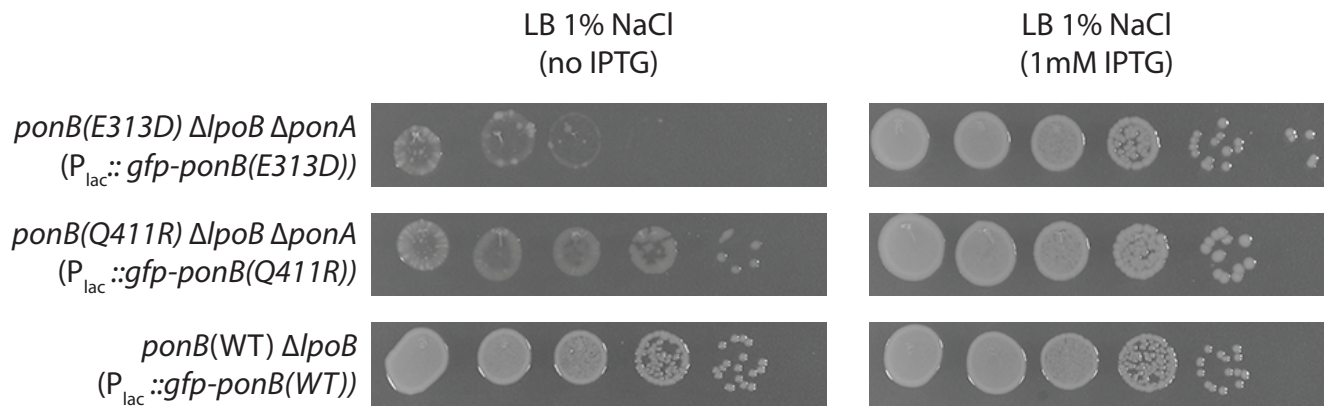

Figure S3

Figure S3. Overproduction of PBP1b* variants allows the simultaneous deletion of ponA and lpoB. Cells of JLB56(attHKMM81) [ponB(E313D) ΔponA ΔlpoB (Plac:: gfp-ponB(E313D)], JLB57(attHKMM79) [ponB(Q411R) ΔponA ΔlpoB (Plac:: gfp-ponB(Q411R)], and MM40 (attHKMM6) [ΔlpoB (Plac:: gfp-ponB(WT)] were grown overnight in LB 0.5% NaCl supplemented with 1 mM IPTG at 30oC. The resulting cultures were washed once in LB 0% NaCl, serial diluted and plated on LB 1% NaCl with or without supplementation with 1 mM IPTG as indicated and grown at 30oC overnight before being photographed.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Suppression of the PBP1a$^-$ LpoB$^-$ synthetic lethal phenotype by ectopically produced GFP-PBP1b* fusions. Cells of TU121(*att*λTB309) [Δ*ponA* (P_{ara}::*ponA*)], MM33(*att*λTB309) [Δ*ponA* Δ*lpoB* (P_{ara}::*ponA*)], and MM33(*att*λTB309) derivatives harboring the indicated chromosomally integrated expression constructs: (*att*HKMM6) [P_{lac}::gfp-*ponB*(WT)], (*att*HKMM79) [P_{lac}::gfp-*ponB*(Q411R)], (*att*HKMM80) [P_{lac}::gfp-*ponB*(I202F)], (*att*HKMM81) [P_{lac}::gfp-*ponB*(E313D)], (*att*HKMM89) [P_{lac}::gfp-*ponB*(Q447K)], grown and plated on the indicated media as described in Figure 2.

