SUPPORTING INFORMATION FOR:

A conserved mechanism of cell wall synthase regulation revealed by the identification of a new PBP activator in *Pseudomonas aeruginosa*

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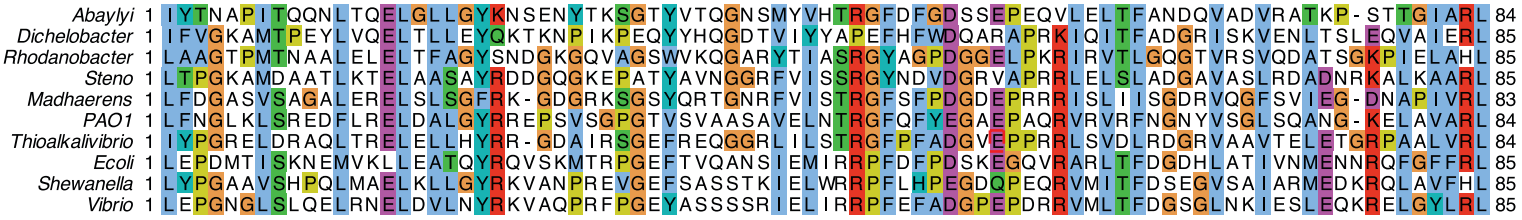

Figure S1. Figure S1. Alignment of UB2H domain sequences from several gram-negative species. The UB2H domain sequences from the PBP1b proteins of *Acinetobacter baylyi* ADP1(Abaylyi), *Dichelobacter nodosus* (Dichelobacter), *Rhodanobacter denitrificans* (Rhodanobacter), *Stenotrophomonas maltophilia* (Steno), *Marinobacter adhaerens* (Madhaerens), *Pseudomonas aeruginosa* (PAO1), *Thioalkalivibrio sulfidiphilus* (Thioalkalivibrio), *Escherichia coli* (Ecoli), *Shewanella oneidensis* (Shewanella) and *Vibrio cholerae* (Vibrio) were aligned with the Clustal Omega program (**https://www.ebi.ac.uk/Tools/msa/clustalo/**). The alignments were modified with Jalview 2.10.2. The aminoacids are colored as follows: blue = hydrophobic, red = positively charged, magenta = negatively charged, green = polar, pink = cysteines, orange= glycine, yellow = prolines, cyan = aromatic, white = unconserved. Note that the UB2H domain of *P. aeruginosa* PAO1 is 26% identical and 58% similar to that of *E. coli*.

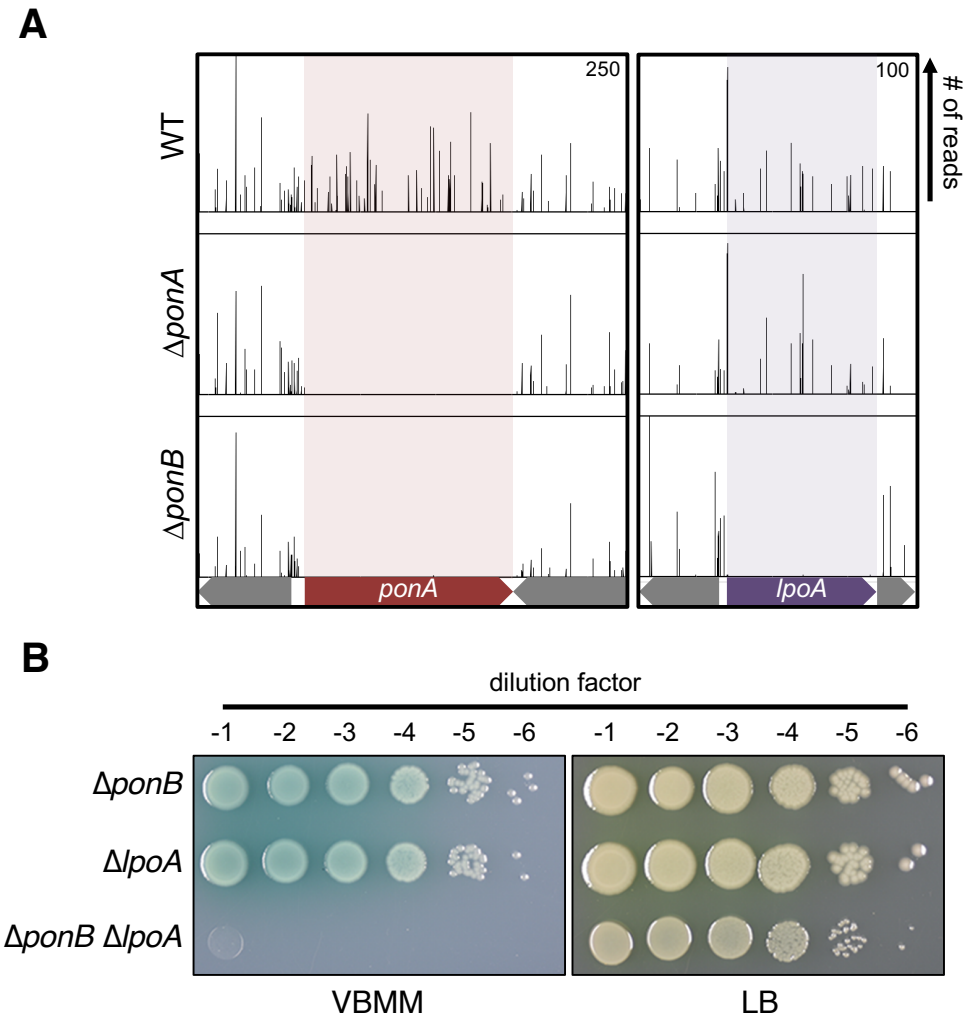

Figure S2. Genetic connections between ^{Pa}PBP1a and ^{Pa}LpoA. (A) Shown are Tn-Seq profiles for the *P. aeruginosa ponA* and *lpoA* loci from analyses performed in the PAO1 wildtype (WT) strain and derivatives lacking either PBP1a (Δ*ponA*) or PBP1b (Δ*ponB*). Individual lines above the locus map represent unique transposon insertion sites and the height of each bar reflects the number of sequencing reads at that site. Colored boxes indicate genes with significantly fewer reads in either mutant as compared to the WT. (B) Cells of PA105 [Δ*ponB*], PA684 [Δ*lpoA*], and PA686 [Δ*ponB* Δ*lpoA*] were grown and spotted on agar as described for Fig. 1B except that PA686 [Δ*ponB* Δ*lpoA*] was grown overnight in LB without NaCl where its growth was more robust than LB with NaCl. For WT growth under these conditions please refer to Figure 1B.

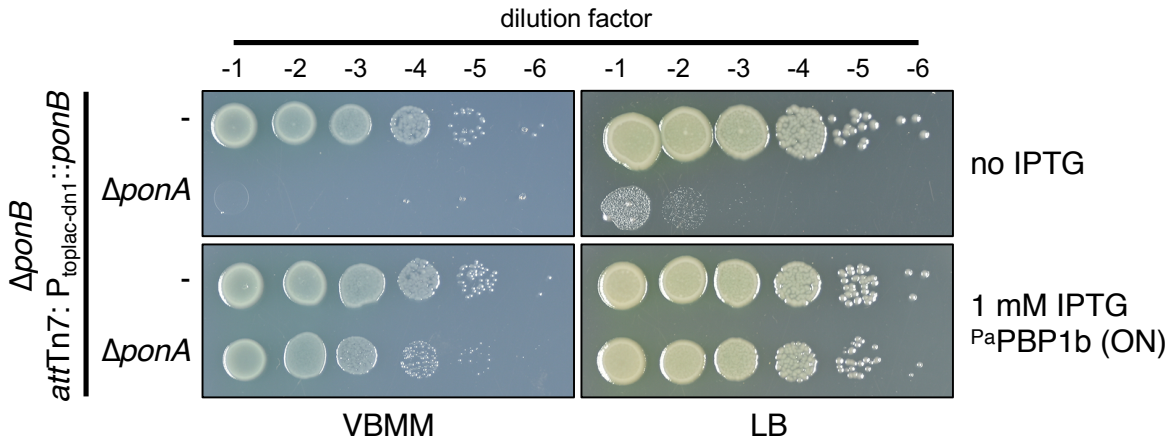

Figure S3. Validation of the synthetic lethal relationship between ^{Pa}PBP1a and ^{Pa}PBP1b.
Cells of PA614 [Δ*ponB* $P_{toplac\text{-}dn1}$::*ponB*] or its Δ*ponA* [PA615] derivative were grown as in Fig. 1B, except that overnight cultures were supplemented with 1 mM IPTG and dilutions were plated on indicated media without or with 1 mM IPTG. Plates were photographed following overnight incubation at 37ºC.

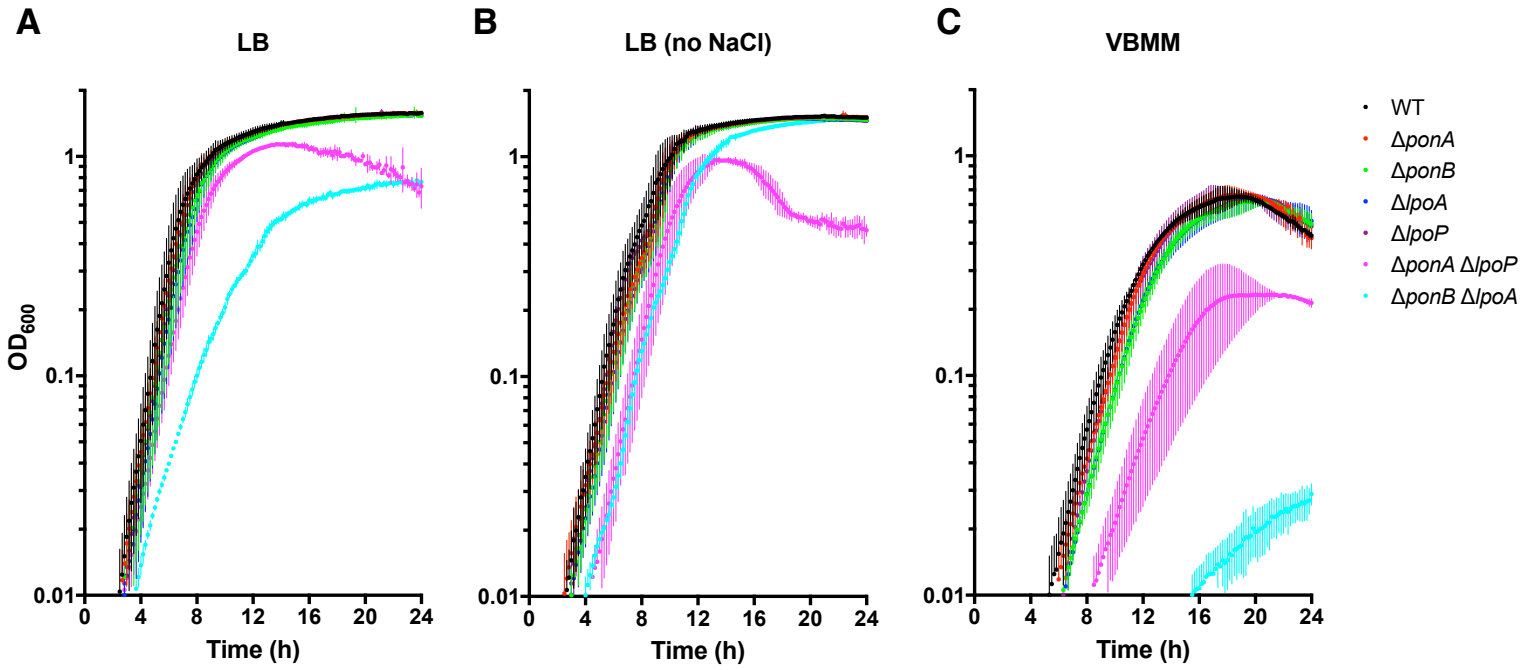

Figure S4. Growth curves of mutant strains.
Cells of PA95 [WT], PA104 [Δ*ponA*], PA105 [Δ*ponB*], PA684 [Δ*lpoA*], PA550 [Δ*lpoP*] and PA685 [Δ*ponA* Δ*lpoP*] were grown at 30°C overnight in LB and PA686 [Δ*ponB* Δ*lpoA*] was grown in LB without NaCl. The next morning, all cultures were washed twice with VBMM and normalized to OD_{600} = 0.2. Five microliters of each culture was added to separate wells of a 96-well plate containing 195 µL of either (A) LB, (B) LB without NaCl or (C) VBMM. Growth was monitored using a Versamax microplate reader by measuring OD_{600} in 10 minute intervals at 30°C with shaking. The OD_{600} values from control wells containing media only were subtracted from each value. The data are presented as the mean ± standard deviation of 3 independent experiments. Note that in each panel the results from WT, Δ*ponA*, Δ*ponB*, Δ*lpoA* and Δ*lpoP* overlap.

