Supplemental Material for:

The cell wall amidase AmiB is essential for *Pseudomonas aeruginosa* cell division, drug resistance, and viability

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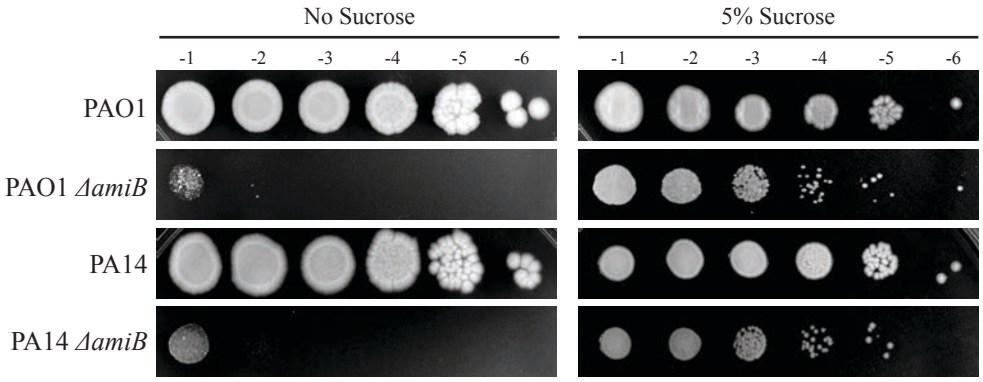

Figure S1. *amiB* is conditionally essential both in PAO1 and in PA14.

Overnight cultures of PAO1 [WT], BPA157 [PAO1 *ΔamiB*], PA14 [WT], and BPA427 [PA14 *ΔamiB*] grown from isolated colonies in LB supplemented with 5% sucrose were treated as for Fig. 1B. 5µL of 10^{-1}-10^{-6} dilutions of each strain were spotted onto LB plates either containing or lacking 5% sucrose. The plates were incubated at 30°C for 2d prior to imaging.

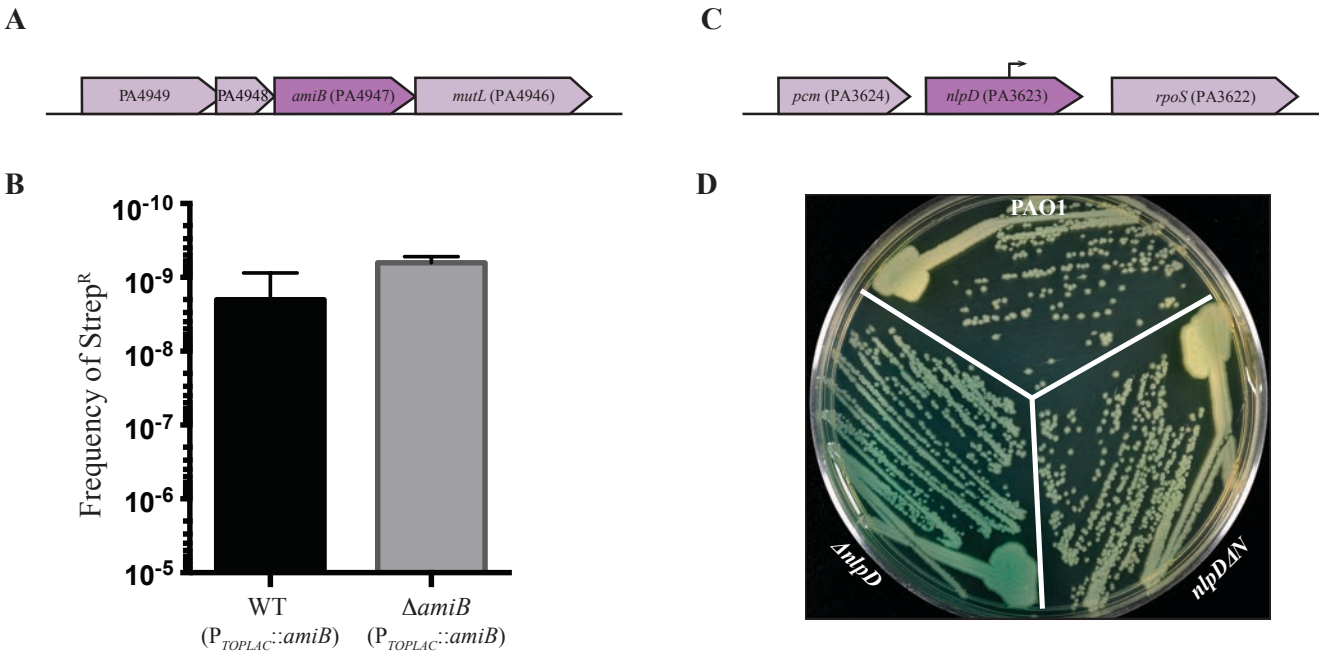

Figure S2. Assessment of polarity of *amiB* and *nlpD* deletions.

A. Genomic context of *amiB* in PAO1 genome. *amiB* (*PA4947*) is predicted to be encoded in the same operon as *mutL* (*PA4946*), which encodes a component of the DNA mismatch repair machinery. The start codon of *mutL* overlaps the stop codon of *amiB*, suggesting translational coupling.

B. Mutation rate does not increase upon *amiB* deletion. Three biological replicates of BPA35 [P_{TOPLAC}*::amiB*] and BPA46 [*ΔamiB* P_{TOPLAC}*::amiB*] overnight cultures grown from isolated colonies in LB supplemented with 1mM IPTG were centrifuged and resuspended in 1/100th of the original volume. The cells were then plated on LB supplemented with 1mM IPTG and 500µg/mL streptomycin, with two technical replicates per biological sample. The remaining cells were subjected to serial dilution, and 10^{-8} and 10^{-9} dilutions were plated on LB supplemented with 1mM IPTG. After 2d of growth at 30°C, colonies were counted to assess the incidence of spontaneous streptomycin resistance-conferring mutations in each culture. The experiment was repeated three times. Loss of *mutL* function is expected to yield a 2-log increase in the mutation rate (1), which is not observed in BPA46.

C. Genomic context of *nlpD* in PAO1 genome. As in *E. coli*, *P. aeruginosa nlpD* (*PA3623*) gene is located upstream of *rpoS (PA3622)*, which encodes the stationary phase sigma factor. One of the promoters driving *rpoS* expression is predicted to be within the coding region of *nlpD*.

D. PAO1 [WT], BPA12 [*ΔnlpD*], and BPA70 [*nlpDΔN*] were streaked out onto an LB plate and grown overnight at 37°C. Since *rpoS* negatively regulates the production of the blue-green pigment pyocyanin (2), a polar mutation in *nlpD* that disrupts *rpoS* transcription is expected to cause pyocyanin overproduction. This is observed for the mutant with completely deleted *nlpD* gene, but not for the *nlpDΔN* mutant, which lacks only the 5' end of the gene encoding the signal peptide, but retains the predicted *rpoS* promoter.

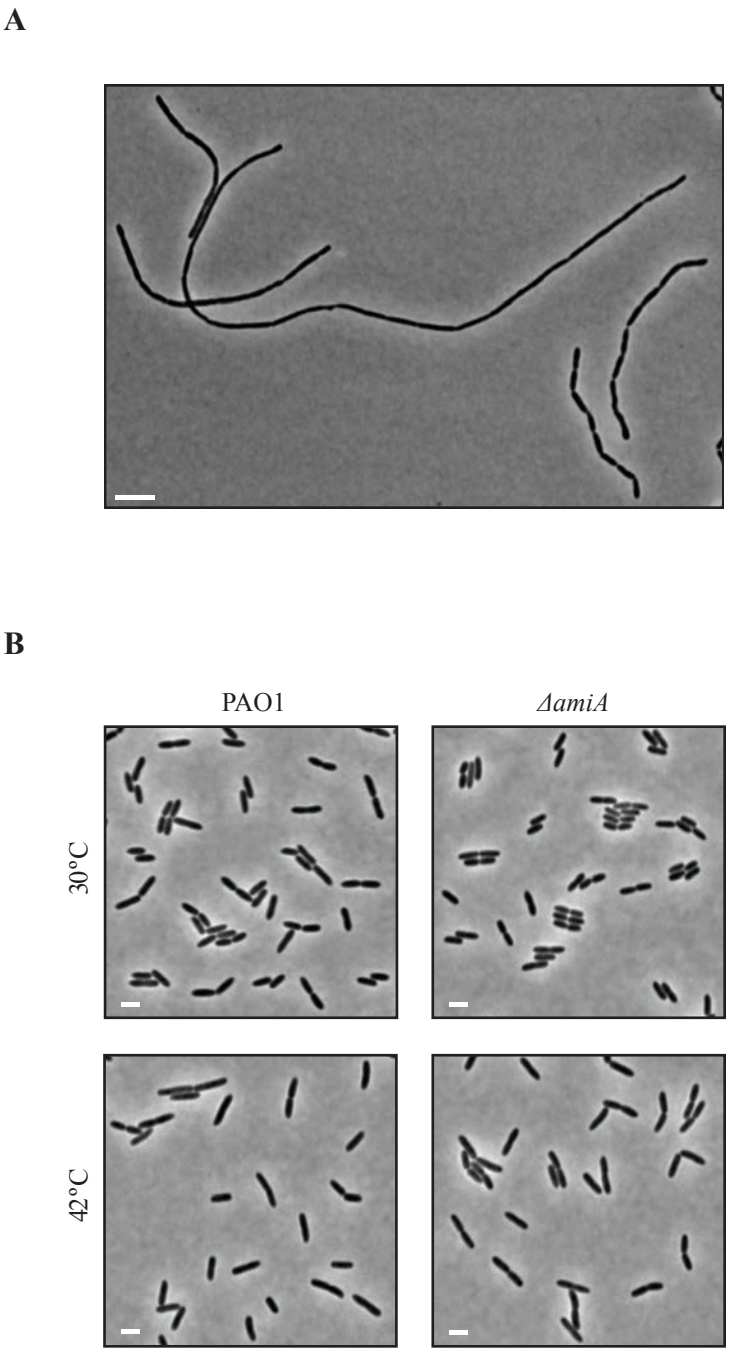

Figure S3. Phenotypic analysis of *P. aeruginosa* amidase mutants.