Figure S2. Conditional growth of cells producing PBP1b(E313D) in the absence of PBP1a and LpoB. (A) Cells of MG1655 [WT], MM49 [*ponB(E313D)* Δ*lpoB*], MM120 [*ponB(E313D)* Δ*ponA*], and MM119 [*ponB(E313D)* Δ*ponA* Δ*lpoB*] were grown overnight in LB without added salt (LB0N) at 30°C. The resulting cultures were diluted 1:100 in the same medium, grown to midlog at 37°C, and diluted to an OD_{600} of 0.025 in either LB0N or LB with 1% NaCl as indicated. Growth at 37°C was followed by regular OD_{600} measurements. Growth of strains harboring a single mutation (*ponB(E313D)* or Δ*ponA* or Δ*lpoB*) was similar to WT in both conditions, but the growth curves were omitted from the panel so that the salt-dependant phenotype of MM119 could be more easily observed. (B) Cells of MM119 [*ponB(E313D)* Δ*ponA* Δ*lpoB*] were grown similarly to those in (A). Cells were removed at the indicated time points, fixed, and visualized using phase-contrast optics. Bar equals 4 microns. Arrows point to membrane blebs and lysed cell ghosts observed in MM119 [*ponB(E313D)* Δ*ponA* Δ*lpoB*] cells grown in LB with 1% NaCl.

Figure S3. Overproduction of PBP1b* variants allows the simultaneous deletion of *ponA* and *lpoB*. Cells of JLB56(*att*HKMM81) [*ponB(E313D)* Δ*ponA* Δ*lpoB* (P_{lac}:: *gfp-ponB(E313D)*], JLB57(*att*HKMM79) [*ponB(Q411R)* Δ*ponA* Δ*lpoB* (P_{lac}:: *gfp-ponB(Q411R)*], and MM40 (*att*HKMM6) [Δ*lpoB* (P_{lac}:: *gfp-ponB(WT)*] were grown overnight in LB 0.5% NaCl supplemented with 1 mM IPTG at 30°C. The resulting cultures were washed once in LB 0% NaCl, serial diluted and plated on LB 1% NaCl with or without supplementation with 1 mM IPTG as indicated and grown at 30°C overnight before being photographed.

Table S1. List of ponB mutations identified in the LpoB bypass selection.

*ponB** isolate[a]	Amino acid substitution[b]	Base change
LIN39	Q411R	CAG ⟶ C<u>G</u>G
LIN65[c]	I202F	ATC ⟶ <u>T</u>TC
LIN222	E313D	GAG ⟶ GA<u>T</u>
LIN414[c]	I202F	CAG ⟶ <u>A</u>AG
LIN425[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN426[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN440	Q411K	CAG ⟶ <u>A</u>AG
LIN496[c]	I202F	ATC ⟶ <u>T</u>TC
LIN498[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN519	E313D	GAG ⟶ GA<u>T</u>
LIN528[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN536[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN567[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN583[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN698[c]	Q447K	CAG ⟶ <u>A</u>AG
LIN699[c]	Q447K	CAG ⟶ <u>A</u>AG

[a]Isolates LIN39-222 were identified in the initial selection/screen. Isolates LIN414-699 were identified in a separate independent selection/screen.
[b]Position relative to first codon of full-length *ponB* gene ATG codon.
[c]LpoB-bypass phenotype is temperature sensitive.

Table S2. Strains used in this study.