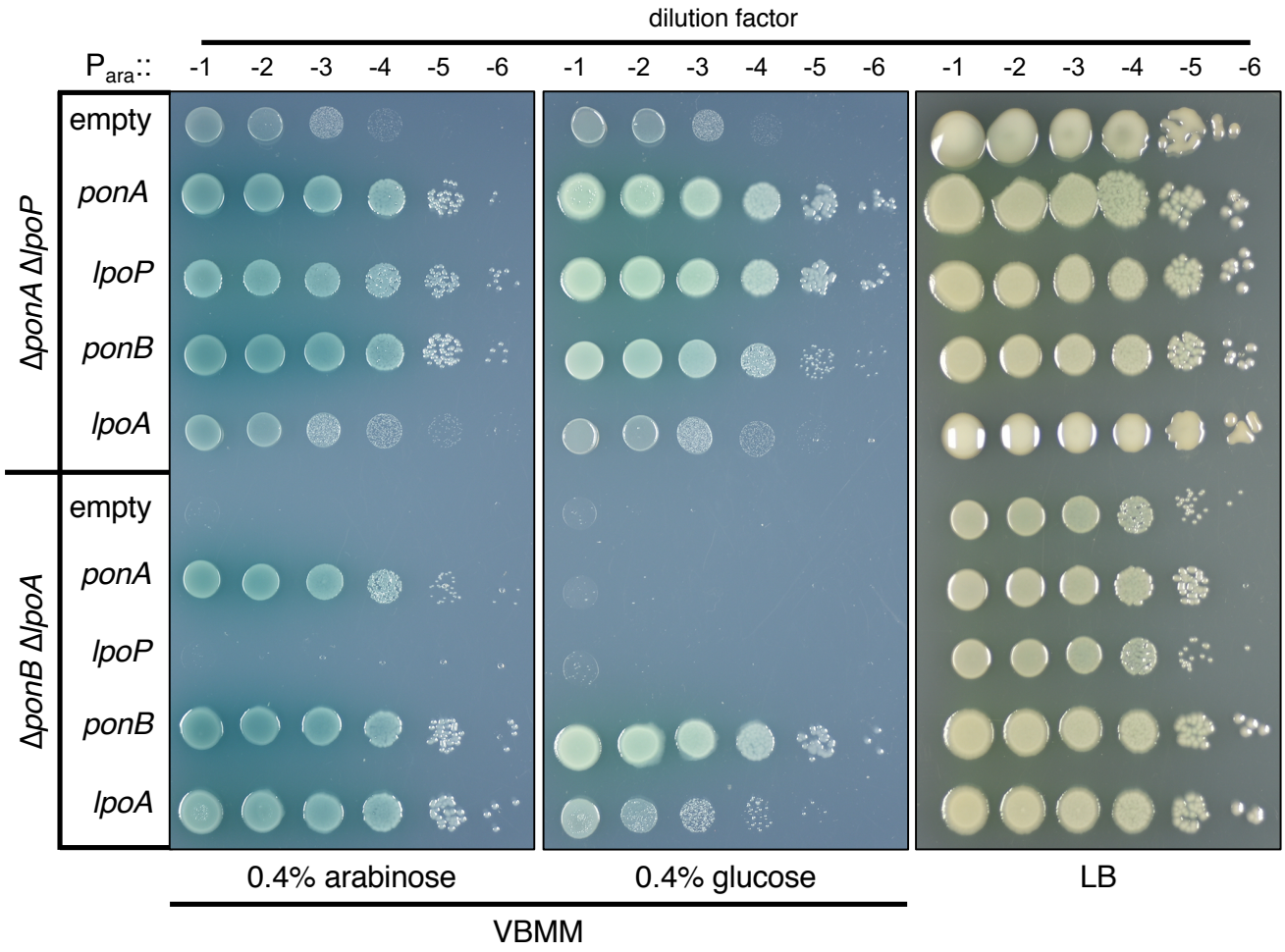

Figure S5. Complementation analysis of *lpo* mutant phenotypes.
Cells of PA685 [Δ*ponA* Δ*lpoP*] or PA686 [Δ*ponB* Δ*lpoA*] harboring a multicopy plasmid with the indicated *P. aeruginosa* genes under control of an arabinose-inducible promoter (P_{ara}) were grown and spotted on agar as described for Fig. 1B with the exception that all PA686 [Δ*ponB* Δ*lpoA*] derivatives were grown overnight in LB without NaCl because their growth was superior in this medium relative to normal LB. The VBMM plates used were supplemented with either 0.4% arabinose or 0.4% glucose, as indicated. Plasmids used were: pJN105 [empty vector], pNG59 [P_{ara}::*ponA*], pNG46 [P_{ara}::*lpoP*], pNG64 [P_{ara}::*ponB*], and pNG45 [P_{ara}::*lpoA*]. Note that due to leaky expression, inducer is not required for the plasmid expressed gene to complement its corresponding deletion (e.g. the *lpoP* construct in the Δ*ponA* Δ*lpoP* mutant). In cases where the unmutated PBP is being overproduced the inducer requirement varies. Leaky expression of *ponB* in the Δ*ponA* Δ*lpoP* is sufficient to restore growth whereas *ponA* must be induced to restore growth to Δ*ponB* Δ*lpoA* cells on VBMM. This difference in inducer requirement is most likely due to the difference in the severity of the phenotype being complemented.

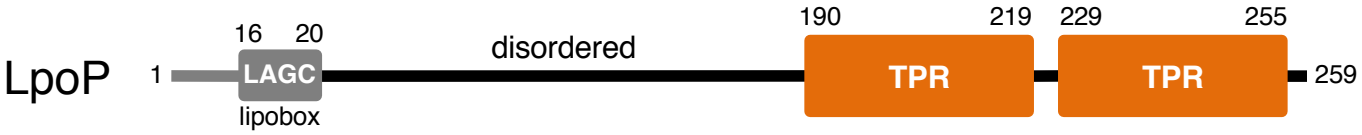

Figure S6. ^{Pa}LpoP primary structure. Shown is a schematic representation of the domain organization of *P. aeruginosa* LpoP. Numbers above denote the boundaries of each predicted domain. Indicated are the N-terminal signal sequence with highlighted lipobox sequence (LAGC) and two tandem tetratricopeptide repeat (TPR) domains at the C-terminus. The intervening sequence between the lipobox and first TPR is predicted to be disordered (IUPred) (1).

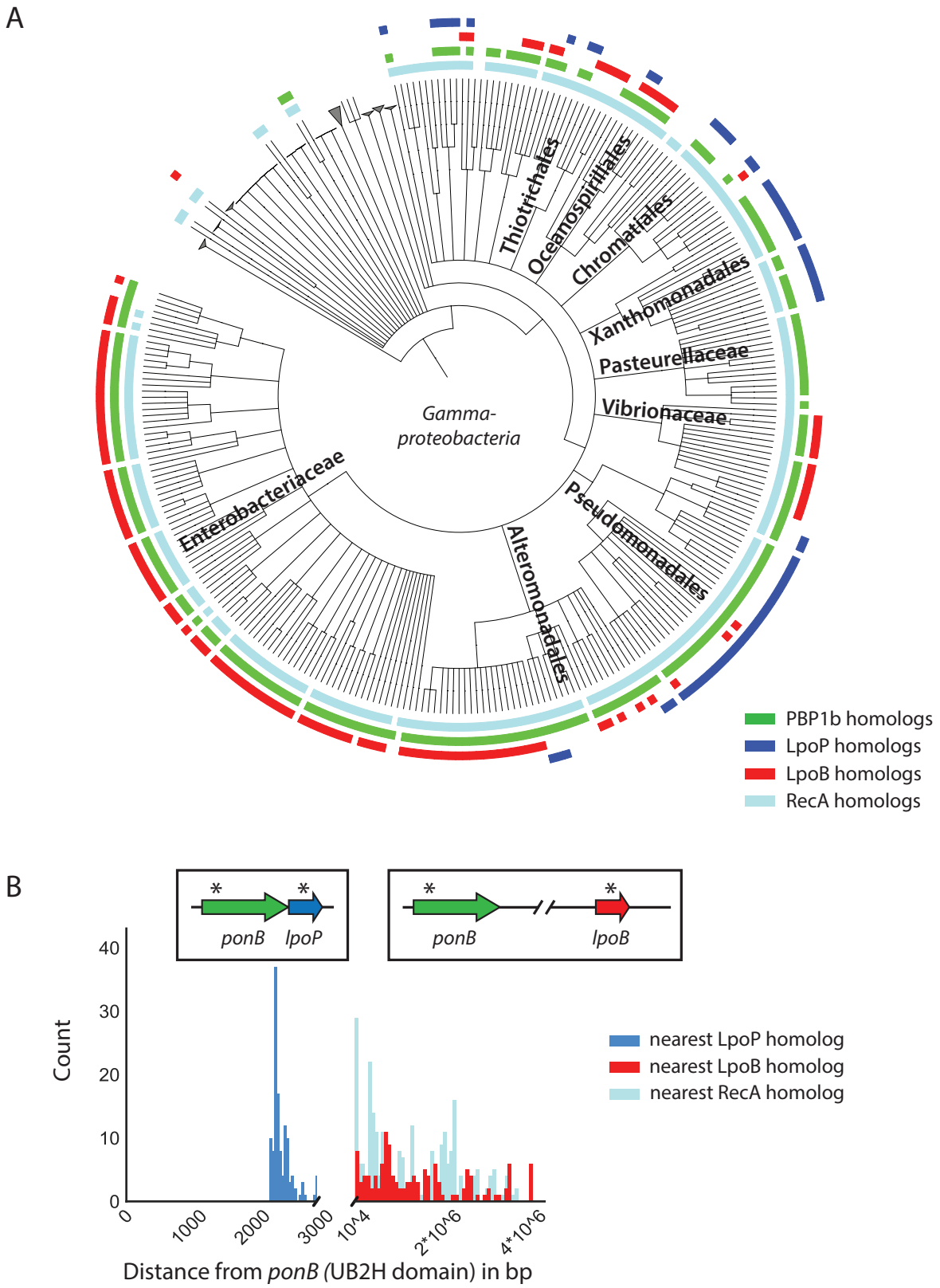

Figure S7. Phylogenetic distribution of LpoP and *ponB-lpo* gene linkage. (A) Shown is a phylogenetic tree depicting the occurrence of PBP1b (green), LpoP (dark blue) and LpoB (red) in the Gammaproteobacteria as indicated by the colored regions at the outer edge of the tree. The tree was constructed in PhyLoT (http://phylot.biobyte.de) and visualized in iTOL (2) with a diversity set of 1773 strains. RecA occurrence (light blue) is also indicated as a control. Names of relevant bacterial orders or families are indicated in the tree. (B) Histogram showing the genetic distance between 205 *ponB* loci (green) and the nearest *lpoP* or *lpoB* locus (blue and red, respectively). The distance is measured between the asterisks (from the center of the UB2H domain to the center of the lipoprotein). *lpoP* loci are commonly observed in an operon with *ponB*, while *lpoB* loci are typically unlinked from the *ponB* gene. Distances between *ponB* and the nearest *recA* gene are shown in light blue as a negative control. The *ponB* and lipoprotein genes were identified using tblastn with 7 diverse representatives of *lpoP* or 3 representatives of *lpoB* used as the query sequence.