A. Phase image of the terminal AmiB depletion phenotype. BPA46 [*ΔamiB* P_{TOPLAC}*::amiB*] cells were treated as in **Fig. 2**, and the aliquot of cells chemically fixed at the 7h time point was imaged on an agarose pad. A larger field of view than seen in **Fig. 2B** is presented. The scale bar is 5μm.

B. Phase images of *amiA* deletion cells. Overnight cultures of BPA60 [*ΔamiA*] and PAO1 [WT] were either diluted 1:400 and allowed to grow at 30°C for 5.5h or diluted 1:2000 and allowed to grow at 42°C for 4h. Then, cells were imaged with phase optics. The scale bars are 2μm.

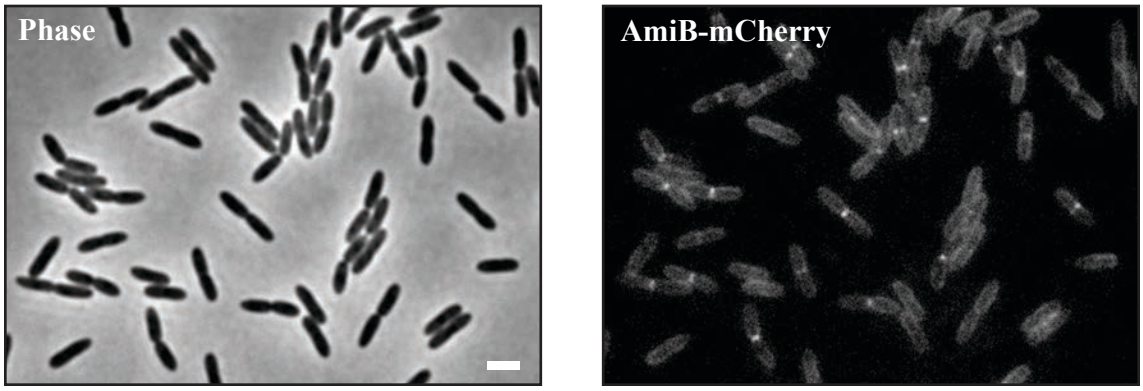

Figure S4. AmiB localizes to the division plane.

Overnight culture of BPA346 [P_{amiB}::*amiB-mCherry*] was diluted 1:100 and grown at 30°C for 4h. The cells were placed on an agarose pad and imaged with phase (Phase) and DsRed epifluorescence (AmiB-mCherry) optics. The space bar is 2μm.

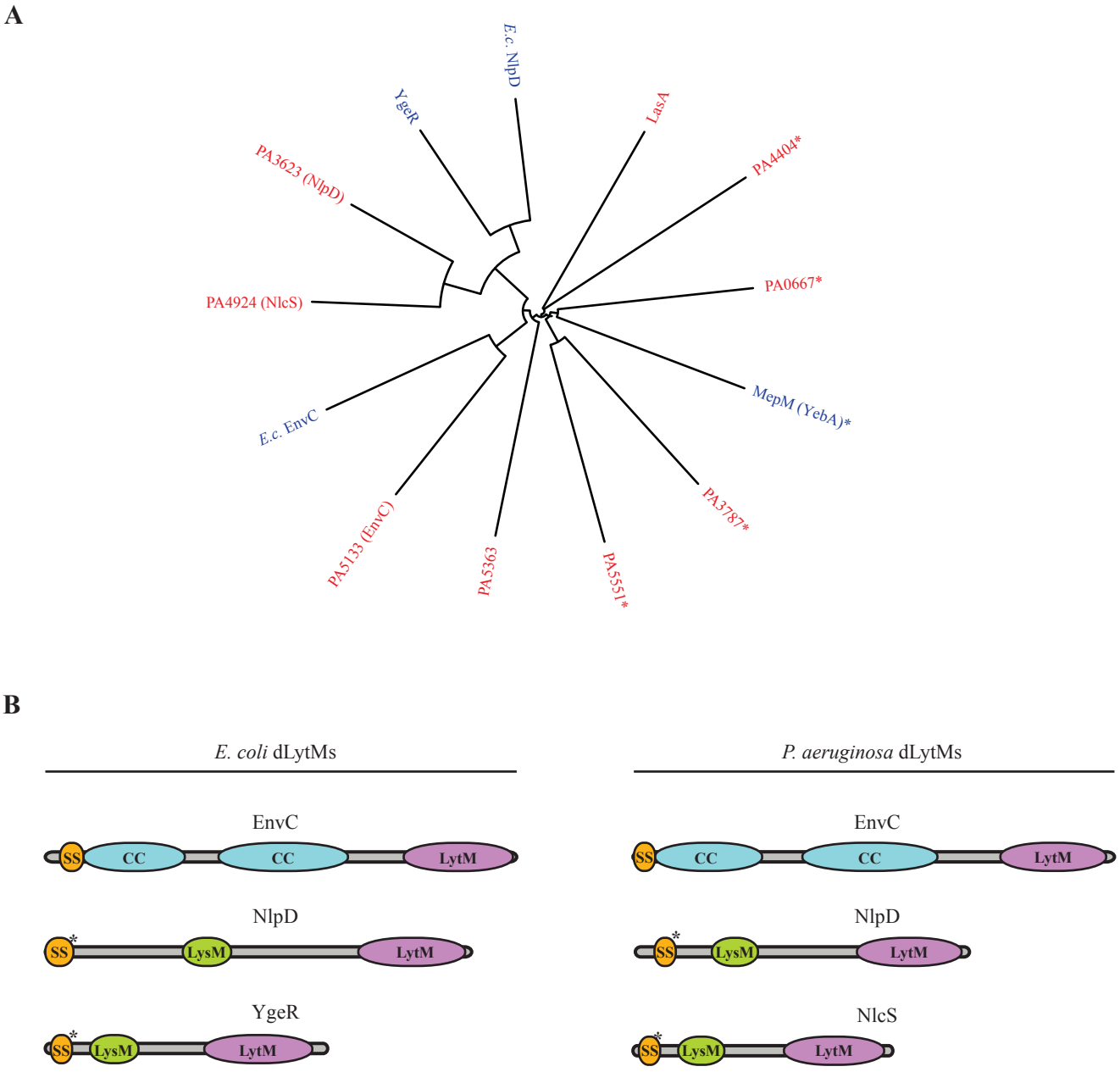

Figure S5. Comparison of the *Pseudomonas* and *E. coli* dLytM proteins.

A. Phylogenetic tree of *P. aeruginosa* and *E. coli* dLytM proteins. Whole-protein alignment of factors containing LytM domain was performed via ClustalW and plotted via iTOL (Interactive Tree of Life) programs. The *E. coli* proteins are presented in blue and the *P. aeruginosa* proteins are presented in red. Asterisks denote the factors that retain residues for Zn^{2+} coordination.

B. Domain architecture of dLytM proteins. Abbreviations are as follows: LytM, LytM domain; LysM, LysM PG-binding domain; CC, coiled coil; SS, signal sequence; SS*, lipoprotein signal sequence.

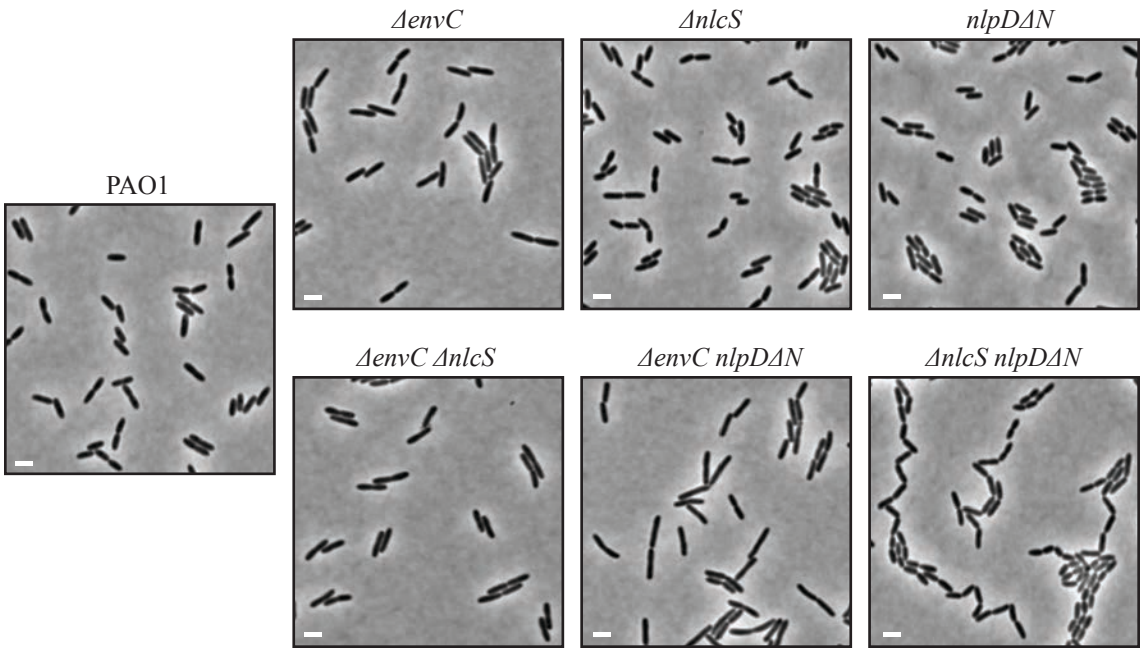

Figure S6. Phenotypic analysis of dLytM factor mutants at 30°C.

Overnight cultures of PAO1 [WT], BPA107 [*ΔenvC*], BPA14 [*ΔnlcS*], BPA70 [*nlpDΔN*], BPA57 [*ΔenvC ΔnlcS*], BPA109 [*ΔenvC nlpDΔN*], and BPA72 [*ΔnlcS nlpDΔN*] were grown in LB at 30°C. The cells were diluted 1:400 in LB, grown for 5.5 h at 30°C, placed on agarose pads, and imaged with phase optics. All space bars are 2μm.