Strain	Genotype[a]	Source/Reference[b]
DH5α	*F– hsdR17 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 ϕ80dlacZΔM15*	Gibco BRL
DH5α(λp*ir*)	DH5α harboring a λ lysogen producing pir for the replication of R6K vectors	de Boer lab stock
Rosetta(λDE3)	*ompT* rB- mB- *gal dcm* (P_{lac}UV5::T7*gene1*)	Novagen
MG1655	*rph-1 ilvG rfb-50*	(1)
JW5157	BW25113 Δ*lpoB(ycfM)*::Kan^R	(2)
JW3359	BW25113 Δ*ponA(mrcA)*::Kan^R	(2)
CAG12025	MG1655 *yadC*::Tn*10*	(3)
TB28	MG1655 *ΔlacIZYA*::*frt*	(4)
CB2	MG1655 Δ*ponB (mrcB)*::Kan^R	(5)
CB4(*att*λTB309)	TB28 *ΔponA (mrcA)*::*frt* Δ*lpoB*::Kan^R (P_{ara}::*ponA*)	(5)
CB7	MG1655 Δ*lpoB*::Kan^R	(5)
TU121(*att*λTB309)	TB28 *ΔponA (mrcA)*::*frt* (P_{ara}::*ponA*)	(5)
MG5	TB28 Δ*pal*::Kan^R	(6)
TU182	MG1655 Δ*ponA (mrcA)*::Kan^R	P1(JW3359) x MG1655
MM33(*att*λTB309)	TB28 *ΔponA (mrcA)*::*frt* Δ*lpoB*::*frt* (P_{ara}::*ponA*)	CB4(*att*λTB309)/pCP20
LIN39	MM33(*att*λTB309) *ponB*(Q411R)	cefsulodin resistant isolate
LIN65	MM33(*att*λTB309) *ponB*(I202F)	cefsulodin resistant isolate
LIN222	MM33(*att*λTB309) *ponB*(E313D)	cefsulodin resistant isolate
LIN425	MM33(*att*λTB309) *ponB*(Q447K)	cefsulodin resistant isolate
MM36(*att*λTB309)	MM33(*att*λTB309) *ponB*(Q411R) *yadC*::Tn*10*	P1(CAG12025) x LIN39
MM37(*att*λTB309)	MM33(*att*λTB309) *ponB*(I202F) *yadC*::Tn*10*	P1(CAG12025) x LIN65
MM38(*att*λTB309)	MM33(*att*λTB309) *ponB*(E313D) *yadC*::Tn*10*	P1(CAG12025) x LIN222
MM84(*att*λTB309)	MM33(*att*λTB309) *ponB*(Q447K) *yadC*::Tn*10*	P1(CAG12025) x LIN425
MM41	MG1655 *ponB*(Q411R) *yadC*::Tn*10*	P1[MM36(*att*λTB309)] x CB2
MM42	MG1655 *ponB*(I202F) *yadC*::Tn*10*	P1[MM37(*att*λTB309)] x CB2
MM43	MG1655 *ponB*(E313D) *yadC*::Tn*10*	P1[MM38(*att*λTB309)] x CB2

Strain	Genotype[a]	Source/Reference[b]
MM87	MG1655 *ponB*(Q447K) *yadC*::Tn*10*	P1[MM86(*att*λTB309)] x CB2
MM44	MG1655 *ponB*(Q411R) Δ*lpoB*::Kan^R *yadC*::Tn*10*	P1(JW5157) x MM41
MM45	MG1655 *ponB*(I202F) Δ*lpoB*::Kan^R *yadC*::Tn*10*	P1(JW5157) x MM42
MM46	MG1655 *ponB*(E313D) Δ*lpoB*::Kan^R *yadC*::Tn*10*	P1(JW5157) x MM43
MM118	MG1655 *ponB*(Q447K) Δ*lpoB*::Kan^R *yadC*::Tn*10*	P1(JW5157) x MM87
MM47	MG1655 *ponB*(Q411R) Δ*lpoB*::*frt yadC*::Tn*10*	MM44/pCP20
MM48	MG1655 *ponB*(I202F) Δ*lpoB*::*frt yadC*::Tn*10*	MM45/pCP20
MM49	MG1655 *ponB*(E313D) Δ*lpoB*::*frt yadC*::Tn*10*	MM46/pCP20
MM121	MG1655 *ponB*(Q447K) Δ*lpoB*::*frt yadC*::Tn*10*	MM118/pCP20
MM51	MG1655 Δ*lpoB*::*frt*	CB7/pCP20
MM66	MG1655 *ponB*(E313D) Δ*lpoB*::*frt yadC*::Tn*10* Δ*ponA*::Kan^R	P1(TU182) x MM49
MM88(*att*λTB309)	TB28 *ΔponA (mrcA)*::*frt* Δ*lpoB*::*frt ponB*(Q411R) *yadC*::Tn*10* (P_{ara}::*ponA*)	P1(CAG12025) x LIN39
MM89(*att*λTB309)	TB28 *ΔponA (mrcA)*::*frt* Δ*lpoB*::*frt ponB*(I202F) *yadC*::Tn*10* (P_{ara}::*ponA*)	P1(CAG12025) x LIN65
MM107	MG1655 *ponB*(E313D) *yadC*::Tn*10* Δ*ponA*::Kan^R	P1(TU182) x MM43
MM108	MG1655 Δ*ponA*::*frt*	TU182/pCP20
MM100(*att*λTB309)	MM33(*att*λTB309) *ponB*(Q447K) *yadC*::Tn*10*	P1[MM84(*att*λTB309)] x MM33(*att*λTB309)
MM102(*att*λTB309)	MM33(*att*λTB309) *ponB*(Q411R) *yadC*::Tn*10*	P1[MM36(*att*λTB309)] x MM33(*att*λTB309)
MM104(*att*λTB309)	MM33(*att*λTB309) *ponB*(I202F) *yadC*::Tn*10*	P1[MM37(*att*λTB309)] x MM33(*att*λTB309)
MM105(*att*λTB309)	MM33(*att*λTB309) *ponB*(E313D) *yadC*::Tn*10*	P1[MM38(*att*λTB309)] x MM33(*att*λTB309)
MM109(attHKMM92)	MG1655 Δ*pal*::Kan^R (P_{lac}::*lpoB*)	P1(MG5) x MG1655(attHKMM92)
MM115(attHKMM92)	MG1655 *ponB*(E313D) Δ*lpoB*::*frt yadC*::Tn*10* Δ*pal*::Kan^R (P_{lac}::*lpoB*)	P1(MG5) x MM49(attHKMM92)
MM117(attHKMM92)	MG1655 Δ*lpoB*::*frt* Δ*pal*::Kan^R (P_{lac}::*lpoB*)	P1(MG5) x MM51(attHKMM92)