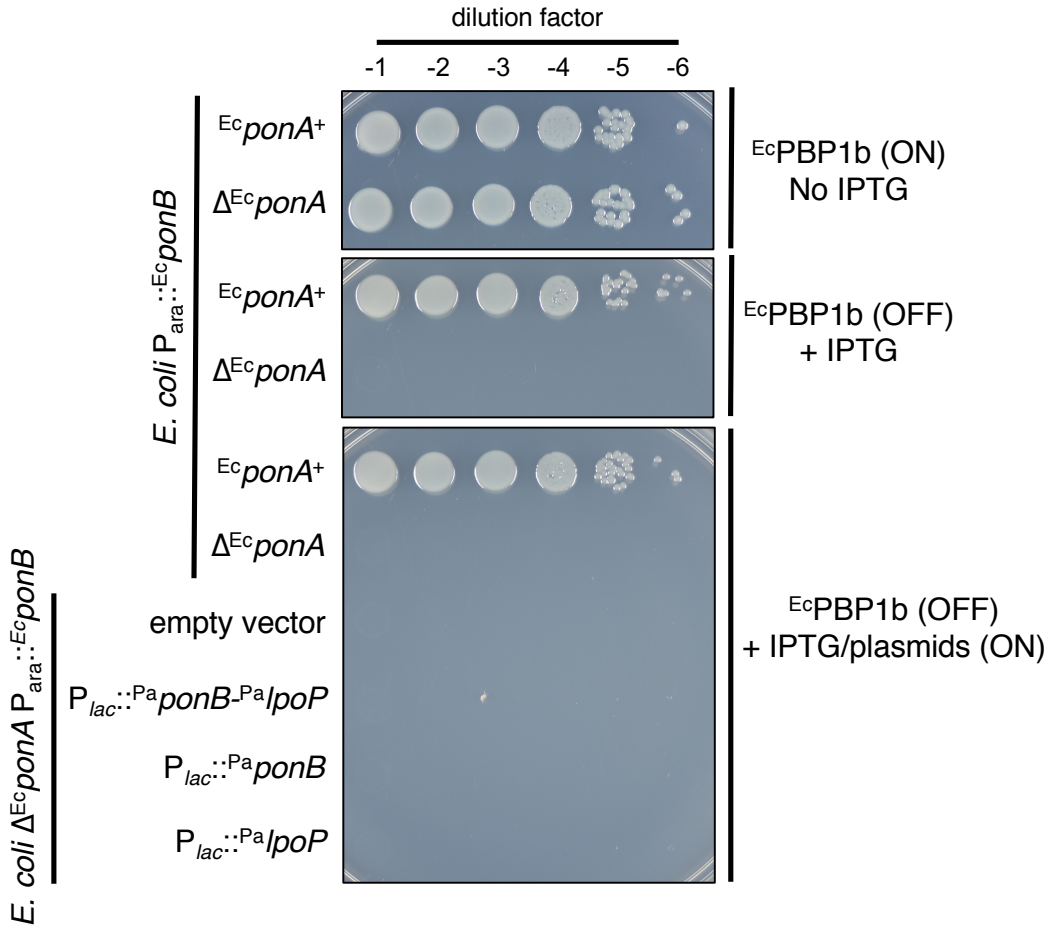

Figure S8. Additional controls for the demonstration of ^{Pa}PBP1b-^{Pa}LpoP activity in *E. coli*. Shown are spot dilutions of *E. coli* strains MM11 [P_{ara}::^{Ec}*ponB*] and MM10 [Δ^{Ec}*ponA* P_{ara}::^{Ec}*ponB*] on M9 agar supplemented with either 0.2% arabinose (^{Ec}PBP1b ON) or 0.2% maltose (^{Ec}PBP1b OFF) as well as 1 mM IPTG, where indicated, to confirm the dependence of strain MM10 on ^{Ec}PBP1b expression for viability in the absence of ^{Ec}PBP1a. Also, the strains from Fig. 3 were spotted on M9 agar without inducer (IPTG) to show that ^{Pa}*ponB*-^{Pa}*lpoP* required induction to suppress the ^{Ec}*ponA* ^{Ec}*ponB* synthetic lethality in MM10. Cells were grown and plated as described in Fig. 3.

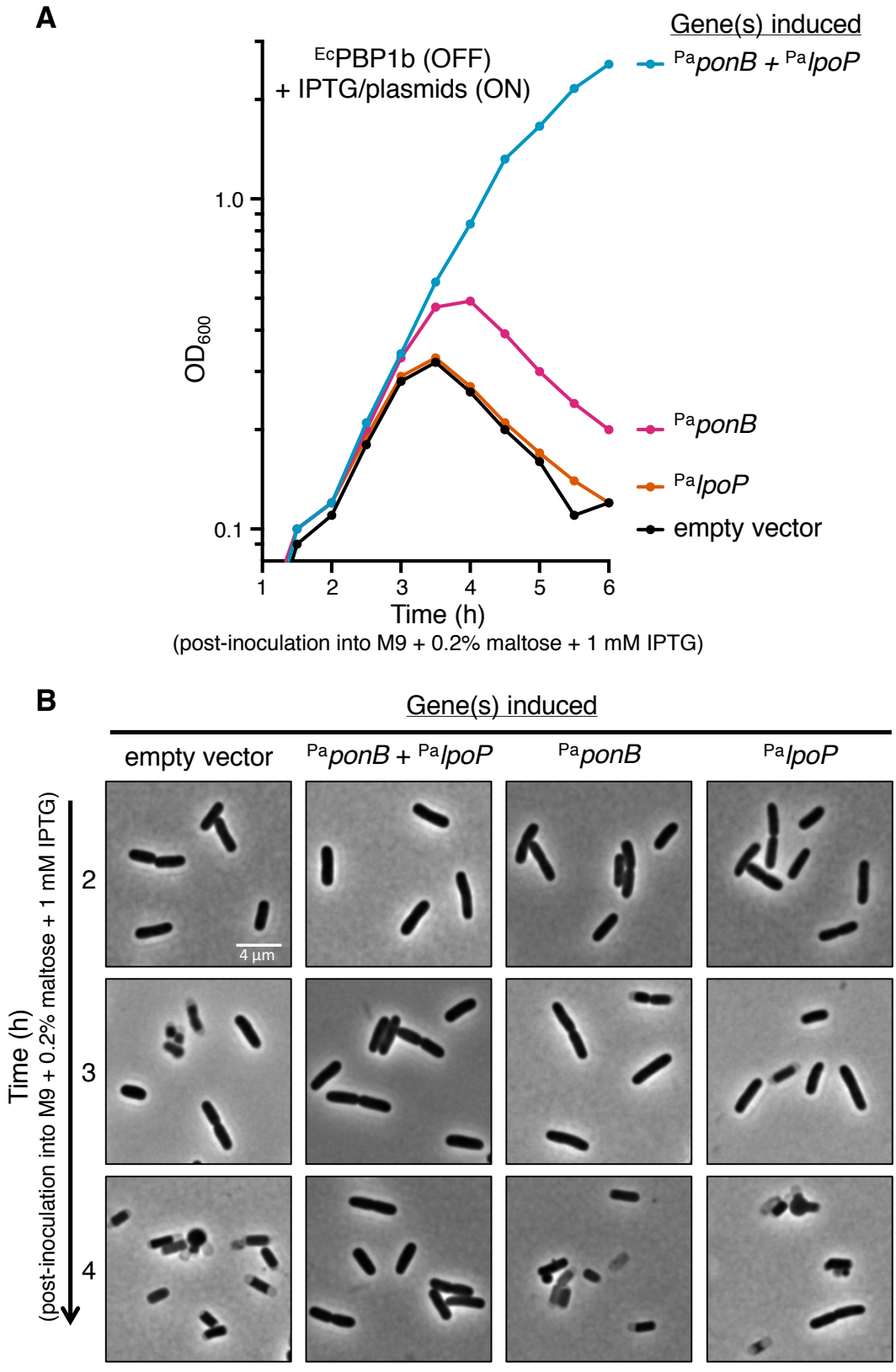

Figure S9. ^{Pa}PBP1b-^{Pa}LpoP maintains cell shape and integrity in *E. coli* cells depleted for endogenous aPBPs. (A) The MM10 [*E. coli* Δ^{Ec}*ponA* P_{ara}::^{Ec}*ponB*] derivatives harboring the integrated plasmid expression constructs pMT116 [empty vector], pNG66 [P_{lac}::^{Pa}*ponB*-^{Pa}*lpoP*], pNG68 [P_{lac}:: ^{Pa}*ponB*], or pNG51 [P_{lac}::^{Pa}*lpoP*] from Fig. 3 were grown in M9 + 0.2% arabinose medium overnight at 37ºC. The next morning, each overnight culture was diluted 1:100 in the same medium and grown at 37ºC for approximately 2.5 hours. Cells were washed three times with M9 lacking a carbon source and diluted into M9 + 0.2% maltose + 1 mM IPTG at an OD_{600} = 0.01. Cultures were incubated with shaking at 37ºC and growth was monitored by measuring OD_{600} every 30 minutes. (B) At the 2, 3 and 4 hour time points in the growth curve from (A), samples from each culture were removed, applied to agarose pads and cells were visualized using phase-contrast microscopy. The white bar in the top left panel equals 4 microns.