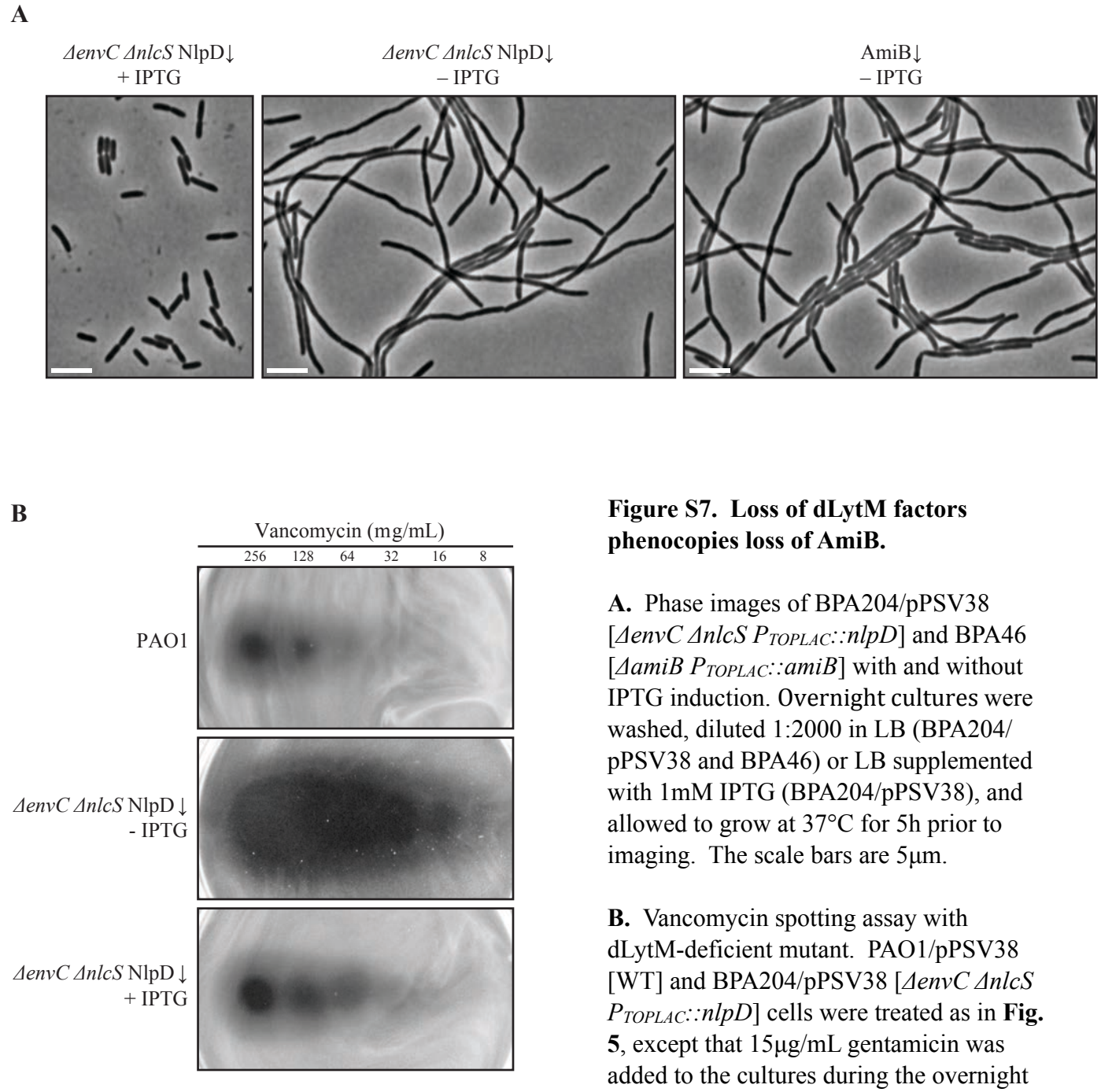

Figure S7. Loss of dLytM factors phenocopies loss of AmiB.

A. Phase images of BPA204/pPSV38 [*ΔenvC ΔnlcS* P_{TOPLAC}*::nlpD*] and BPA46 [*ΔamiB* P_{TOPLAC}*::amiB*] with and without IPTG induction. Overnight cultures were washed, diluted 1:2000 in LB (BPA204/pPSV38 and BPA46) or LB supplemented with 1mM IPTG (BPA204/pPSV38), and allowed to grow at 37°C for 5h prior to imaging. The scale bars are 5μm.

B. Vancomycin spotting assay with dLytM-deficient mutant. PAO1/pPSV38 [WT] and BPA204/pPSV38 [*ΔenvC ΔnlcS* P_{TOPLAC}*::nlpD*] cells were treated as in **Fig. 5**, except that 15μg/mL gentamicin was added to the cultures during the overnight and the initial 3h of growth with the inducer. 10μL of vancomycin solutions at the indicated concentrations were spotted onto the plates, which were then incubated for 2d at RT.

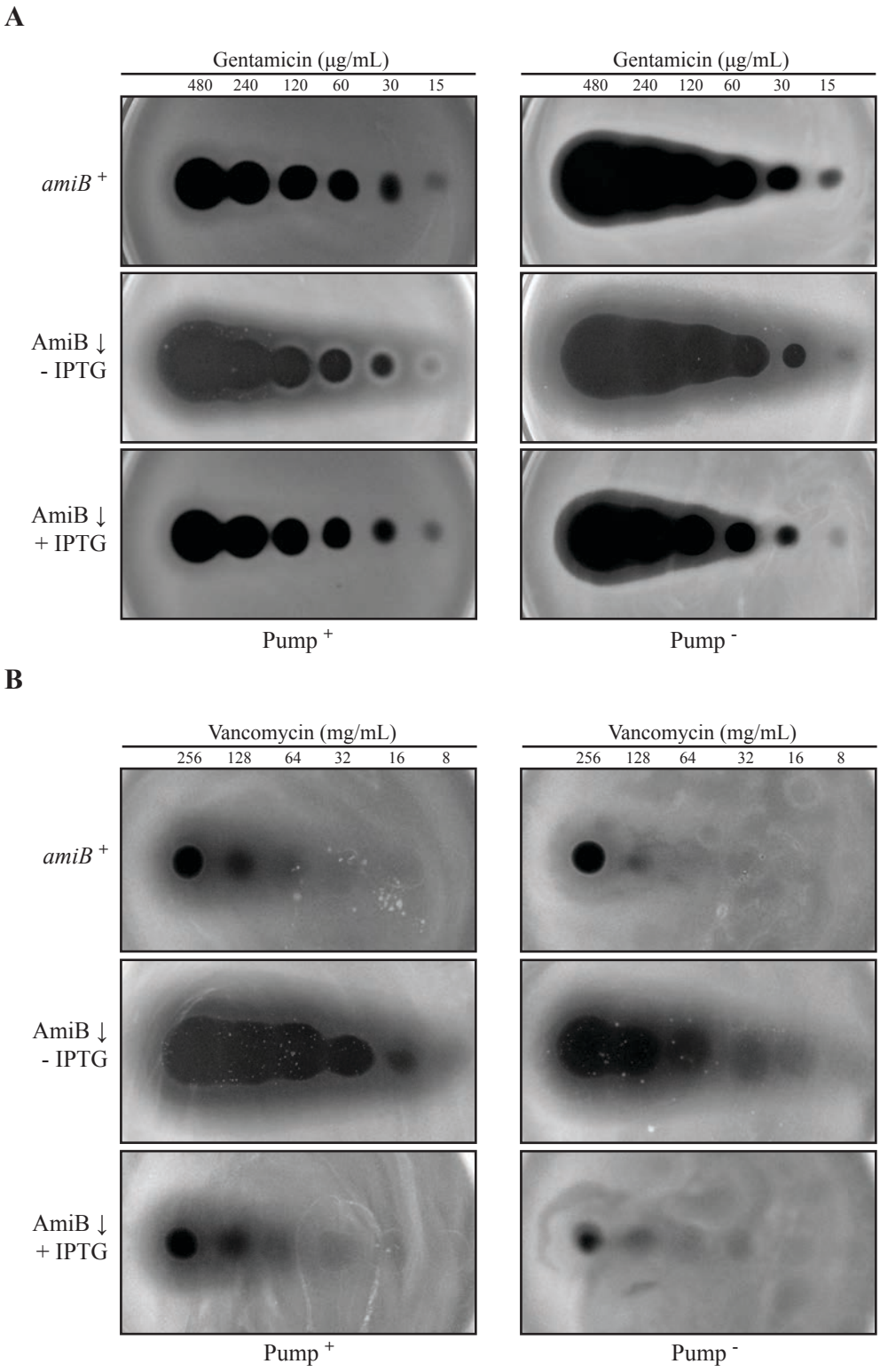

Figure S8. Antibiotic susceptibility of efflux pump mutant upon AmiB depletion.

A-B. Gentamicin (A) and vancomycin (B) spotting assays. PAO1 [WT], BPA46 [*ΔamiB* P_{TOPLAC}*::amiB*], PAO397 [*ΔmexAB-oprM ΔmexCD-oprJ ΔmexJKL ΔmexXY ΔmexEF-oprN ΔopmH362*], and BPA378 [*ΔmexAB-oprM ΔmexCD-oprJ ΔmexJKL ΔmexXY ΔmexEF-oprN ΔopmH362 ΔamiB* P_{TOPLAC}*::amiB*] cells were treated as in **Fig. 5**. 5µL of gentamicin or 10µL of vancomycin solutions of the indicated concentrations were spotted onto the surface of top agar inoculated with bacteria. The plates were incubated for 2d at RT prior to imaging.