Strain	Genotype[a]	Source/Reference[b]
MM119	MG1655 *ponB*(E313D) Δ*lpoB*::*frt yadC*::Tn*10* Δ*ponA*::*frt*	MM66/pCP20
MM120	MG1655 *ponB*(E313D) *yadC*::Tn*10* Δ*ponA*::*frt*	MM107/pCP20
MM127	MG1655 *yadC*::Tn*10*	P1(CAG12025) x MG1655
MM131	MG1655 *ponB*(Q411R) *yadC*::Tn*10*	P1[MM88(*att*λTB309)] x CB2
MM133	MG1655 *ponB*(I202F) *yadC*::Tn*10*	P1[MM89(*att*λTB309)] x CB2
MM135	MG1655 Δ*lpoB*::*frt yadC*::Tn*10*	P1(CAG12025) x MM51
MM139	MG1655 *ponB*(Q411R) Δ*lpoB*::Kan^R *yadC*::Tn*10*	P1(JW5157) x MM131
MM140	MG1655 *ponB*(I202F) Δ*lpoB*::Kan^R *yadC*::Tn*10*	P1(JW5157) x MM133
MM141	MG1655 Δ*ponB (mrcB)*::*frt yadC*::Tn*10*	MM144/pCP20
MM142	MG1655 *ponB*(Q411R) Δ*lpoB*::*frt yadC*::Tn*10*	MM139/pCP20
MM143	MG1655 *ponB*(I202F) Δ*lpoB*::*frt yadC*::Tn*10*	MM140/pCP20
MM144	MG1655 Δ*ponB (mrcB)*::Kan^R *yadC*::Tn*10*	P1(CAG12025) x CB2
JLB56(*att*HKMM81)	MG1655 *ponB*(E313D) Δ*lpoB*::*frt* Δ*ponA*:: Kan^R *yadC*::Tn*10* (P_{lac}::*gfp-ponB*(E313D)	P1(TU182) x MM49(*att*HMMM81)
JLB56(*att*HKMM79)	MG1655 *ponB*(Q411R) Δ*lpoB*::*frt* Δ*ponA*:: Kan^R *yadC*::Tn*10* (P_{lac}::*gfp-ponB*(Q411R)	P1(TU182) x MM47(*att*HMMM79)

[a] The Kan^R and Cm^R cassettes are flanked by *frt* sites for removal by FLP recombinase. An *frt* scar remains following removal of the cassette using FLP expressed from pCP20.

[b] Strain constructions by P1 transduction are described using the shorthand: P1(donor) x recipient.

Table S3. Plasmids used in this study.