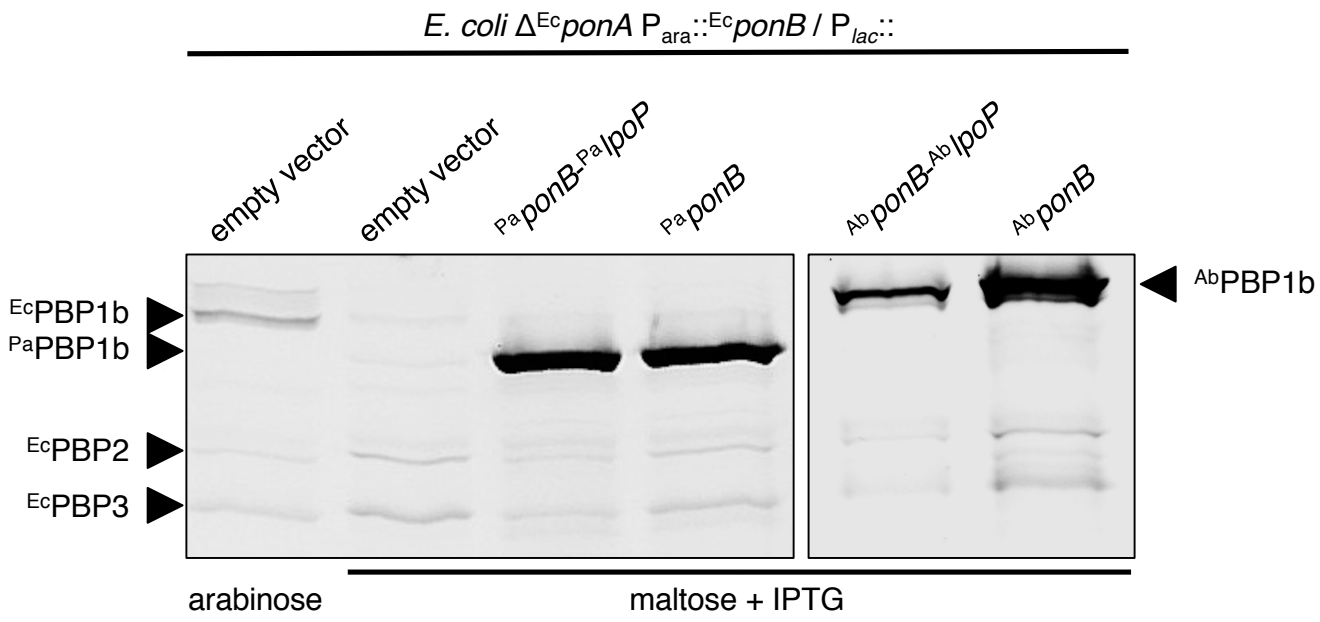

Figure S10. Measurement of relative PBP1b concentration by Bocillin binding. Overnight cultures of *E. coli* MM10 [Δ*Ec*ponA P_{ara}::*Ec*ponB] harboring the integrated plasmid expression constructs pMT116 [empty vector], pNG66 [P_{lac}::*Pa*ponB-*Pa*lpoP] and pNG68 [P_{lac}::*Pa*ponB] or pNG75 [P_{lac}::*Ab*ponB-*Ab*lpoP] and pNG74 [P_{lac}::*Ab*ponB] were diluted 1:100 in M9 medium with 0.2% arabinose and grown at 37ºC for approximately 2.5 hours. Cells were washed three times with M9 lacking a carbon source and diluted into M9 arabinose (for the empty vector) or maltose + 1 mM IPTG (for all strains) at an OD_{600} = 0.01. Cultures were incubated 2.5 hours with shaking at 37ºC until the OD600 reached 0.2–0.3. Cells were collected and labeled with Bocillin (see Methods). All results are representative of at least duplicate experiments.

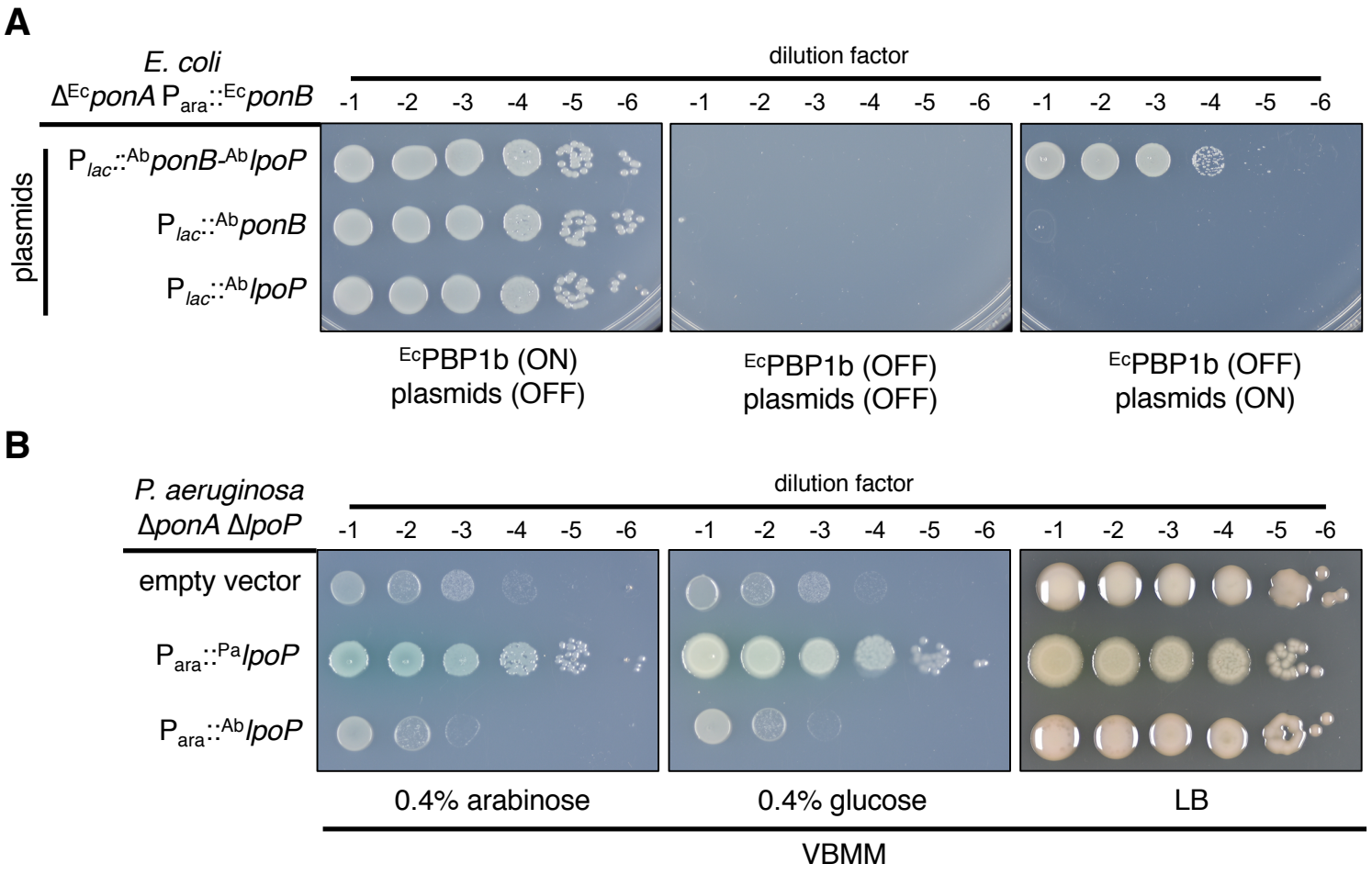

Figure S11. ^{Ab}LpoP-^{Ab}PBP1b from *Acinetobacter baylyi* complements the loss of aPBP activity in *E. coli* but ^{Ab}LpoP cannot cross-complement *P. aeruginosa* ^{Pa}PBP1b. (A) Cells of *E. coli* MM10 [Δ^{Ec}*ponA* P$_{ara}$::^{Ec}*ponB*] and its derivatives harboring the integrated plasmid expression constructs pNG75 [P$_{lac}$::^{Ab}*ponB*-^{Ab}*lpoP*], pNG74 [P$_{lac}$::^{Ab}*ponB*], and pNG134 [P$_{lac}$::^{Ab}*lpoP*] were grown and plated as described in Fig. 3. The ^{Ab}*ponB*-^{Ab}*lpoP* genes used were (ACIAD2234-ACIAD2235) from *Acinetobacter baylyi* ADP1. (B) *P. aeruginosa* strain PA685 [Δ*ponA* Δ*lpoP*] harboring the multicopy plasmid pJN105 alone [empty vector] or with an arabinose-inducible copy of *lpoP* from either *P. aeruginosa* (pNG46 [P$_{ara}$::^{Pa}*lpoP*]) or *A. baylyi* (pNG62 [P$_{ara}$::^{Ab}*lpoP*]) was grown and spotted on VBMM or LB agar as described for Fig. 1B. As indicated, the VBMM plates used were supplemented with either 0.4% arabinose or 0.4% glucose.

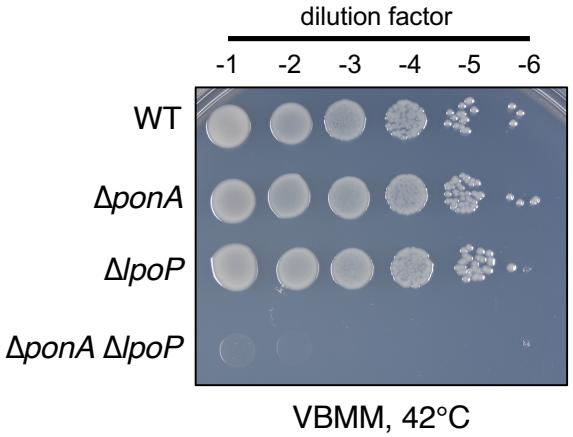

Figure S12. ^{Pa}PBP1a and ^{Pa}LpoP are synthetically lethal on VBMM at 42ºC. The indicated strains were grown and spot dilutions performed as described in Fig. 1B except the plate was incubated at 42ºC overnight.

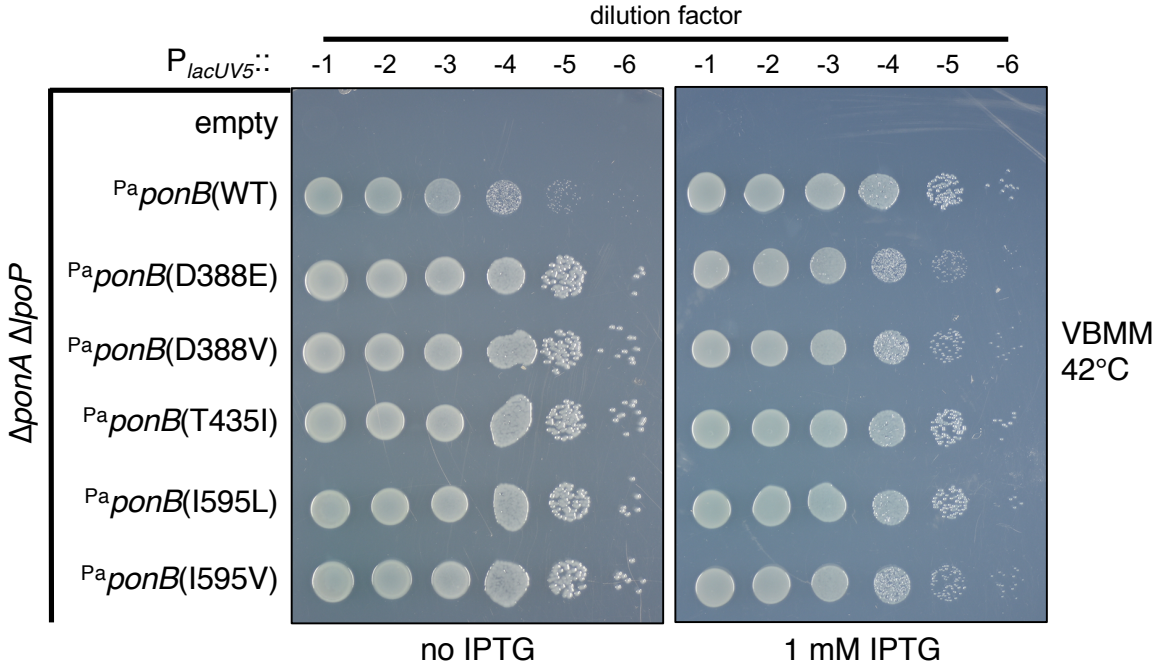

Figure S13. ^{Pa}PBP1b variants suppress Δ*ponA* Δ*lpoP* synthetic lethality in *P. aeruginosa* under non-permissive conditions. Cells of *P. aeruginosa* strain PA685 [Δ*ponA* Δ*lpoP*] harboring plasmids expressing the indicated ^{Pa}*ponB* alleles under control of the *lacUV5* promoter (P_{lacUV5}) were grown overnight at 37ºC in LB + 30 µg/mL gentamicin to maintain the plasmid. The next morning, cells from each culture were washed twice in VBMM and spot dilutions were performed as described in Fig. 1B except that dilutions were plated on VBMM without or with 1 mM IPTG and the plates were incubated at 42ºC overnight. Plasmids used were: pPSV38 [empty vector], pNG92 [P_{lacUV5}::^{Pa}*ponB*(WT)], pNG97 [P_{lacUV5}::^{Pa}*ponB*(T435I)], pNG98 [P_{lacUV5}::^{Pa}*ponB*(I595V)], pNG99 [P_{lacUV5}::^{Pa}*ponB*(I595L)], pNG100 [P_{lacUV5}::^{Pa}*ponB*(D388E)], pNG101 [P_{lacUV5}::^{Pa}*ponB*(D388V)]. Note that leaky expression provides enough ^{Pa}PBP1b(WT) to partially restore growth to Δ*ponA* Δ*lpoP* in the absence of inducer, yet under the same conditions all PBP1b variants confer a more robust growth phenotype.