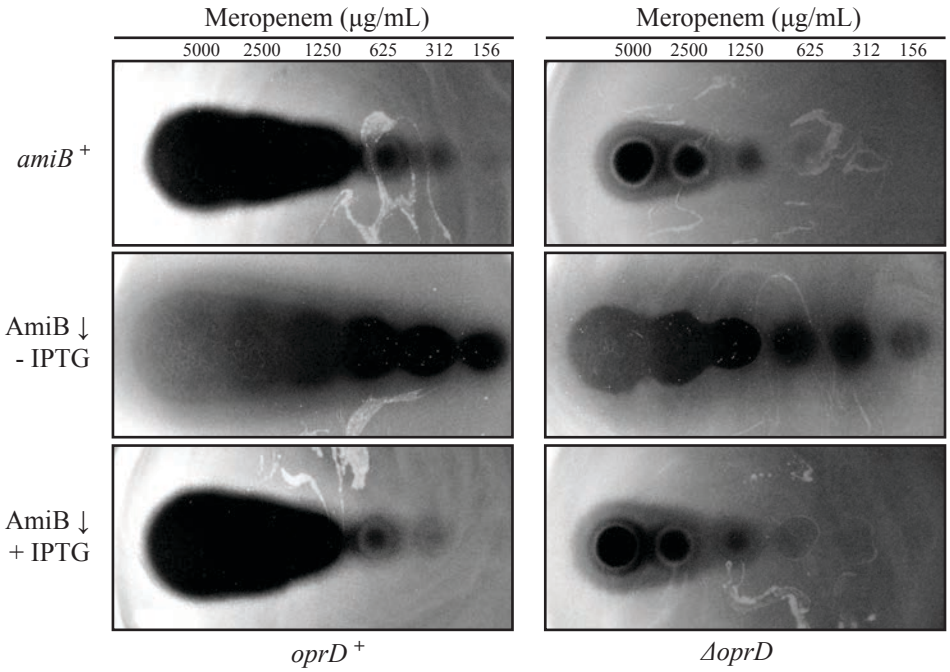

Figure S9. β-lactam susceptibility of *oprD* mutant upon AmiB depletion.

PAO1 [WT], BPA46 [*ΔamiB* P_{TOPLAC}*::amiB*], BPA162 [*ΔoprD*], and BPA164 [*ΔoprD ΔamiB* P_{TOPLAC}*::amiB*] cells were treated as in **Fig. 5.** 5μL of meropenem solutions at the indicated concentrations were spotted onto the surface of top agar impregnated with the bacteria. The plates were incubated for 2d at RT prior to imaging.

Table S1. *P. aeruginosa* strains used in this work

Strain	Genotype	Reference
BPA12	PAO1 *ΔnlpD*	This study
BPA14	PAO1 *ΔnlcS*	This study
BPA28	PAO1 *attTn7::P_{TOPLAC}::amiB aacC1*	This study
BPA35	PAO1 *attTn7::P_{TOPLAC}::amiB*	This study
BPA46	PAO1 *ΔamiB attTn7::P_{TOPLAC}::amiB*	This study
BPA57	PAO1 *ΔenvC ΔnlcS*	This study
BPA60	PAO1 *ΔamiA*	This study
BPA70	PAO1 *nlpDΔN*	This study
BPA72	PAO1 *ΔnlcS nlpDΔN*	This study
BPA107	PAO1 *ΔenvC*	This study
BPA109	PAO1 *ΔenvC nlpDΔN*	This study
BPA157	PAO1 *ΔamiB*	This study
BPA158	PAO1 *ΔamiA ΔamiB*	This study
BPA162	PAO1 *ΔoprD*	This study
BPA164	PAO1 *ΔamiB ΔoprD attTn7::P_{TOPLAC}::amiB*	This study
BPA166	PAO1 *ΔenvC attTn7::$lacI^q$ aacC1*	This study
BPA170	PAO1 *ΔenvC attTn7::$lacI^q$*	This study
BPA186	PAO1 *ΔenvC attTn7::$lacI^q$ P_{nlpD}::T4term::P_{TOPLAC}::nlpD*	This study
BPA204	PAO1 *ΔenvC ΔnlcS attTn7::$lacI^q$ P_{nlpD}::T4term::P_{TOPLAC}::nlpD*	This study
BPA215	PAO1 *P_{zapA}::zapA-mCherry ΔamiB attTn7::P_{TOPLAC}::amiB*	This study
BPA220	PAO1 *P_{zapA}::zapA-mCherry ΔamiA ΔamiB attTn7::P_{TOPLAC}::amiB*	This study
BPA231	PAO1 *P_{zapA}::zapA-mCherry ΔamiA ΔamiB cpxA(A154S) attTn7::P_{TOPLAC}::amiB*	This study
BPA237	PAO1 *cpxA(A154S)*	This study
BPA245	PAO1 *P_{zapA}::zapA-mCherry ΔamiA ΔamiB ΔcpxR attTn7::P_{TOPLAC}::amiB*	This study
BPA247	PAO1 *P_{zapA}::zapA-mCherry ΔamiA ΔamiB cpxA(A154S) ΔcpxR attTn7::P_{TOPLAC}::amiB*	This study

Strain	Genotype	Reference
BPA294	PAO1 *ΔamiB cpxA(A154S) attTn7::P_{TOPLAC}::amiB*	This study
BPA346	PAO1 *P_{amiB}::amiB-mCherry*	This study
BPA366	PAO1 *ΔmexAB-oprM nfxB ΔmexCD-oprJ ΔmexJKL ΔmexXY ΔmexEF-oprN ΔopmH362 attTn7::P_{TOPLAC}::amiB aacC1*	This study
BPA372	PAO1 *ΔmexAB-oprM nfxB ΔmexCD-oprJ ΔmexJKL ΔmexXY ΔmexEF-oprN ΔopmH362 attTn7::P_{TOPLAC}::amiB*	This study
BPA378	PAO1 *ΔmexAB-oprM nfxB ΔmexCD-oprJ ΔmexJKL ΔmexXY ΔmexEF-oprN ΔopmH362 attTn7::P_{TOPLAC}::amiB frt ΔamiB*	This study
BPA427	PA14 *ΔamiB*	This study
PA14	Wild-type	3
PAO1	Wild-type	4
PAO397	PAO1 *ΔmexAB-oprM nfxB ΔmexCD-oprJ ΔmexJKL ΔmexXY ΔmexEF-oprN ΔopmH362*	5

Table S2. *E. coli* strains used in this work

Strain	Genotype	Reference
AAY4	*amiA::frt amiB::frt attHK::P_{BAD}::amiA cpxR::Kan*	This study
AAY5	*amiA::frt amiB::frt attHK::P_{BAD}::amiA rcsB::Kan*	This study
AAY6	*amiA::frt amiB::frt attHK::P_{BAD}::amiA cpxR::frt*	This study
AAY7	*amiA::frt amiB::frt attHK::P_{BAD}::amiA rcsB::frt*	This study
AAY8	*amiA::frt amiB::frt attHK::P_{BAD}::amiA cpxR::frt amiC::Kan*	This study
AAY9	*amiA::frt amiB::frt attHK::P_{BAD}::amiA rcsB::frt amiC::Kan*	This study
AAY10	*amiA::frt amiB::frt attHK::P_{BAD}::amiA cpxR::frt rcsB::Kan*	This study
AAY12	*amiA::frt amiB::frt attHK::P_{BAD}::amiA cpxR::frt rcsB::frt*	This study
AAY14	*amiA::frt amiB::frt attHK::P_{BAD}::amiA amiC::Kan*	This study
AAY15	*amiA::frt amiB::frt attHK::P_{BAD}::amiA cpxR::frt rcsB::frt amiC::Kan*	This study
SM10(λpir)	*Kan^R thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu attλ::pir*	6
TB171	*rph1 ilvG rfb-50 ΔlacZYA::frt amiA::frt amiC::frt amiB::Kan*	7
TU207	*amiA::frt amiB::frt*	8
TU207(attHKNP115)	*amiA::frt amiB::frt attHK::P_{BAD}::amiA*	This study

Table S3. Plasmids used in this work

Strain	Genotype*	Reference
pAAY1	*aacC1 sacB oriT 'surE-pcm-nlpDΔ(2-74)-rpoS'*	This study
pAAY12	*aacC1 sacB oriT* P_{TOPLAC}	This study
pAAY16	*aacC1 sacB oriT 'surE-pcm::T4term::*P_{TOPLAC}	This study
pAAY17	*aacC1 sacB oriT 'surE-pcm::T4term::*P_{TOPLAC}*::nlpD*	This study
pAAY18	*aacC1 sacB oriT 'PA0959-oprDΔ(12-439)-PA0957-proS'*	This study
pAAY24	*aacC1 sacB oriT 'PA5226-zapA-mCherry::ssrS-PA5228'*	This study
pAAY28	*aacC1 sacB oriT 'PA3205-cpxA(A154S)*	This study
pAAY30	*aacC1 sacB oriT PA3202-PA3203-cpxRΔ(5-219)-PA3205-cpxA'*	This study
pAAY38	*aacC1 sacB oriT mCherry::PA5537-PA5536-PA5535'*	This study
pAAY39	*aacC1 sacB oriT 'amiA-mCherry::PA5537-PA5536-PA5535'*	This study
pAAY53	*aacC1 sacB oriT PA5535'-PA5536-PA5537::mutL'*	This study
pAAY54	*aacC1 sacB oriT amiB-mCherry::mutL'*	This study
pAAY65	*aacC1* P_{syn135}*::*^{ss}*dsbA-mCherry*	This study
pCP20	*bla cat flp cI857* λP_R*::*Rep^{Ts} [plasmid for Flp recombination]	9
pDY75	*tetA tetR attHK022 araC* P_{BAD}*::*^{ss}*dsbA-amiB*	10
pEXG2	*aacC1 sacB oriT* [vector for allelic exchange]	11
pFLP2	*bla sacB flp cI oriT* [plasmid for Flp recombinase expression]	12
pHRM151	*aacC1 sacB oriT 'PA5539-amiAΔ(2-397)-'PA5537-PA5536-PA5535'*	This study
pHRM153	*aacC1 sacB 'PA4949-PA4948-amiBΔ(2-475)-mutL'* [PAO1]	This study
pHRM154	*aacC1 sacB 'PA4949-PA4948-amiBΔ(2-475)-mutL'* [PA14]	This study
pHRM155	*aacC1 sacB 'surE-pcm-nlpDΔ(2-297)-rpoS'*	This study
pHRM157	*aacC1 sacB 'PA4925-nlcSΔ(2-231)-PA4923-azu'*	This study
pHRM159	*aacC1 sacB PA5132-envCΔ(2-428)-PA5134'*	This study
pHRM164	*aacC1 sacB oriT 'PA5539-amiAΔ(2-391)- PA5537-PA5536-PA5535'*	This study