Plasmid	Genotype[a]	ori	Source/Reference
pMM6	*bla lacI* P_{lac}*::gfp-ponB(WT)*	R6K	This study
pMM79	*bla lacI* P_{lac}*::gfp-ponB(Q411R)*	R6K	This study
pMM80	*bla lacI* P_{lac}*::gfp-ponB(I202F)*	R6K	This study
pMM81	*bla lacI* P_{lac}*::gfp-ponB(E313D)*	R6K	This study
pMM89	*bla lacI* P_{lac}*::gfp-ponB(Q447K)*	R6K	This study
pMM82	*bla lacI* P_{T7}*::h-sumo-ponB(Q411R)*	pBR/colE1	This study
pMM83	*bla lacI* P_{T7}*::h-sumo-ponB(I202F)*	pBR/colE1	This study
pMM84	*bla lacI* P_{T7}*::h-sumo-ponB(E313D)*	pBR/colE1	This study
pMM90	*bla lacI* P_{T7}*::h-sumo-ponB(WT)*	pBR/colE1	This study
pMM92	*bla lacI* P_{lac}*::lpoB*	R6K	This study

[a] A 6xHis tag for purification is indicated by the letter *h*. P_{lac} and P_{T7} indicate the lactose, phage T7 promoters, respectively.

SUPPLEMENTAL METHODS AND MATERIALS

Media, bacterial strains, and plasmids. Cells were grown in LB (1% tryptone, 0.5% yeast extract, and 0.5% NaCl unless otherwise indicated) or minimal M9 media (7) supplemented with 0.2% Casamino Acids and 0.2% sugar (glucose or arabinose as indicated). Unless otherwise indicated, antibiotics were used at 25 (chloramphenicol; Cm), 25 (kanamycin; Kan), 12.5 (tetracycline; Tet), or 15 (ampicillin; Amp) µg/ml. µg/mL.

The bacterial strains used in this study are listed in **Table S2**. All *E. coli* strains used in the reported experiments are derivatives of MG1655 (1). Mutants containing multiple deletions were constructed by successive rounds of P1 transduction (7) and the removal of the Kan-resistance marker (Kan^R) from cassettes flanked by *frt* sites using plasmid pCP20 as described previously (8). The desired chromosomal modification was confirmed using diagnostic PCR each time a Kan^R cassette was transduced or cured. For strains containing multiple lesions, diagnostic PCR was used to confirm the status of each lesion.

To construct strain MM66 [*ponB*(E313D) Δ*lpoB*::*frt yadC*::Tn*10* Δ*ponA*::Kan^R] the ΔponA::KanR allele was transduced into strain MM49 [*ponB*(E313D) Δ*lpoB*::*frt yadC*::Tn*10*]. The transductions were plated on LB0N, LB 0.5% NaCl, or LB 1% NaCl at 30°C. Transductants only grew on the LB0N and LB 0.5% NaCl plates. Similar transductions were performed to generate equivalent strains with the *ponB*(I202F) or *ponB*(Q411R) mutants, but the no Kan^R transductants grew on any of the LB plates.

Plasmids used are listed in **Table S3**. Vectors with R6K origins are all derivatives of CRIM plasmids developed by Haldimann and Wanner (9). The vectors were either maintained in the

cloning strain DH5α(*λpir*), where they replicate as plasmids, or they were integrated into phage attachment sites (HK022 or λ) by using the helper vectors pTB102 or pINT-ts, respectively, as previously described (9). Single-copy integrants were identified by using diagnostic PCR (9). Integrated vectors were transferred between strains by P1-mediated transduction. For all plasmid constructions, PCR was performed using KOD polymerase (Novagen) according to the manufacturer's instructions. Plasmid DNA was purified using either the Qiagen spin miniprep kit (Qiagen) or the Zippy miniprep kits (Zymo Research) while PCR fragments were purified using a Qiaquick PCR purification kit (Qiagen). Site-directed mutagenesis was performed using the QuikChange method (Stratagene).