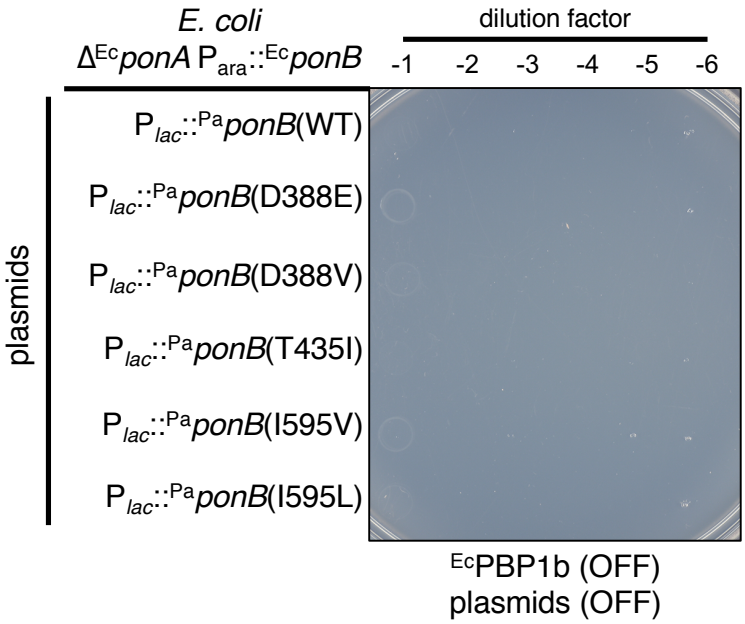

Figure S14. Additional controls for the demonstration of ^{Pa}LpoP-independent activity of ^{Pa}PBP1b variants in *E. coli*. The strains from Fig. 4B were also spotted on M9 + 0.2% maltose agar without IPTG to show that ^{Pa}*ponB* variants require IPTG induction to suppress the ^{Ec}*ponA* ^{Ec}*ponB* synthetic lethality in MM10. Cells were grown and plated as described in Fig. 3.

Table S1. List of ^{Pa}*ponB* mutations isolated in selection for ^{Pa}LpoP bypass

Locus	Base change	Amino acid substitution	Number of isolates
ponB	ACC → A<u>T</u>C	T435I	1
ponB	GAC → GA<u>G</u>	D388E	2
ponB	GAC → G<u>T</u>C	D388V	1
ponB	ATC → <u>C</u>TC	I595L	2
ponB	ATC → <u>G</u>TC	I595V	3
intergenic region; *ponB*-PA4701	G → C*	NA	1

*131 bp upstream of *ponB* start codon
NA = not applicable

Table S2. Strains used in this study

Strain	Genotype	Source/Reference
Escherichia coli		
BL21(λDE3)	*F– ompT dcm hsdS (rB- mB-) gal (λDE3)*	Novagen
DH5α	*F⁻ hsdR17 deoR recA1 endA1 phoA supE44 thi-1 gyrA96 relA1 Δ(lacZYA-argF)U169 ϕ80dlacZΔM15*	Gibco BRL
DH5α(λpir)	DH5α harboring a λ lysogen producing pir for the replication of R6K vectors	de Boer lab
Lemo21(λDE3)	*fhuA2 [lon] ompT gal (λDE3) [dcm] ΔhsdS/ pLemo*	(3)
MG1655	*rph-1 ilvG rfb-50*	(4)
MM10	TB28 Δ*ponA*::*frt* (Kan^R *araC* P_{ara})::*ponB*	(5)
MM11	TB28 (*frt araC* P_{ara})::*ponB*	(5)
SM10(λpir)	Kan^R *thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu attλ::pir*	(6)
TB28	MG1655 *ΔlacIZYA*::*frt*	(7)
Pseudomonas aeruginosa		
PAO1	wildtype	(8)
PA104	Δ*ponA*	This study
PA105	Δ*ponB*	This study
PA550	Δ*lpoP*	This study
PA614	Δ*ponB att*Tn7::$P_{toplac\text{-}dn1}$::*ponB*	This study
PA615	Δ*ponB att*Tn7::$P_{toplac\text{-}dn1}$::*ponB* Δ*ponA*	This study
PA684	Δ*lpoA*	This study
PA685	Δ*ponA* Δ*lpoP*	This study
PA686	Δ*ponB* Δ*lpoA*	This study
PA687	Δ*ponA* Δ*lpoP ponB*(T435I)	This study (isolated suppressor)
PA688	Δ*ponA* Δ*lpoP ponB*(I595V)	This study (isolated suppressor)
PA689	Δ*ponA* Δ*lpoP ponB*(I595L)	This study (isolated suppressor)

Strain	Genotype	Source/Reference
PA690	Δ*ponA* Δ*lpoP ponB*(D388E)	This study (isolated suppressor)
PA691	Δ*ponA* Δ*lpoP ponB*(D388V)	This study (isolated suppressor)

Table S3. Plasmids used in this study

Plasmid	Genotype[a]	Origin	Source or Reference
pBTK30	*aacC1 bla oriT mariner* C9	R6K	(9)
pCB39	*bla lacI* P_{T7}::*h-lpoB*(21-213)	pBR/ColE1	(5)
pCF288	*aacC1 sacB oriT* 'PA4422-*lpoA*Δ(2-604)-PA4424-PA4425'	pBR/ColE1	This study
pCF291	*aacC1 sacB oriT* '*pilM-ponA*Δ(2-822)-PA5046'	pBR/ColE1	This study
pCF352	*aacC1 sacB oriT* '*lpoP-ponB*Δ(2-774)-PA4701'	pBR/ColE1	This study
pCF526	*aacC1 sacB oriT* 'PA4697-PA4698-*lpoP*Δ(3-259)-*ponB*'	pBR/ColE1	This study
pCF964	*kan lacI* P_{T7}::*h-lpoP*(20-259)	pBR/ColE1	This study
pCF972	*bla lacI* P_{T7}::*h-sumo-*^{Pa}*ponB*	pBR/ColE1	This study
pEXG2	*aacC1 sacB oriT* [facilitates allelic exchange in *P. aeruginosa*]	pBR/ColE1	(10)
pFLP2	*bla sacB flp cI oriT* [Flp recombinase expression]	pRO1600	(11)
pJN105	*aacC1 araC* P_{ara} [replicating arabinose-inducible expression vector for *P. aeruginosa*]	pBBR1	(12)
pKHT104	*aacC1 bla* Tn7 *lacI*^{q} $P_{toplac\text{-}dn1}$ [facilitates insertion of $P_{toplacdn1}$-regulated sequences into the *att*Tn7 attachment site of *P. aeruginosa*]	pUC18	Dove lab
pNG41	*aacC1 bla* Tn7 *lacI*^{q} $P_{toplac\text{-}dn1}$::RBS_{native}-*ponB*	pUC18	This study
pNG45	*aacC1 araC* P_{ara}::RBS_{native}-*lpoA*	pBBR1	This study
pNG46	*aacC1 araC* P_{ara}::RBS_{native}-*lpoP*	pBBR1	This study
pNG51	*att*HK022 *tetA tetR lacI*^{q} P_{lac}::^{Pa}*lpoP*	R6K	This study
pNG59	*aacC1 araC* P_{ara}::RBS_{native}-*ponA*	pBBR1	This study
pNG62	*aacC1 araC* P_{ara}::RBS_{native}-^{Ab}*lpoP*	pBBR1	This study
pNG64	*aacC1 araC* P_{ara}::RBS_{native}-*ponB*	pBBR1	This study
pNG66	*att*HK022 *tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB*-^{Pa}*lpoP*	R6K	This study
pNG68	*att*HK022 *tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB*	R6K	This study
pNG74	*att*HK022 *tetA tetR lacI*^{q} P_{lac}::^{Ab}*ponB*	R6K	This study
pNG75	*att*HK022 *tetA tetR lacI*^{q} P_{lac}::^{Ab}*ponB*-^{Ab}*lpoP*	R6K	This study

Plasmid	Genotype[a]	Origin	Source or Reference
pNG92	*aacC1 lacI*^{q} P_{lacUV5}::^{Pa}*ponB*	pBR322/ pRO1614	This study
pNG97	*aacC1 lacI*^{q} P_{lacUV5}::^{Pa}*ponB* T435I	pBR322/ pRO1614	This study
pNG98	*aacC1 lacI*^{q} P_{lacUV5}::^{Pa}*ponB* I595V	pBR322/ pRO1614	This study
pNG99	*aacC1 lacI*^{q} P_{lacUV5}::^{Pa}*ponB* I595L	pBR322/ pRO1614	This study
pNG100	*aacC1 lacI*^{q} P_{lacUV5}::^{Pa}*ponB* D388E	pBR322/ pRO1614	This study
pNG101	*aacC1 lacI*^{q} P_{lacUV5}::^{Pa}*ponB* D388V	pBR322/ pRO1614	This study
pNG117	*tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB*	pBR/ColE1	This study
pNG118	*tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB* T435I	pBR/ColE1	This study
pNG119	*tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB* I595V	pBR/ColE1	This study
pNG120	*tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB* I595L	pBR/ColE1	This study
pNG121	*tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB* D388E	pBR/ColE1	This study
pNG122	*tetA tetR lacI*^{q} P_{lac}::^{Pa}*ponB* D388V	pBR/ColE1	This study
pNG134	*att*HK022 *tetA tetR lacI*^{q} P_{lac}::^{Ab}*lpoP*	R6K	This study
pMM90	*bla lacI* P_{T7}::*h-sumo-*^{Ec}*ponB*	pBR/ColE1	(13)
pMT116	*att*HK022 *tetA tetR lacI*^{q} P_{lac} [empty vector]	R6K	This study
pPSV38	*aacC1 lacI*^{q} P_{lacUV5} [replicating IPTG-inducible expression vector for *P. aeruginosa*]	pBR322/ pRO1614	(14)
pTB102	*cat repA(ts) cI857(ts)* $P_{\lambda R}$*::int(HK022)*	pSC101	(15)
pTNS2	*bla oriR6K tnsABCD* [Tn7 transposase expression]	R6K	(16)

[a] P_{ara}, P_{lac}, $P_{\lambda R}$ and P_{T7} indicate the arabinose, lactose, λR and phage T7 promoters, respectively. $P_{toplac\text{-}dn1}$ is a derivative of P_{toplac} (Dove lab) with a C→T mutation immediately upstream of the -35 sequence resulting in decreased expression. A 6xHis tag for purification is indicated by the letter *h*. Where indicated, the native ribosome binding site (RBS_{native}), representing 20 bp upstream of the start codon, was used. In all other cases, the RBS sequences used are described in *Supplemental Materials and Methods*.