Strain	Genotype*	Reference
pHRM166	*aacC1 bla Tn7 lacI^q P$_{TOPLAC}$::exSD-amiB*	This study
pJN105	*aacC1 araC P$_{BAD}$* [replicating arabinose-inducible expression vector]	13
pKD46	*bla repA101ts exo bet gam* [plasmid for *E. coli* λ recombineering]	14
pKHT103	*aacC1 bla Tn7 lacI^q P$_{TOPLAC}$* [vector for insertion of P$_{TOPLAC}$-regulated sequences into the *Tn*7 attachment site of *Pseudomonas*]	Dove Lab
pNP3	*bla attHK022 lacI^q P$_{lac}$::^{ss}dsbA-amiB(23-445)-mCherry*	10
pNP115	*tetA tetR attHK022 araC P$_{BAD}$::^{ss}dsbA-amiA(34-289)*	This study
pPSV38	*aacC1 bla Tn7 lacI^q P$_{TOPLAC}$* [replicating IPTG-inducible expression vector]	15
pTB102	*cat repA^{Ts} cI857 λP$_R$::intHK022* [plasmid for HK integrase expression]	16
pTNS2	*bla oriR6K tnsABCD* [plasmid for *Tn*7 transposase expression]	17
pTU175	*aadA P$_{syn135}$::^{ss}dsbA-mCherry*	8

* For the plasmids used to construct deletions in *P. aeruginosa*, apostrophes (‘ and ’) are used to denote 5’ and 3’ gene truncations, respectively.

***P. aeruginosa* strain construction**

BPA12 – To create the *ΔnlpD* strain, pHRM155 was conjugated into PAO1 recipient from SM10(λpir) donor. For this purpose, PAO1 was patched on an LB plate and grown overnight at 42°C while SM10(λpir) was similarly grown at 37°C. Both the donor and the recipient were scraped, patched together onto an LB plate, and incubated at 37°C for ~8h. All subsequent growth steps were performed at 30°C. The cells were scraped, resuspended in 500µL of LB, diluted 1:20, and 100µL of the resulting suspension was plated on PIA (Pseudomonas Isolation Agar) supplemented with 60µg/mL gentamicin, incubating the plates at 30°C. The ex-conjugants were grown overnight in LB with 15µg/mL gentamicin; then, the overnight culture was diluted 1:250 in plain LB, allowed to grow for ~8h without antibiotic to allow for the second plasmid recombination event, and 100µL of the 1:10 dilution of the resulting culture were finally plated on LB supplemented with 5% sucrose to select for the loss of the plasmid-encoded *sacB* gene. Sucrose-resistant colonies were then patched onto LB plates either containing or lacking 30µg/mL gentamicin. Gentamicin-sensitive colonies were further tested by PCR with *nlpD*-flanking primers `5′-TACTCGACTCGGTACGTTTCGTCC-3′` and `5′-GTGCCATGTCGTTATCCCTTGCAT-3′` to confirm gene deletion. This strain lacks the entire coding region of *nlpD* between the start and stop codons.

BPA14 – *nlcS* was deleted from PAO1 by integration and re-circularization of pHRM157 plasmid. The same procedure that was used for construction of BPA12 was employed for this process. The sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *nlcS*-flanking primers `5′-AACAAGGTATAGTGCGGGCCT-3′` and `5′-CCTGCTCGAAGTGAACGGTTTCTA-3′`. The deletion spans the entire coding region of *nlcS* between the start and stop codons.

BPA28 – Construction of the strain with P_{TOPLAC}-regulated copy of *amiB* integrated at Tn7 locus was based on the protocol described in (18). In short, pHRM166 plasmid, which encodes a P_{TOPLAC}-regulated copy of *amiB* flanked by Tn7 transposon inverted repeats, and pTNS2 plasmid, which encodes Tn7 transposase, were co-electroporated into PAO1, selecting transformants on LB plates supplemented with gentamicin. The integration of the transposon at the Tn7 attachment locus was confirmed by diagnostic PCR with PTn7R and PglmS-down primers (18).

BPA35 – To eliminate the gentamicin resistance cassette from BPA28 by Flp-mediated excision, pFLP2 plasmid was electroporated into BPA28, and transformants were selected for growth on LB medium supplemented with 200µg/mL carbenicillin, as described in (18). Carbenicillin-resistant transformants were grown overnight in liquid LB medium lacking antibiotics and streaked out onto a plain LB plate. The isolated colonies were patched onto LB supplemented with gentamicin, carbenicillin, or no antibiotic to check for the loss of both the gentamicin-resistance cassette and the pFLP2 plasmid.

BPA46 – To make the AmiB depletion strain, pHRM153 was conjugated into BPA35. The same procedure that was used for construction of BPA12 was employed in this process, except that the cells were grown in the presence of 1mM IPTG at every step to ensure the expression of AmiB. The sucrose-resistant, gentamicin-sensitive colonies were screened by PCR with *amiB*-flanking

primers 5′-ACCGCCGATCTGGACATTACCATT-3′ and 5′-TGCTCGACCTCCACATCAATGC-3′. The deletion spans the entire coding region of *amiB* between the start and stop codons.

BPA57 – For the *envC nlcS* double mutant, pHRM159 was conjugated into BPA14, and allelic replacement protocol was carried out as for BPA12. Gentamicin-sensitive, sucrose-resistant colonies were tested by diagnostic PCR with *envC*-flanking primers 5′-ATCTGCTGCTGTTCACCTAC-3′ and 5′-AAGCAATGCAGCATGTTCAG-3′. The *envC* deletion spans the entire coding region of *envC* between the start and stop codons.

BPA60 – To make the *amiA* deletion strain, pHRM164 was conjugated into PAO1, and allelic replacement protocol was carried out as for BPA12. Gentamicin-sensitive, sucrose-resistant colonies were tested by diagnostic PCR with *amiA*-flanking primers 5′-TGGAGAACTCGAAGAAGGGTAG-3′ and 5′-GCCTCAGCCGCAACATC-3′. The deletion preserves the start codon and the 6 C-terminal residues of AmiA and does not perturb the coding region of the adjacent gene, *PA5537*.

BPA70 – To create the *nlpDΔN* strain that expresses a variant of NlpD lacking the N-terminal secretion signal sequence, pAAY1 was conjugated into PAO1, and allelic replacement protocol was carried out as for BPA12. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *nlpD*-flanking primers used for BPA12. The deletion leaves intact the start codon and the 223 C-terminal residues of NlpD, thereby preserving the promoter of the downstream *rpoS* gene.

BPA72 – For the *ΔnlcS nlpDΔN* double mutant, pAAY1 was conjugated into BPA14, and allelic replacement protocol was carried out as for BPA12. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *nlpD*-flanking primers used for BPA12.

BPA107 – To make the *envC* deletion strain, pHRM159 was conjugated into PAO1, and allelic replacement protocol was carried out as for BPA12. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *envC*-flanking primers used for BPA57.

BPA109 – To construct the *ΔenvC nlpDΔN* double mutant, pHRM159 was conjugated into BPA70, and allelic replacement protocol was carried out as for BPA12. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *envC*-flanking primers used for BPA57.

BPA157 – To create the *amiB* deletion mutant, pHRM153 was conjugated into PAO1, and ex-conjugants were selected on PIA plates supplemented with 60μg/mL gentamicin. The allelic replacement protocol was modified from that described for BPA12, with the conjugation step carried out at 30°C. Additionally, the sucrose-resistant second recombinants were patched onto LB supplemented either with 5% sucrose alone or with the sugar and 15μg/mL gentamicin. The gentamicin-sensitive colonies were tested by diagnostic PCR with *amiB*-flanking primers used for BPA46.

BPA158 – For the *ΔamiA ΔamiB* double mutant strain, pHRM153 was conjugated into BPA60, and allelic replacement protocol was carried out as for BPA157. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *amiB*-flanking primers used for BPA46.

BPA162 – To make the *oprD* deletion mutant, pAAY18 was conjugated into PAO1, and allelic replacement protocol was carried out as for BPA12. Gent^S Suc^R colonies were tested by diagnostic PCR with *oprD*-flanking primers 5′-TTTT***GAATTC***CAATTCCCAAGCGAGAGCC-3′ and 5′-TTTT***AAGCTT***CGACCCGCTGGAATACCATG-3′. The deletion retains the 11 N-terminal and the 4 C-terminal residues of OprD.

BPA164 – To construct *oprD* deletion in AmiB-depletion genetic background, pAAY18 was conjugated into BPA46, and allelic replacement protocol was carried out as for BPA12, except that all growth media were supplemented with 1mM IPTG. Gent^S Suc^R colonies were screened by diagnostic PCR with *oprD*-flanking primers used for BPA162.

BPA166 – To create the strain with *envC* deletion and *lacI*^{q}-carrying Tn7 transposon integrated at the Tn7 attachment locus, pKHT103 was co-electroporated with pTNS2 into BPA107. The transformants were selected for growth on LB plates supplemented with 30μg/mL gentamicin, and the integration of the transposon was confirmed by diagnostic PCR with PTn7R and PglmS-down primers (18).

BPA170 – In order to eliminate the gentamicin-resistance cassette from BPA166, the strain was electroporated with pFLP2 plasmid, and the transformants were selected for growth on LB plates supplemented with 200μg/mL carbenicillin. Carb^R colonies were grown overnight in liquid LB medium lacking antibiotics and streaked out onto a plain LB plate. The isolated colonies were patched onto LB supplemented with gentamicin, carbenicillin, or no antibiotic to ensure the loss of both the gentamicin-resistance cassette and the pFLP2 plasmid.

BPA186 – For making the NlpD depletion strain in *envC*-deletion genetic background, pAAY17 was conjugated into BPA170, and allelic replacement protocol was carried out as for BPA12, except that all growth media were supplemented with 1mM IPTG. Gent^S Suc^R colonies were tested by diagnostic PCR with *nlpD*-flanking primers used for BPA12. The resulting mutant contains the entire coding region of the adjacent *pcm* gene followed by the T4 intrinsic transcriptional terminator to prevent the expression of *nlpD* from its native promoter, the exogenous P_{TOPLAC} promoter, and the *nlpD* gene with its native Shine-Dalgarno sequence all at the endogenous genomic locus of *nlpD*.