For pMM79 [*att*HK022 *bla lacI*^{q} P_{lac}::*gfp-ponB(Q411R)*], pMM80 [*att*HK022 *bla lacI*^{q} P_{lac}::*gfp-ponB(I202F)*], pMM81 [*att*HK022 *bla lacI*^{q} P_{lac}::*gfp-ponB(E313D)*], and pMM89 [*att*HK022 *bla lacI*^{q} P_{lac}::*gfp-ponB(Q447K)*] the *ponB** genes were amplified from genomic DNA using the primers 5'-GTACGGATCCC CGCGCAAAGGTAAGGG-3' and 5'-GTCACTCGAGATGGGATGTTATTTTACCGGATGG C-3'. The PCR products were then digested with BamHI and XhoI and ligated into BamHI/SalI digested pTB183 [*att*HK022 *bla* P_{lac}::*gfp-zapA*] (10) to replace the *zapA* gene.

For pMM82 [P_{T7}::*h-sumo-ponB(Q411R)*], pMM83 [P_{T7}::*h-sumo-ponB(I202F)*], pMM84 [P_{T7}::*h-sumo-ponB(E313D)*], and pMM90 [P_{T7}::*h-sumo-ponB(WT)*], the relevant *ponB* PCR products generated as above were inserted into BamHI/SalI digested pTD54, a H-SUMO fusion vector essentially identical to pTD68 (11).

For pMM92 [*att*HK022 *bla lacI*^{q} P_{lac}::*lpoB*], the *lpoB*-containing XbaI-HindIII fragment from pCB25 [*bla lacI*^{q} P_{lac}::*lpoB*] (5) was used to replace the corresponding *gfp-zapA* fragment from pTB183 [*att*HK022 *bla* P_{lac}::*gfp-zapA*] (10).

Selection and screen for LpoB-bypass mutants. Cells of MM33(*att*λTB309) [*ΔlpoB ΔponA* (P_{ara}::*ponA*)] were grown overnight in M9 minimal medium containing 0.2% arabinose at 30°C. The next morning, the cultures were subcultured 1:100 into 50 ml of the same medium and grown to saturation (OD_{600} = 1.0) at 30°C. Serial dilutions were plated onto permissive conditions (M9 0.2% arabinose) to determine the concentration of viable cells. For the selection, 5 ml and 10 ml volumes of culture were pelleted, resuspended in 100 µl, and plated on M9 0.2% arabinose with 0.01 µg/ml cefsulodin. Resistant mutants arose at an average frequency of approximately 10^{-7} based on two independent selections. Colonies were then patched on M9 0.2% arabinose and M9 0.2% glucose agar to identify arabinose-independent isolates, which were considered good candidates for mutants encoding PBP1b* variants that function in the absence of LpoB (see Results section for details on the rationale). The *ponB* gene from each isolate was amplified and sequenced. All isolates harbored mutations in *ponB*. A list of the isolates and the mutations identified in the *ponB* gene are presented in **Table S1**.

Culture and plating conditions. Specific conditions used to monitor cell growth on solid or liquid media are provided in the relevant figure legends.

Protein purification. PBP1b (wild-type or variant) and Lpo proteins were purified as previously described (5).

Ether-permeabilized cell assay. This assay was performed as described previously in (5) with ether-treated cells prepared from cultures of the relevant strains grown to an OD_{600} of 0.50 at 30˚C.

PGT Assay. Glycan synthesis was assayed as described previously (5). Wild-type PBP1b or PBP1b variants (400 nM) were preincubated with or without lipoproteins (400 nM) at room temperature for 5 min. Reactions were initiated upon addition of proteins to lipid II (4 μM) and incubated at room temperature for 30 min prior to heat inactivation (90°C, 10 min). Gel electrophoresis analysis was carried out as described previously (12). Briefly, samples were dried and resuspended in sample buffer (125 mM Tris-HCl pH 6.8, 40% glycerol, 9% SDS, 0.004% bromophenol blue) before being loaded on a 20 x 20 cm gel (9% acrylamide, 1 mm thick) and separated at 30 mA until the dye front was 1.5 cm from the bottom. Gels were dried overnight and exposured to a phosphorimager screen for 1 week. Imaging was performed with a Typhoon phosphorimager (GE Healthcare).

SUPPORTING REFERENCES

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