SUPPLEMENTAL MATERIALS AND METHODS

Media, Bacterial Strains and Plasmids

Cells were grown in either LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, unless otherwise indicated), minimal M9 medium (17) supplemented with 0.2% casamino acids and 0.2% sugar, as indicated or Vogel-Bonner minimal medium (VBMM) (16). Where appropriate, antibiotics were used at the following concentrations, unless otherwise specified: ampicillin (50 μg/mL), carbenicillin (200 μg/mL), chloramphenicol (25 μg/mL), gentamicin (15 μg/mL for *E. coli*, 50 μg/mL for *P. aeruginosa*), kanamycin (50 μg/mL) and tetracycline (5 μg/mL). The bacterial strains used in this study are listed in **Table S2**. All *P. aeruginosa* strains used in the reported experiments are derivatives of PAO1 and *E. coli* strains are derivatives of MG1655. Plasmids used in this study are listed in **Table S3**. PCR was performed using Phusion or the Q5 polymerase (New England Biolabs) according to the manufacturer's instructions. Plasmid DNA was purified with Zippy miniprep kits (Zymo Research) while PCR fragments were purified using Qiaquick PCR purification kit (Qiagen). Unless otherwise indicated, plasmids were constructed using the Gibson isothermal assembly method (18) and the reactions were incubated at 50°C for 30 minutes.

To construct pCF288 [*aacC1 sacB oriT* 'PA4422-*lpoA*Δ(2-604)-PA4424-PA4425'], which is used for deletion of *lpoA*, the ~800bp region upstream of *lpoA* (PA4424-PA4425') was amplified from PAO1 gDNA using 5'-AAATGTAAAGCAAGCTTCTGCAGGTCGACTCTAACACGTCGCGCATGTCCGCCAGGCTTTCCAGCA-3' and 5'-CCCTTGTCCCGGGAGCTTTCCCTATCGGATAGGGCTTCTCATGGCGCATTCACGAAGAGT-3' primers. The ~800bp region downstream of the gene ('PA4422) was amplified with 5'-

ACTCTTCGTGAATGCGCCATGAGAAGCCCTATCCGATAGGGAAAGCTCCCGGGACAAGGG -3' and 5'-GAATTCGAGCTCGAGCCCGGGGATCCTCTAGGATCTGCTTGGAGAAGACTTCGTTGTAGC TGT-3' primers. The two resulting fragments were then combined by isothermal assembly into pEXG2, which was previously digested with XbaI.

To construct pCF291 [*aacC1 sacB oriT* '*pilM-ponA*Δ(2-822)-PA5046'], which is used for deletion of *ponA*, the ~800bp region upstream of *ponA* (PA5046') was amplified from PAO1 gDNA using 5'-AAATGTAAAGCAAGCTTCTGCAGGTCGACTCTTTCGCGAACCTCGACGTTTTCTTTCCGAC AGG-3' and 5'-CGTCGCCAACGAAGCGCAGGCGCCGGCCGGTGGGTAGATTCGGATTTCCAGGAAAAAGA AAG-3' primers. The ~800bp region downstream of the gene ('*pilM*) was amplified with 5'-CTTTCTTTTTCCTGGAAATCCGAATCTACCCACCGGCCGGCGCCTGCGCTTCGTTGGCGA CG-3' and 5'-GAATTCGAGCTCGAGCCCGGGGATCCTCTATGGGCGCCAAGGTCGAGAACATCTACATGA -3' primers. The two resulting fragments were then combined by isothermal assembly into pEXG2, which was previously digested with XbaI.

To construct pCF352 [*aacC1 sacB oriT* '*lpoP-ponB*Δ(2-774)-PA4701'], which is used for deletion of *ponB*, the ~800bp region upstream of *ponB* (PA4701') was amplified from PAO1 gDNA using 5'-AAATGTAAAGCAAGCTTCTGCAGGTCGACTCTTTGAGCAGCGGGCGAACCTGCTCGAAAT TCTG-3' and 5'-

CTTCTTCACGTCAGGTCCTCTCGGAGGCGGCGCATTATACGTACTTTATCCACAG-3' primers. The ~800bp region downstream of the gene ('*lpoP*) was amplified with 5'-CTGTGGATA AAGTACGTATAATGCGCCGCCTCCGAGAGGACCTGACGTGAAGAAG-3' and 5'-GAATTCGAGCTCGAGCCCGGGGATCCTCTAAAGGCGCTGTCGCTTTCGAGAATCAGCTT-3' primers. The two resulting fragments were then combined by isothermal assembly into pEXG2, which was previously digested with XbaI.

To construct pCF526 [*aacC1 sacB oriT* 'PA4697-PA4698-*lpoP*Δ(3-259)-*ponB*'], which is used for deletion of *lpoP*, the ~800bp region upstream of *lpoP* (*ponB'*) was amplified from PAO1 genomic DNA (gDNA) using 5'-AAATGTAAAGCAAGCTTCTGCAGGTCGACTCTACGATCTTCCTGTACCAGGGCCTG-3' and 5'-CCAGCGCATAGACCCGTCGATCCACTTCACGTCAGGTCCTCTCGGATCAA-3' primers. The ~800bp region downstream of the gene ('PA4697-PA4698) was amplified with 5'-TTGATCCGAGAGGACCTGACGTGAAGTGGATCGACGGGTCTATGCGCTGG-3' and 5'-GAATTCGAGCTCGAGCCCGGGGATCCTCTAAAGGTGCGCAAGCGACCACGGTGAATACC CA-3' primers. The two resulting fragments were then combined by isothermal assembly into pEXG2, which was previously digested with XbaI.

For pCF964 [P_{T7}::*h-lpoP*(20-259)], *lpoP* lacking its signal sequence was PCR amplified from PAO1 gDNA with 5'-TGGACAGCAAATGGGTCGCGGATCCGAATTCGCCAGCCCGCAGCACGGGGCGATCCCG GTG-3' and 5'-TGGTGGTGGTGGTGCTCGAGTGCGGCCGCATCAGGAGCTGACCTTGGCCTTCTGC-3'

primers, digested with EcoRI and HindIII and ligated into the H-fusion vector pET28-*amiD* (19) digested in a similar manner to replace *amiD*.

For pCF972 [*bla lacI* P_{T7}::*h-sumo-*^{Pa}*ponB*], the *ponB* PCR product was amplified from PAO1 gDNA with 5'-ATTATTGAGGCTCACAGAGAACAGATTGGTGGTATGACGCGTCCCCGATCCCCCCGTTCCCGT-3' and 5'-GATCCCCTTCCTGCAGTCACCCGGGCTCGATCAATTCAGCCAGCCACGTACCCAGTCCAT-3' primers, digested with SapI and XhoI and ligated into similarly digested pTB146 (20), which is a H-SUMO fusion vector.

For pNG41 [*aacC1 bla* Tn7 *lacI*^{q} $P_{toplac\text{-}dn1}$::RBS_{native}-*ponB*], the *ponB* gene was amplified along with its putative native RBS sequence (20 nucleotides upstream of ATG start codon) from PAO1 gDNA with primers 5'-ATTAAGCTTAGTCGACAGCTAGCCGAGTACGTATAATGCGCCGCCATG-3' and 5'-CTAAAGCTTGCATGCGGTACCCGGGTCAATTCAGCCAGCCACGTAC-3'. The resulting product was cloned into BamHI-digested pKHT104 (Dove lab) using isothermal assembly. The *toplac-dn1* promoter is a weakened derivative of *toplac*.

For pNG45 [*aacC1 araC* P_{ara}::*lpoA*], pNG46 [*aacC1 araC* P_{ara}::*lpoP*], pNG59 [*aacC1 araC* P_{ara}::*ponA*] and pNG64 [*aacC1 araC* P_{ara}::*ponB*], each gene was amplified along with its putative native RBS sequence (20 nucleotides upstream of ATG start codon) from PAO1 gDNA with the following primers (restriction sites are underlined): *lpoA* 5'-ATATGAATTCTGCGCCATGAGAAGCCCTATA-3' and 5'-

TATATCTAGAATCAAAAGCTGCTGGTCCCC-3'; *lpoP* 5'-

ATATGAATTCTTGATCCGAGAGGACCTGACG-3' and 5'-

TATATCTAGAATCAGGAGCTGACCTTGGCC-3'; *ponA* 5'-

TTTTGAATTCCTGGAAATCCGAATCTACCCATGCG-3' and 5'-

TTTTTCTAGAACGGTTCAGAACAGGTCGATCG-3'; *ponB* 5'-

GCGCGAATTCAGTACGTATAATGCGCCGCCATG-3' and 5'-

ATGCTCTAGAATCAATTCAGCCAGCCACGTAC-3'. Each PCR product was digested with EcoRI and XbaI and ligated into similarly digested pJN105 (12).

For pNG51 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*lpoP*], pNG66 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*-^{Pa}*lpoP*] and pNG68 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*] the following primers were used to amplify each insert from PAO1 gDNA (restriction sites are underlined, RBS sequences are bolded): ^{Pa}*lpoP* 5'-GCTATCTAGA**TTTAAGAAGGAGATATACAT**ATGAAGAAGATTTTCGTATTCGCCGC-3' and 5'-GCTAAAGCTTTCAGGAGCTGACCTTGGCC-3'; ^{Pa}*ponB*-^{Pa}*lpoP* 5'-GCTATCTAGA**TTTAAGAAGGAGATATACAT**ATGACGCGTCCCCGATCC-3' and 5'-GCTAAAGCTTTCAGGAGCTGACCTTGGCC-3'; ^{Pa}*ponB* 5'-GCTATCTAGA**TTTAAGAAGGAGATATACAT**ATGACGCGTCCCCGATCC-3' and 5'-GCTAAAGCTTTCAATTCAGCCAGCCACGTAC-3'. Each PCR product was digested with XbaI and HindIII and ligated into similarly digested pHC943 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::*msfgfp-pbpA*] (21) to replace the *msfgfp-pbpA* gene.

For pNG62 [*aacC1 araC* P$_{ara}$::^{Ab}*lpoP*], ^{Ab}*lpoP* (ACIAD2235) was amplified along with its putative native RBS sequence (20 nucleotides upstream of ATG start codon) from *Acinetobacter baylyi* ADP1 gDNA with the following primers (restriction sites are underlined): 5'-GCGCGAATTCTAACTACTAACTAGCGTAATTTGATGAGAG-3' and 5'-

ATGCTCTAGAACTAGAGAGACTTCAGTGCTTG-3'. Each PCR product was digested with EcoRI and XbaI and ligated into similarly digested pJN105 (12).

For pNG74 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::^{Ab}*ponB*], pNG75 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::^{Ab}*ponB*-^{Ab}*lpoP*] and pNG134 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::^{Ab}*lpoP*] the following primers were used to amplify each insert from *Acinetobacter baylyi* ADP1 gDNA (RBS sequences are bolded): ^{Ab}*ponB* (ACIAD2234) 5'-TCTAGA**TTTAAGAAGGAGATATACAT**ATGAAGTTTGAACGCGGCATAG-3' and 5'-CCCGGATATTATCGTGAGATCGATACGCTAGTTAGTAGTTATAACTACCACTTG-3'; ^{Ab}*ponB*-^{Ab}*lpoP* (ACIAD2234-2235) 5'-TCTAGA**TTTAAGAAGGAGATATACAT**ATGAAGTTTGAACGCGGCATAG-3' and 5'-CCCGGATATTATCGTGAGATCGATACTAGAGAGACTTCAGTGCTTGTTG-3'; ^{Ab}*lpoP* (ACIAD2235) 5'-TCTAGA**TTTAAGAAGGAGATATACA**TTGATGAGAGATACTATGTTGAAAAAAAGTC-3' and 5'-CCCGGATATTATCGTGAGATCGATACTAGAGAGACTTCAGTGCTTGTTG-3'. Each PCR product was cloned into pHC943 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::*msfgfp-pbpA*] (21) digested with NdeI and HindIII to replace the *msfgfp-pbpA* gene.