BPA204 – To create the NlpD depletion strain in *ΔenvC ΔnlcS* double mutant genetic background, pHRM157 was conjugated into BPA186, and allelic replacement protocol was carried out as for BPA12, except that all growth media were supplemented with 1mM IPTG. Gent^S Suc^R colonies were tested by diagnostic PCR with *nlcS*-flanking primers used for BPA14.

BPA215 – To construct the AmiB depletion strain with a ZapA-mCherry fusion encoded at the endogenous locus, pAAY24 was conjugated into BPA46, and allelic replacement protocol was carried out as for BPA12, except that all growth media were supplemented with 1mM IPTG. Gent^S Suc^R colonies were tested for the presence of the *mCherry* gene by diagnostic PCR with 5′-CTCGAGGGTCCGGCTGGTCTGTCCAAG-3′ and 5′-CTTCGCCGGCATCGTTATTAGGATCCGCCAGCACCTTTG-3′ primers.

BPA220 – To make the AmiB depletion strain with *amiA* deletion and ZapA-mCherry fusion, pHRM164 was conjugated into BPA215, and allelic replacement protocol was carried out as for BPA12, except that all growth media were supplemented with 1mM IPTG. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *amiA*-flanking primers used for BPA60.

BPA231 – For making the AmiB-depletion strain with *cpxA*(*A154S*) mutation and *amiA* deletion, pAAY28 was conjugated into BPA220, and allelic replacement protocol was carried out as for BPA12, but with two modifications. Firstly, all growth media were supplemented with 1mM IPTG. Secondly, following the conjugation step, the ex-conjugants were selected on VBMM plates supplemented with 1mM IPTG and 60µg/mL gentamicin. $Gent^S$ Suc^R colonies were subjected to colony PCR with `5′- CCATGCGTTCACTCTTCTGGC-3′` and `5′-TTTG`***`TCTAGA`***`GTCATGCCGGATGCTCCTGG-3′` primers, and the presence of the *cpxA*(*A154S*) allele was confirmed by sequencing of the resulting PCR products with the `5′-CCATGCGTTCACTCTTCTGGC-3′` primer.

BPA237 – To make the *cpxA*(*A154S*) mutant in wild-type genetic background, pAAY28 was conjugated into PAO1, and allelic replacement protocol was carried out as for BPA12, except that the ex-conjugants were selected on VBMM plates supplemented with 60µg/mL gentamicin. $Gent^S$ Suc^R colonies were screened for the presence of *cpxA*(*A154S*) mutation by sequencing, as described for BPA231.

BPA245 – To create *cpxR* deletion in AmiB-depletion background, pAAY30 was conjugated into BPA220, and allelic replacement protocol was carried out as for BPA12, but with two modifications. Firstly, all growth media were supplemented with 1mM IPTG. Secondly, the ex-conjugants following conjugation were selected on VBMM plates supplemented with 1mM IPTG and 60µg/mL gentamicin. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *cpxR*-flanking primers `5′- TTTG`***`TCTAGA`***`GTCATGCCGGATGCTCCTGG-3′` and `5′-TTTT`***`GAGCTC`***`GTAGCTGCCCTGTTCTTCG-3′`. The deletion retains the 4 N-terminal and the 6 C-terminal residues of CpxR.

BPA247 – To construct the AmiB-depletion strain with both *cpxA*(*A154S*) mutation and *cpxR* deletion, pAAY30 was conjugated into BPA231, and allelic replacement protocol was carried out as for BPA12, but with two modifications. Firstly, all growth media were supplemented with 1mM IPTG. Secondly, the ex-conjugants were selected on VBMM plates supplemented with 1mM IPTG and 60µg/mL gentamicin. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *cpxR*-flanking primers used for BPA245.

BPA 294 – For making AmiB-depletion strain with *cpxA*(*A154S*) mutation and wild-type *amiA*, pAAY28 was conjugated into BPA46, and allelic replacement protocol was carried out as for BPA12, but with two modifications. Firstly, all growth media were supplemented with 1mM IPTG. Secondly, following conjugation, the ex-conjugants were selected on VBMM plates supplemented with 1mM IPTG and 60µg/mL gentamicin. $Gent^S$ Suc^R colonies were subjected to colony PCR with `5′- CCATGCGTTCACTCTTCTGGC-3′` and `5′-TTTG`***`TCTAGA`***`GTCATGCCGGATGCTCCTGG-3′` primers, and the presence of the *cpxA*(*A154S*) allele was determined by sequencing of the resulting PCR products with the `5′-CCATGCGTTCACTCTTCTGGC-3′` primer.

BPA346 – To construct the strain with an AmiB-mCherry fusion encoded at the native locus, pAAY54 was conjugated into PAO1, and allelic replacement protocol was carried out as for BPA12, except that the ex-conjugants were selected on VBMM plates supplemented with 60μg/mL gentamicin. $Gent^S$ Suc^R colonies were tested for the presence of *mCherry* gene by diagnostic PCR with `5′-CTGGCGGCCCAGCTCGAGGGTCCGGCTGGTCTG-3′` and `5′-GCTTCACTCATCA`***`GGATCC`***`GCCAGCACCTTTGTACAGC-3′` primers.

BPA366 – To create the strain with disruptions of the five primary efflux pumps and with an IPTG-inducible copy of *amiB* integrated at an exogenous locus, pHRM166 was co-electroporated with pTNS2 into PAO397. The transformants were selected for growth on LB plates supplemented with 30μg/mL gentamicin, and the integration of the transposon was confirmed by diagnostic PCR with PTn7R and PglmS-down primers (18).

BPA372 – In order to excise the gentamicin-resistance cassette from BPA366, the strain was electroporated with pFLP2 plasmid, and the transformants were selected for growth on LB plates supplemented with 200μg/mL carbenicillin. $Carb^R$ colonies were grown overnight in liquid LB medium lacking antibiotics and streaked out onto a plain LB plate. The isolated colonies were patched onto LB supplemented with gentamicin, carbenicillin, or no antibiotic to ensure the loss of both the gentamicin-resistance cassette and the pFLP2 plasmid.

BPA378 – To construct the AmiB-depletion stain with disruption of all five major efflux pumps, pHRM153 was conjugated into BPA372, and allelic replacement protocol was carried out as for BPA12, but with two modifications. Firstly, all growth media were supplemented with 1mM IPTG. Secondly, the ex-conjugants following conjugation were selected on VBMM plates supplemented with 1mM IPTG and 60μg/mL gentamicin. $Gent^S$ Suc^R colonies were tested by diagnostic PCR with *amiB*-flanking primers used for BPA46.

BPA427 -- To create the *amiB* deletion mutant in PA14 genetic background, pHRM154 was conjugated into PA14, and ex-conjugants were selected on VBMM plates supplemented with 60μg/mL gentamicin. The allelic replacement protocol was modified from that described for BPA12, with the conjugation step carried out at 30°C. Additionally, the sucrose-resistant second recombinants were patched onto LB supplemented either with 5% sucrose alone or with the sugar and 15μg/mL gentamicin. The gentamicin-sensitive colonies were tested by diagnostic PCR with *amiB*-flanking primers `5′-ACCGGCGTCAAGGCTGTTT-3′` and `5′-ACCGCCGATCTGGACATTACCATT-3′`. The deletion spans the entire coding region of *amiB* between the start and stop codons.

E. coli strain construction

AAY4 – Made by P1 transduction of *cpxR::Kan* from the Keio collection *cpxR::Kan* donor (19) to TU207(attHKNP115) recipient. Transductants were selected on M9 plates supplemented with arabinose, casamino acids, and kanamycin.

AAY5 – Made by P1 transduction of *rcsB::Kan* from the Keio collection *rcsB::Kan* donor (19) to TU207(attHKNP115) recipient. Transductants were selected on M9 plates supplemented with arabinose, casamino acids, and kanamycin.

AAY6 – pCP20, which encodes lambda recombineering proteins, was electroporated into AAY4, and transformants were selected at 30°C on M9 plates supplemented with arabinose, casamino acids, and ampicillin. To allow for plasmid loss, the transformants were re-streaked on M9 plates with arabinose, casamino acids, and no antibiotics and incubated at 37°C. Isolated colonies were tested for loss of the plasmid and the kanamycin-resistance cassette by patching onto M9 plates supplemented with arabinose, casamino acids and either kanamycin, ampicillin, or no antibiotic. The absence of *cpxR* or the *cpxR::Kan* insertion was confirmed by PCR with 5′-ACGTAAAGTCATGGATTAGC-3′ and 5′-GAATCGAGCTTGGGTAACATC-3′ primers, which flank the *cpxR* gene. Additionally, improper site-specific recombination between non-adjacent *frt* sites was ruled out by diagnostic PCR with *amiA*-flanking primers 5′-GTCACCGTTTACCTCAGCGAC-3′ and 5′-CACCATTACGCAACACCCGAC-3′ and *amiB*-flanking primers 5′-GGTGTTCTTCCTGACCCGGAT-3′ and 5′-GTGCGTGAAGTGAGCGTCAG-3′.

AAY7 – Lambda recombineering was performed using the same procedure as for construction of AAY6. The absence of *rcsB* or the *rcsB::Kan* insertion was confirmed by diagnostic PCR with 5′-CGTACGGGAAATTGGGCCTG-3′ and 5′-CTGGTCGTGGATGATCATCCG-3′ primers.

AAY8, AAY9, AAY14, and AAY15 – These strains were constructed by P1 transduction of *amiC::Kan* from the Keio collection *amiC::Kan* donor (19) to AAY6, AAY7, TU207(attHKNP115), and AAY12 recipients, respectively. Transductants were selected on M9 plates supplemented with arabinose, casamino acids, and kanamycin.

AAY10 – This strain was constructed by P1 transduction of *rcsB::Kan* from the Keio collection *rcsB::Kan* donor (19) to AAY6 recipient. Transductants were selected on M9 plates supplemented with arabinose, casamino acids, and kanamycin.