For pNG92 [*aacC1 lacI*^{q} P$_{lacUV5}$::^{Pa}*ponB*], pNG97 [*aacC1 lacI*^{q} P$_{lacUV5}$::^{Pa}*ponB*(T435I)], pNG98 [*aacC1 lacI*^{q} P$_{lacUV5}$::^{Pa}*ponB*(I595V)], pNG99 [*aacC1 lacI*^{q} P$_{lacUV5}$::^{Pa}*ponB*(I595L)], pNG100 [*aacC1 lacI*^{q} P$_{lacUV5}$::^{Pa}*ponB*(D388E)] and pNG101 [*aacC1 lacI*^{q} P$_{lacUV5}$::^{Pa}*ponB*(D388V)] the *ponB* genes were amplified from PAO1 gDNA or appropriate Δ*ponA* Δ*lpoP* suppressor colonies [PA687 for ^{Pa}*ponB*(T435I), PA688 for ^{Pa}*ponB*(I595V), PA689 for ^{Pa}*ponB*(I595L), PA690 for ^{Pa}*ponB*(D388E) and PA691 for ^{Pa}*ponB*(D388V)] using primers 5'-AATTGAATTC**GAGGAGGATACAT**ATGACGCGTCCCCGATCC-3' and 5'-AATTCCCGGGTCAATTCAGCCAGCCACGTACC-3' (restriction sites are underlined, synthetic RBS is bolded). The PCR products were digested with EcoRI and XmaI and ligated into similarly digested pPSV38 (14).

For pNG117 [*tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*], pNG118 [*tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*(T435I)], pNG119 [*tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*(I595V)], pNG120 [*tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*(I595L)], pNG121 [*tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*(D388E)] and pNG122 [*tetA tetR lacI*^{q} P$_{lac}$::^{Pa}*ponB*(D388V)] the *ponB* genes were amplified from PAO1 genomic DNA or the relevant plasmids above (pNG97, pNG98, pNG99, pNG100, pNG101, respectively) using primers 5'-GCTATCTAGA**TTTAAGAAGGAGATATACAT**ATGACGCGTCCCCGATCC-3' and 5'-GCTAAAGCTTTCAATTCAGCCAGCCACGTAC-3' (restriction sites are underlined, synthetic RBS is bolded). The PCR products were digested with XbaI and HindIII and ligated into similarly digested pMT74 [*tetA tetR lacI*^{q} P$_{lac}$::*sulA*] (22) to replace the *sulA* gene.

For pMT116 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::empty], the complementary oligonucleotides 5'-AGCTTGAGTCTGACTAGCT-3' and 5'-CTAGAGCTAGTCAGACTCA-3' were annealed together and the resulting product was ligated directly into XbaI/HindIII-digested pNP20 [*att*HK022 *tetA tetR lacI*^{q} P$_{lac}$::*nlpD-mCherry*] (23) to replace the *nlpD-mCherry* gene.

***P. aeruginosa* strain construction.**

Briefly, plasmids were transferred into *P. aeruginosa* by conjugation from an *E. coli* donor [SM10(λpir)] on LB plates. Counterselection against *E. coli* and selection of exconjugants was accomplished on Vogel-Bonner minimal medium (VBMM) (16) supplemented with 50 µg/ml gentamicin, unless otherwise indicated.

To construct PA104 [Δ*ponA*], pCF291 [*aacC1 sacB oriT* '*pilM-ponA*Δ(2-822)-PA5046'] was conjugated from the SM10(λpir) donor into the recipient strain PAO1. For this purpose, PAO1

was patched on an LB plate and grown overnight at 42°C while SM10(λpir) carrying pCF526 was similarly grown at 37°C. Donor and recipients were scraped up using a toothpick, patched together onto an LB plate, and incubated at 37°C for ~5h. The conjugation mixture was scraped up, resuspended in 500 µL of VBMM, diluted 1:10, and 100 µL of the resulting suspension was plated on VBMM supplemented with 50 µg/mL gentamicin. Plates were incubated at 37°C overnight. Exconjugants were purified on LB supplemented with 50 µg/mL gentamicin. A few single colonies were allowed to grow for ~6h in LB broth to allow for plasmid recombination to take place and 100 µL of the resulting culture was plated on LB supplemented with 5% sucrose to select for the loss of the plasmid by virtue of the encoded *sacB* gene. Sucrose-resistant colonies were patched onto LB plates either containing or lacking 50 µg/mL gentamicin. Gentamicin-sensitive colonies were further tested by PCR with *ponA*-flanking primers 5'-AACTCAAGGAGCTTTACCGAGGTCGAGCT-3' and 5'-TACATCATTCCGAAGCCGATGGACAAGCGC-3' to confirm gene deletion. The deletion retains the first and last codons of the *ponA* reading frame.

For PA105 [Δ*ponB*], *ponB* was deleted from PAO1 by integration and re-circularization of pCF352 [*aacC1 sacB oriT* 'PA4699-*ponB*Δ(2-774)-PA4701'] as described above for PA104 construction. Sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *ponB*-flanking primers 5'-TTCCCGTAACAGCAAAGCACGC-3' and 5'-AAAGTCTGGATCGGTGCGGAATTGGCGC-3'. The deletion retains the first and last codons of the *ponB* reading frame.

For PA550 [Δ*lpoP*], *lpoP* was deleted from PAO1 by integration and re-circularization of pCF526 [*aacC1 sacB oriT* 'PA4697-PA4698-*lpoP*Δ(3-259)-*ponB*'] as described above for

PA104 construction. The sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *lpoP*-flanking primers 5'-TTCGGTGTACAGTCAATTGCCGTCGT-3' and 5'-TTGAAGCAAAGAAAAACCCGGCCTG-3'. The deletion retains the first two and last codons of the *lpoP* reading frame.

Construction of PA614 [Δ*ponB att*Tn7::$P_{toplac\text{-}dn1}$::*ponB*] was carried out following a previously described method (16). Briefly, PA105 [PAO1 Δ*ponB*] was co-electroporated with pNG41 [*aacC1 bla* Tn7 *lacI*^{q} $P_{toplacdn1}$::RBS_{native}-*ponB*] and pTNS2 [*bla oriR6K tnsABCD*] to incorporate the $P_{toplac\text{-}dn1}$::*ponB* construct into the chromosomal *att*Tn7 site of PA105, thus creating a strain where the sole copy of *ponB* is IPTG-inducible. Co-transformants were selected on LB + 30 µg/mL gentamicin and resistant colonies were subject to diagnostic PCR with primers PTn7R and P*glmS*-down, as described (16). The gentamicin resistance cassette was removed from the resulting strain by electroporation with pFLP2. Transformants were selected on LB supplemented with 200 µg/mL carbenicillin and carbenicillin-resistant mutants were grown in plain LB broth to allow for Flp-mediated excision of the gentamicin resistance cassette, as described (16). Cells from this culture were subsequently isolated on LB plates supplemented with 5% sucrose to select for loss of the pFLP2 plasmid. Sucrose-resistant colonies were then patched on LB, LB + 30 µg/mL gentamicin and LB + 200 µg/mL carbenicillin to screen for loss of the gentamicin resistance cassette and pFLP2.

For PA615 [Δ*ponB att*Tn7::$P_{toplac\text{-}dn1}$::*ponB* Δ*ponA*], *ponA* was deleted from PA614 [Δ*ponB att*Tn7::$P_{toplac\text{-}dn1}$::*ponB*] by integration and re-circularization of pCF291 [*aacC1 sacB oriT* '*pilM-ponA*Δ(2-822)-PA5046'] as described above for PA104 construction, except that all media were supplemented with 2 mM IPTG to ensure *ponB* expression during strain construction and,

when necessary, gentamicin was included at a concentration of 30 µg/mL. Sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *ponA*-flanking primers 5'-TAGATACCAGAGCCGAAGGCC-3' and 5'-CCCTGCCGTATCCGAAGAACT-3'. The deletion retains the first and last codons of the *ponA* reading frame.

For PA684 [Δ*lpoA*], *lpoA* was deleted from PAO1 by integration and re-circularization of pCF288 [*aacC1 sacB oriT* 'PA4422-*lpoA*Δ(2-604)-PA4424-PA4425'] as described above for PA104 construction except when necessary, gentamicin was included at a concentration of 30 µg/mL. Sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *lpoA*-flanking primers 5'-CCTCGGGAGCTATCTGAAAAGG-3' and 5'-GACAGGTCCAATTCTTGCCAAG-3'. The deletion retains the first and last codons of the *lpoA* reading frame.

For PA685 [Δ*ponA* Δ*lpoP*], *lpoP* was deleted from PA104 [Δ*ponA*] by integration and re-circularization of pCF526 [*aacC1 sacB oriT* 'PA4697-PA4698-*lpoP*Δ(3-259)-*ponB*'] as described above for PA104 construction except that when necessary, gentamicin was included at a concentration of 30 µg/mL and sucrose counterselection was performed on LB lacking NaCl. Sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *lpoP*-flanking primers 5'-TTCGGTGTACAGTCAATTGCCGTCGT-3' and 5'-TTGAAGCAAAGAAAAACCCGGCCTG-3'. The deletion retains the first two and last codons of the *lpoP* reading frame.

For PA686 [Δ*ponB* Δ*lpoA*], *lpoA* was deleted from PA105 [Δ*ponB*] by integration and re-circularization of pCF288 [*aacC1 sacB oriT* 'PA4422-*lpoA*Δ(2-604)-PA4424-PA4425'] as

described above for PA104 construction except that when necessary, gentamicin was included at a concentration of 30 μg/mL. Sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *lpoA*-flanking primers 5'-CCTCGGGAGCTATCTGAAAAGG-3' and 5'-GACAGGTCCAATTCTTGCCAAG-3'. The deletion retains the first and last codons of the *lpoA* reading frame.

Transposon mutagenesis

P. aeruginosa PAO1 and its Δ*ponA* and Δ*ponB* derivatives were mutagenized with the mini-transposon pBTK30 (9) using the following mating protocol. The donor strain SM10(λpir) carrying pBTK30 was grown in LB broth supplemented with 50 μg/mL ampicillin at 37°C and each recipient strain was grown in LB broth at 42°C overnight. The next morning, cultures were concentrated and adjusted to an OD_{600} of 5 for the donor and 10 for each recipient. Equal volumes of donor and recipient were mixed together and 50 μL aliquots were spotted on pre-warmed LB plates. Matings were allowed to proceed at 37°C for one hour prior to resuspension in VBMM supplemented with 30 μg/mL gentamicin. Transconjugants were selected on VBMM supplemented with 30 μg/mL gentamicin at 30°C for 24 hours. Donor only and recipient only controls were performed in parallel to ensure counterselection against *E. coli* donor and selection of *P. aeruginosa* transconjugants, respectively. Approximate complexity (i.e. number of transconjugants collected) of each mutant library is as follows: WT (~1.7×10^6 CFU), Δ*ponA* (~9.2×10^5 CFU) and Δ*ponB* (~1.5×10^6 CFU). Colonies were isolated from agar plates by suspension in VBMM broth supplemented with 30 μg/mL gentamicin. Cell pellets of each library consisting of approximately 5×10^9 CFU were collected and frozen at -80°C.

Transposon sequencing

Genomic DNA from thawed cell pellets of each mutant library was extracted, fragmented, poly-C tailed and purified as previously described (24). The resulting DNA was used as the template to amplify transposon-chromosome junctions in a PCR reaction using Easy-A Hi-Fi Cloning System (Agilent Technologies). The primers used were the poly-C tail-specific 5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGG-3' and the transposon-specific 5'-GGTTCTGGACCAGTTGCGTGAG-3'. The transposon-chromosome junctions were further amplified in a second, nested PCR reaction that also serves to add specific barcode sequences to each mutant library. The primers used were NEBNext Multiplex Oligos for Illumina (NEB) and the transposon-specific primer 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTGTTTTCTGGAAGGCGAGCATCGTTTG-3'. Equal amounts of each barcoded library were mixed, separated on a 2% agarose gel and the region between 200 and 500 basepairs was excised. DNA was purified from the gel using the QIAquick Gel Extraction Kit (Qiagen). The resulting library was sequenced using a MiSeq reagent kit v3 (150-cycle) (Illumina) with the custom primer 5'-CTAGAGACCGGGGACTTATCAGCCAACCTGTTA-3'. De-multiplexed sequencing reads were trimmed to 25 nucleotides at the 5' end and mapped to the *P. aeruginosa* PAO1 genome (NC_002516). The resulting reads were subsequently mapped to chromosomal TA dinucleotides (*mariner* insertion site specificity) on the PAO1 genome prior to normalization for total read counts. Differences in the total number of reads at any given TA site between wildtype and Δ*ponA* or Δ*ponB* were determined using a Mann-Whitney U test. The mapped sequencing reads for each library were visualized using the Sanger Artemis Genome Browser and Annotation tool.

Protein purification

E. coli and *P. aeruginosa* PBP1b, LpoB and LpoP proteins were purified using previously published protocols (5, 13). Briefly, the PBP1b proteins were overexpressed and purified with a 6xHis-SUMO (H-SUMO) tag fused to their N-termini (25, 26). *P. aeruginosa* PBP1b (^{Pa}PBP1b) and *E. coli* PBP1b (^{Ec}PBP1b) were purified from Lemo21(λDE3)/pCF972 and Lemo21(λDE3)/pMM90, respectively. Overnight cultures were grown in LB supplemented with 100 µg/mL ampicillin and 0.4% glucose. The cultures were diluted 1:100 into 4 L of LB supplemented with 100 µg/mL ampicillin and 0.2% glucose and incubated at 37°C to an OD_{600} of 0.6. The cultures were cooled for 30 minutes at room temperature before addition of IPTG to 1 mM. Cultures were then grown overnight at 16°C and the cells were harvested by centrifugation. Cell pellets were stored at -80°C. Thawed cell pellets were resuspended in buffer A [20 mM Tris-HCl pH 8.0, 500 mM NaCl and protease inhibitor cocktail (Roche)] and disrupted by passing them through a French pressure cell two times at 25,000 psi. Cell debris was pelleted twice by centrifugation at 10,000 rpm for 15 min at 4°C. The membrane fraction was collected by high speed centrifugation at 100,000g for 1 hour at 4°C with a 70Ti rotor. Membranes were solubilized in 15 mL of Buffer B (20 mM Tris-HCl pH 8.0, 1 M NaCl, 2% reduced Triton X-100), and incubated overnight at 4°C on a rocker. Unsolubilized membranes were recovered by centrifugation at 100,000g for 1 hour at 4°C with a 70Ti rotor and the supernatant containing H-SUMO-PBP1b proteins was incubated for 1 hour with pre-washed Ni-NTA agarose (Qiagen). The beads were allowed to settle in a gravity column (Bio-Rad) and were washed with buffer C (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1% reduced Triton) containing 50 mM imidazole, and fusion proteins were eluted with buffer C containing 300 mM imidazole. Purified PBP1b proteins were dialyzed against buffer D (20 mM Tris-HCl pH 8.0, 1 M NaCl, 0.1% reduced Triton, 10% glycerol). H-UlpI protease was added to the mixture and incubated overnight at 4°C to remove the H-SUMO tag from purified PBP1b. Cleavage reactions were passed

through Ni-NTA resin to remove free H-SUMO and H-UlpI, yielding pure preparations of untagged PBP1b proteins. Untagged PBP1b was concentrated using Amicon Centrifugal Filter Units (MWCO 10 KDa) and stored at -80°C.

His-tagged versions of LpoP(20-259) and LpoB(21-213) were purified from Lemo21(λDE3)/ pCF964 and BL21(λDE3)/pCB39 cells, respectively. Overnight cultures were grown in LB supplemented with 0.4% glucose and 25 μg/mL kanamycin or 100 μg/mL ampicillin, respectively. The cultures were diluted 1:100 into 1 liter of LB and grown to an OD_{600} of 0.6 at 37°C. IPTG was added to 1 mM and the cultures were grown for 6 hr at 30°C. Cells were harvested by centrifugation and stored at -80°C. The cell pellets were resuspended in 35 mL of buffer A4 [20 mM Tris-HCl pH 8.0, 300 mM NaCl, protease inhibitor cocktail (Roche)] and disrupted by passing them through a French pressure cell two times at 25,000 psi. Cell debris and membranes were collected by centrifugation at 100,000g for 30 min at 4°C with a 70Ti rotor. Cell extracts were then loaded with 1 mL Ni-NTA agarose (Qiagen) and the mixture was allowed to settle in a gravity column before being washed with 50 mL of buffer A4 containing 30 mM imidazole. The H-LpoP and H-LpoB fusion proteins were eluted with buffer A4 containing 300 mM imidazole. Elution fractions were dialyzed against dialysis buffer (30 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol), aliquoted and stored at -80°C.

Phylogenetic tree

A phylogenetic tree showing the distribution of PBP1b, LpoP, LpoB and RecA homologs in a diverse set of 1,773 bacterial taxa. The amino acid sequences of ^{Pa}PBP1b and ^{Pa}RecA were used as a query in a BLASTp search against the NCBI “non redundant” (*nr*) database (27) with an *e*-value cutoff of $1e^{-80}$ for PBP1b (to discriminate from PBP1a) and $1e^{-08}$ for RecA. To

identify LpoB and LpoP homologs, a set of 3 LpoB sequences *(Escherichia.coli* K-12 MG1655: NP_415623*, Shewanella oneidensis*: WP_011071314*, Vibrio cholerae*: NP_231528*)* and a set of 7 LpoP sequences (*Pseudomonas aeruginosa* PAO1: NP_253387, *Acinetobacter baylyi* ADP1: WP_004927845, *Rhodanobacter denitrificans*: WP_015448633, *Dichelobacter nodosus*: WP_012030905, *Stenotrophomonas maltophilia*: WP_005414256, *Marinobacter adhaerens*: WP_014575927, *Thioalkalivibrio sulfidiphilus*: WP_012637976), identified by the presence of a lipoprotein sequence signal, TPR repeat motifs and location downstream of *ponB* were used as a query with an *e*-value cutoff of $1e^{-08}$. A list of all the taxa for which significant BLAST results were found was then sorted. We used a complex and diverse set of 1773 bacterial taxa called "Representative Genomes" that is available on NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/, Representative_Genomes.00.tar.gz). The phylogenetic tree was constructed using PhyloT (http://phylot.biobyte.de/) and BLASTp results were plotted against the tree. The tree was visualized and annotated using iToL (http://itol.embl.de/) (2).

Genetic neighborhood analysis

A database of 1542 fully assembled representative bacterial genomes was created (https://www.ncbi.nlm.nih.gov/assembly). Except for PBP1b, the same queries as for the phylogenetic tree were used in a tBLASTn against this database. For PBP1b, the UB2H consensus domain (pfam14814) served as the query. The start and stop positions of the alignment were sorted for each genome and the distances between *ponB* and *lpoP*, *lpoB* or *recA* were calculated for each organism.

Suppressor selection, whole genome sequencing and variant detection

To select for suppressors of the lethal temperature-sensitive growth defect of Δ*ponA* Δ*lpoP* on VBMM, four colonies of the strain were each inoculated into separate cultures of LB lacking NaCl (LB0N) and grown overnight at 30°C. The following morning, each culture was diluted 1:100 into LB0N and grown at 37°C for 4 hours. Cells from each culture were collected by centrifugation, washed three times with VBMM and concentrated five-fold. A 100 µL aliquot was spread plate on VBMM agar and plates were incubated overnight at 42°C to select for spontaneous mutants capable of growth under the non-permissive condition. Colonies of at least two different sizes appeared after approximately 24 hours of incubation and candidate suppressors were colony-purified on VBMM at 42°C to confirm growth under non-permissive conditions. Isolated suppressor mutants and the parent strain were subjected to whole genome sequencing to identify spontaneous mutations allowing growth under non-permissive conditions.

Genomic DNA was isolated from each strain using the Wizard Genomic DNA Purification Kit (Promega) and further processed with the DNA Clean & Concentrator Kit (Zymo Research). The resulting DNA was sheared for 4 minutes at 20% amplitude with a pulse rate of 15 seconds ON/15 seconds OFF using a Qsonica sonicator. Sequencing libraries were prepared with sonicated DNA using the NEBNext Ultra Kit (NEB), following the manufacturer's instructions. Samples were barcoded with NEBNext Multiplex Oligos for Illumina (NEB), pooled together and sequenced using a MiSeq reagent kit v3 (600-cycle) (Illumina).
De-multiplexed sequencing reads from the parent strain and isolated suppressors were trimmed based on quality, mapped to the PAO1 reference genome (NC_002516) and basic variant detection was performed with the mapped reads using CLC Genomics Workbench 8 software.

Bocillin FL binding assay.

Overnight cultures of *E. coli* MM10 [Δ*Ec ponA* P_{ara}::*Ec ponB*] harboring the integrated plasmid expression constructs pMT116 [empty vector], pNG66 [P_{lac}::*Pa ponB-Pa lpoP*] and pNG68 [P_{lac}::*Pa ponB*] or pNG75 [P_{lac}::*Ab ponB-Ab lpoP*] and pNG74 [P_{lac}::*Ab ponB*] were diluted 1:100 in M9 medium with 0.2% arabinose and grown at 37ºC for approximately 2.5 hours. Cells were washed three times with M9 lacking a carbon source and diluted into M9 arabinose (for the empty vector) or maltose + 1 mM IPTG (for all strains) at an OD_{600} = 0.01. Cultures were incubated 2.5 hours with shaking at 37ºC until the OD_{600} reached 0.2–0.3. Cells were then collected by centrifugation at 4 °C, washed with ice-cold 1× PBS twice, resuspended in 500 µl 1× PBS containing 10 mM EDTA and 15 µM Bocillin FL penicillin (Invitrogen) and incubated at room temperature for 15 min. After incubation, the cell suspensions were washed with 1× PBS twice, resuspended in 500 µl 1× PBS and disrupted by sonication. After a brief spin for 1 min at 4,000g to remove undisrupted cells, membrane fractions were pelleted by ultracentrifugation at 200,000g for 20 min at 4 °C. The membrane fractions were then washed with 1× PBS and resuspended in 50 µl 1× PBS. Resuspended samples were mixed with 50 µl 2× Laemmli sample buffer and boiled for 10 min at 95 °C. After measuring the total protein concentrations of each sample with the NI-protein assay (G-Biosciences), 25 µg of total protein for each sample was then separated on a 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel and the labelled proteins were visualized using a Typhoon 9500 fluorescence imager (GE Healthcare) with excitation at 488 nm and emission at 530 nm.

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