AAY12 – Lambda recombineering used to construct this strain was performed as for AAY6 and AAY7. The strain was checked by diagnostic PCR with the *amiA-, amiB-, cpxR-,* and *rcsB*-flanking primers listed in the descriptions of AAY6 and AAY7 construction processes.

TU207(attHKNP115) – To obtain the strain with a P_{BAD}-regulated copy of *amiA* in *amiA::frt amiB::frt* genetic background, pNP115 and the helper plasmid pTB102 were co-transformed into TU207, using the protocol described in (20). *amiA* is fused to the signal sequence of DsbA to ensure efficient secretion into the periplasm.

Plasmid construction

pAAY1 – To construct pAAY1 plasmid, which is used for deletion of the N-terminal part of NlpD, the ~1000bp region upstream of *nlpD* (*'surE-pcm*) was amplified from PAO1 genomic

DNA (gDNA) using 5′-GCTA***AAGCTT***CCGTGCTCAACGTCAATA-3′ and 5′-CAGGGTGTC***CCATGG***CATCTTCTTCGCTTTCGTTC-3′ primers. Meanwhile, the 3' end of *nlpD* with a 363bp region downstream of the gene (*'nlpD-rpoS'*) was amplified using 5′-CGAAGAAGATG***CCATGG***GACACCCTGTATTCCATTGCC-3′ and 5′-TT***TCTAGA***GACGAGCGAAGTGGACTTCC-3′ primers. The two resulting PCR products were then combined by sewing PCR with 5′-GCTA***AAGCTT***CCGTGCTCAACGTCAATA-3′ and 5′-TT***TCTAGA***GACGAGCGAAGTGGACTTCC-3′ primers. The final PCR product was digested with HindIII and XbaI and ligated into pEXG2, which had been digested with the same enzymes.

pAAY12, pAAY16, and pAAY17 – The construction of pAAY17 plasmid for integration of an IPTG-inducible version of *nlpD* at the native locus involved several steps. Firstly, to create pAAY12, the *P. aeruginosa* suicide vector with the P_{TOPLAC} promoter, the promoter region was excised from pPSV38 with NheI and XhoI and ligated into pEXG2, which had been digested with XbaI and XhoI. Secondly, to construct pAAY16, ~870bp region upstream of *nlpD* (*'surE-pcm*) was amplified from PAO1 gDNA using 5′-TTTT***GGTACC***ACCGGTGAAGGTGGTCAATCC-3′ and 5′-TT***GAATTC***AAAAAAAGGGGACCTCTAGGGTCCCCATAGCTCAGGCGATCGGGCCGTTGAG-3′ primers, simultaneously fusing it to T4 transcriptional terminator. The PCR product was then digested with EcoRI and KpnI and ligated into pAAY12 pre-digested with the same enzymes. Finally, to construct pAAY17, *nlpD* with its native Shine-Dalgarno sequence was amplified from PAO1 gDNA using 5′-AAAA***CTGCAG***CGATTGAACGAAAGCGAAGAAGATGG-3′ and 5′-AAAA***AAGCTT***GAACTCCCGGTCAGCGACGTGG-3′ primers. The PCR product was ligated into pAAY16 via PstI and HindIII restriction sites.

pAAY18 – To construct pAAY18 vector for *oprD* deletion, ~900bp region upstream of *oprD* (*'PA0959*) was amplified from PAO1 gDNA with 5′-TTT***TCTAGA***GGAACAGCTCGGTGACATCC-3′ and 5′-CAGGATCGACAGCAGTGCAATGGCGCTCCACTTCATC-3′ primers. Concurrently, ~900bp region downstream of *oprD* (*PA0957-proS'*) was amplified using 5′-GCCATTGCACTGCTGTCGATCCTGTAATCGACCG-3′ and 5′-GGTCGGTGATCACTTCTTCGTGG-3′ primers. The two PCR products were combined by sewing PCR with 5′-TTT***TCTAGA***GGAACAGCTCGGTGACATCC-3′ and 5′-GGTCGGTGATCACTTCTTCGTGG-3′ primers. Finally, the resulting PCR product was digested with XbaI and EcoRI (the EcoRI site is native to *PA0959-proS'* and does not originate from a primer) and ligated into pEXG2 predigested with the same enzymes.

pAAY24 – The construction of pAAY24 plasmid for introduction of a *zapA-mCherry* C-terminal fusion at the *zapA* native locus required several steps. Firstly, the *zapA* gene (*PA5227*) with 226bp of its upstream region *('PA5226-zapA*) was amplified from PAO1 gDNA using 5′-TTTT***GAATTC***CAATTCCCAAGCGAGAGCC-3′ and 5′-CAGACCAGCCGGACCCTCGAGGGCTTCGCCGGCATCGGCCGGATTC-3′ primers. Meanwhile, the sequence coding for mCherry and LEGPAGL linker was amplified from pNP3 using 5′-CTCGAGGGTCCGGCTGGTCTGTCCAAG-3′ and 5′-CTTCGCCGGCATCGTTATTAGGATCCGCCAGCACCTTTG-3′ primers. The resulting *'PA5226-zapA* and *mCherry* PCR products were combined by sewing PCR with 5′-TTTT***GAATTC***CAATTCCCAAGCGAGAGCC-3′ and 5′-CTTCGCCGGCATCGTTATTAGGATCCGCCAGCACCTTTG-3′ primers. Then, 526bp of *zapA*

downstream region (*ssrS-PA5228'*) were amplified from PAO1 gDNA with 5′-CGGATCCTAATAACGATGCCGGCGAAGCCTGACC-3′ and 5′-TTTT***AAGCTT***CGACCCGCTGGAATACCATG-3′ primers. *'PA5226-zapA-mCherry* and *ssrS-PA5228'* PCR products were combined by sewing PCR with 5′-TTTT***GAATTC***CAATTCCCAAGCGAGAGCC-3′ and 5′-TTTT***AAGCTT***CGACCCGCTGGAATACCATG-3′ primers. The final *'PA5226-zapA-mCherry-ssrS-PA5228'* PCR product was then digested with EcoRI and HindIII and ligated into pEXG2 predigested with the same enzymes.

pAAY28 – To construct pAAY28 vector for replacement of *cpxA* with its A154S allele, *cpxA*(*A154S*) with 412bp of *cpxA* upstream region (*'PA3205-cpxA*(*A154S*)) was amplified from the genomic DNA of a spontaneous suppressor mutant isolated in the *ΔamiA ΔamiB* [BPA158] genetic background. 5′-AGAG***AAGCTT***GATGAAGGAACTGGACCTGACCC-3′ and 5′-TTTG***TCTAGA***GTCATGCCGGATGCTCCTGG-3′ primers were used. The PCR product was cloned into pEXG2 via HindIII and XbaI restriction sites. No mutations other than the one responsible for A154S substitution were present in the cloned region.

pAAY30 – To construct pAAY30 vector for *cpxR* deletion, ~860bp of *cpxR* upstream region (*PA3202-PA3203*) were amplified with 5′- TTTG***TCTAGA***GTCATGCCGGATGCTCCTGG-3′ and 5′- GTAGTAGTAGCCCAGTTCGCTCATCGAGTGTCG-3′ primers. Concurrently, ~880bp of *cpxR* downstream region (*PA3205-cpxA'*) were amplified with 5′-GATGAGCGAACTGGGCTACTACTACAGCCACTGAGC-3′ and 5′-TTTT***GAGCTC***GTAGCTGCCCTGTTCTTCG-3′ primers. *PA3202-PA3203* and *PA3205-cpxA'* were combined by sewing PCR with 5′- TTTG***TCTAGA***GTCATGCCGGATGCTCCTGG-3′ and 5′-TTTT***GAGCTC***GTAGCTGCCCTGTTCTTCG-3′ primers. Finally, the resulting PCR product was cloned into pEXG2 via XbaI and SacI restriction sites.

pAAY38, pAAY39, pAAY53, and pAAY54 – Construction of pAAY54 plasmid for introduction of a *amiB-mCherry* C-terminal fusion at the *amiB* native locus required several steps and involved *amiA-mCherry* plasmid pAAY39 as an intermediate. Firstly, to make pAAY38, mCherry with LEGPAGL linker-coding DNA was amplified from pAAY24 using 5′-CAGCCTCCCGGC***CTCGAG***GGTCCGGCTGGTCTG-3′ and 5′-GCGAAGGCCCTA***GGATCC***GCCAGCACCTTTGTACAGC-3′ primers. Meanwhile, 945bp region downstream of *amiA* (*PA5537-PA5536-PA5535'*) was amplified from PAO1 gDNA with 5′-GCTGGC***GGATCC***TAGGGCCTTCGCTGCAGCCGCTGAC-3′ and 5′-TAGC***TCTAGA***ACAGGTAGGCGTTGGTTCACG-3′ primers. *mCherry* and *PA5537-PA5536-PA5535'* were combined by sewing PCR with 5′- CAGCCTCCCGGC***CTCGAG***GGTCCGGCTGGTCTG-3′ and 5′-TAGC***TCTAGA***ACAGGTAGGCGTTGGTTCACG-3′ primers, and the resulting *mCherry-PA5537-PA5536-PA5535'* PCR product was cloned into pEXG2 via XhoI and XbaI restriction sites. Then, to construct pAAY39, a 930bp fragment of *amiA* 3' coding region (*'amiA*) was amplified from PAO1 gDNA with 5′-TTTT***GGTACC***ATACGCAGTGGCCAACTGG-3′ and 5′-CGGACC***CTCGAG***GCCGGGAGGCTGCGCGCTGAG-3′ primers and cloned into pAAY38 via KpnI and XhoI restriction sites. To construct pAAY53, ~1400bp region downstream of *amiB* (*mutL'*) was amplified from PAO1 gDNA with 5′-gtgctggc***GGATCC***TGATGAGTGAAGCACCGCGTATCC-3′ and 5′-TTTT***GGTACC***GCATGTCCACCAGCACCAGG-3′ primers. pAAY39 was digested with KpnI and BamHI, thereby excising *'amiA-mCherry*, and *mutL'* PCR product pre-digested with the

same enzymes was ligated into this vector. Finally, to make pAAY54, *amiB* was amplified from PAO1 gDNA with 5′-TTTT***GTCGAC***ATGGGTTGGGGCTTGCGTCTG-3′ and 5′-CGGACCCTCGAGCTGGGCCGCCAGGGCGGTGCTC-3′ primers while *mCherry* was amplified from pAAY24 with 5′-CTGGCGGCCCAGCTCGAGGGTCCGGCTGGTCTG-3′ and 5′-GCTTCACTCATCA***GGATCC***GCCAGCACCTTTGTACAGC-3′ primers. *amiB* and *mCherry* PCR products were combined by sewing PCR with 5′-TTTT***GTCGAC***ATGGGTTGGGGCTTGCGTCTG-3′ and 5′-GCTTCACTCATCA***GGATCC***GCCAGCACCTTTGTACAGC-3′ primers, and *amiB-mCherry* was cloned into pAAY53 via SalI and BamHI restriction sites, with a concomitant excision of *PA5537-PA5536-PA5535'*.

pAAY65 – Plasmid pAAY65 for constitutive expression of periplasmic mCherry was constructed by subcloning the DNA sequence encoding mCherry with the periplasmic transport signal peptide of *E. coli* DsbA protein and the constitutive P_{syn135} promoter that controls its expression from pTU175 into pJN105 via EcoRI and SalI restriction sites. The P_{BAD} promoter and the *araC* gene encoded by pJN105 were excised during this cloning process.

pHRM151 and pHRM164 – Construction of pHRM164, the vector for *amiA* deletion, required several steps. Firstly, to construct pHRM151, ~1000bp region upstream of *amiA* (*'PA5539*) was amplified from PAO1 gDNA with 5′-GCTA***AAGCTT***GCATGCAGGCTTTCCGCA-3′ and 5′-GCAGCGAAGGCCCTAT***GCGGCCGC***CATCGACGAAATACCCGATT-3′ primers. Concurrently, ~1000bp region downstream of *amiA* (*'PA5537-PA5536-PA5535'*) was amplified with 5′-GGTATTTCGTCGATG***GCGGCCGC***ATAGGGCCTTCGCTGCAGCCG-3′ and 5′-TAGC***TCTAGA***CACATGGTTGAGCAGCGT-3′ primers. *'PA5539* and *'PA5537-PA5536-PA5535'* were combined by sewing PCR with 5′-GCTA***AAGCTT***GCATGCAGGCTTTCCGCA-3′ and 5′-TAGC***TCTAGA***CACATGGTTGAGCAGCGT-3′ primers, and the resulting PCR product was cloned into pEXG2 via HindIII and XbaI restriction sites. The result was pHRM151, which in addition to *amiA*, deleted the 3' end of the adjacent gene, *PA5537*. To avoid this problem, ~1000bp region downstream of *amiA* plus six codons of *amiA* coding sequence (*'amiA-PA5537-PA5536-PA5535'*) was amplified from PAO1 gDNA with 5′-TAGC***GCGGCCGC***ATAGTCAGCGCGCAGCCTCCCG-3′ and 5′-TAGC***TCTAGA***ACAGGTAGGCGTTGGTTCACG-3′. pHRM151 was digested with NotI and XbaI to excise *'PA5537-PA5536-PA5535'*, and *'amiA-PA5537-PA5536-PA5535'* predigested with the same enzymes was ligated into the vector, yielding pHRM164.

pHRM153 – For construction of the PAO1 *amiB* deletion vector, pHRM153, ~1000bp region upstream of *amiB* (*'PA4949-PA4948*) was amplified from PAO1 gDNA with 5′-GCAT***GAATTC***AGGTCTGGGATGCCGACG-3′ and 5′-GTGCTTCACTCATCATGCGGCCGCCATCGTTTTTCTCATGCTCC-3′ primers. Concurrently, ~1000bp region downstream of *amiB* (*mutL'*) was amplified with 5′-ATGAGAAAAACGATGGCGGCCGCATGATGAGTGAAGCACCGCGT-3′ and 5′-ATGC***GGATCC***AAGTCATGGACCATCCGG-3′ primers. *'PA4949-PA4948* and *mutL'* were combined by sewing PCR with 5′-GCAT***GAATTC***AGGTCTGGGATGCCGACG-3′ and 5′-ATGC***GGATCC***AAGTCATGGACCATCCGG-3′ primers, and the resulting PCR product was cloned into pEXG2 via EcoRI and BamHI restriction sites.

pHRM154 – For construction of the PA14 *amiB* deletion vector, pHRM154, ~1000bp region upstream of *amiB* (*'PA4949-PA4948*) was amplified from PA14 gDNA with 5′-GCAT***GAATTC***AGGTCTGGGATGCCGACG-3′ and 5′-GTGTTTCACTCATCATGCGGCCGCCATCGTTTTTCTCATGCTCC-3′ primers. Concurrently, ~1000bp region downstream of *amiB* (*mutL'*) was amplified with 5′-ATGAGAAAAACGATGGCGGCCGCATGATGAGTGAAACACCGCGT-3′ and 5′-ATGC***GGATCC***AAGTCATGGACCATCCGG-3′ primers. *'PA4949-PA4948* and *mutL'* were combined by sewing PCR with 5′-GCAT***GAATTC***AGGTCTGGGATGCCGACG-3′ and 5′-ATGC***GGATCC***AAGTCATGGACCATCCGG-3′ primers, and the resulting PCR product was cloned into pEXG2 via EcoRI and BamHI restriction sites.

pHRM155 – To make pHRM155, vector for deletion of the entire coding region of *nlpD*, ~1000bp region upstream of the gene (*'surE-pcm*) was amplified from PAO1 gDNA with 5′-GCTA***AAGCTT***CCGTGCTCAACGTCAATA-3′ and 5′-GCGAACTCCCGGTCATGCGGCCGCCATCTTCTTCGCTTTCGTTC-3′ primers. Also, ~1000bp region downstream of *nlpD* (*rpoS'*) was amplified using 5′-AAAGCGAAGAAGATGGCGGCCGCATGACCGGGAGTTCGCCCGCC-3′ and 5′-GCTA***TCTAGA***CCGACCTCTTCCAGCGTG-3′ primers. *'surE-pcm* and *rpoS'* were combined by sewing PCR with 5′-GCTA***AAGCTT***CCGTGCTCAACGTCAATA-3′ and 5′-GCTA***TCTAGA***CCGACCTCTTCCAGCGTG-3′ primers, and the resulting PCR product was cloned into pEXG2 via HindIII and XbaI restriction sites.

pHRM157 – To construct pHRM157 vector for *nlcS* deletion, ~1000bp region upstream of *nlcS* (*'PA4925*) was amplified from PAO1 gDNA with 5′-GCTA***AAGCTT***GATCAACGCGCTCTGGGC-3′ and 5′-TCCGCTCCTCTCTCATGCGGCCGCCACACGGAATCCTTTTCCCG-3′ primers. Meanwhile, *nlcS* downstream region (*PA4923-azu'*) was amplified using 5′-AAAGGATTCCGTGTGGCGGCCGCATGAGAGAGGAGCGGATCACT-3′ and 5′-GCTA***TCTAGA***ACGGTGATGGCATTGGTG-3′ primers. *'PA4925* and *PA4923-azu'* were combined by sewing PCR with 5′-GCTA***AAGCTT***GATCAACGCGCTCTGGGC-3′ and 5′-GCTA***TCTAGA***ACGGTGATGGCATTGGTG-3′ primers, and the resulting PCR product was cloned into pEXG2 via HindIII and XbaI restriction sites.

pHRM159 – To construct pHRM159 plasmid for deletion of *envC*, the ~1000bp region upstream of the gene (*PA5132*) was amplified from PAO1 gDNA using 5′-GCTA***AAGCTT***CTTTTCCATCTGCCCTAC-3′ and 5′-CGAGTTCGGCGCCTAGGATCCCATGGGGTGGCGGGCACCTG-3′ primers. Meanwhile, the ~1000bp region downstream of *envC* (*PA5134'*) was amplified using 5′-GCCCGCCACCCCATGGGATCCTAGGCGCCGAACTCGTGATT-3′ and 5′-GCAT***TCTAGA***GCTGTCGGTGCCCATCAG-3′ primers. The two resulting PCR products were then combined by sewing PCR, using 5′-GCTA***AAGCTT***CTTTTCCATCTGCCCTAC-3′ and 5′-GCAT***TCTAGA***GCTGTCGGTGCCCATCAG-3′ primers. The final PCR product was digested with HindIII and XbaI and ligated into pEXG2, which had been digested with the same enzymes.

pHRM166 – To construct pHRM164, the vector for insertion of a P_{TOPLAC}-regulated copy of *amiB* at the Tn7 integration locus, *amiB* with an exogenous Shine-Dalgarno sequence was

amplified from PAO1 gDNA with 5′-ATCG***GGATCC***GAGGAGGATACATATGGGTTGGGGCTTGCGT-3′ and 5′-ATGC***GGTACC***TCACTGGGCCGCCAGGGC-3′ primers. The resulting PCR product was cloned into pKHT103 via BamHI and KpnI restriction sites.

pNP115 – To construct pNP115, the vector for insertion of a P_{BAD}-regulated copy of *amiA* at the HK022 attachment site of *E. coli*, *amiA* was amplified from MG1655 gDNA using 5′-GTCA***GGATCC***GCCAAAGACGAACTTTTAAAAACCAGC-3′ and 5′-GATC***AAGCTT***ATTA***CTCGAG***TCGCTTTTTCGAATGTGCTTTCTGGTTG-3′ primers. The resulting PCR product was cloned into pDY75 via BamHI and HindIII restriction sites.

References for Supplemental Materials:

1. Oliver A, Baquero F, Blázquez J. The mismatch repair system (*mutS, mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol Microbiol.* 43(6): 1641-50. 2002.

2. Suh S-J, Silo-Suh L, Woods DE, Hassett DJ, West SHE, Ohman DE. Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa. J Bacteriol.* 181(13): 3890-7. 1999.

3. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. Common virulence factors for bacterial pathogenicity in plants and animals. *Science*. 268(5219): 1899-902. 1995.

4. Stover CK, *et al.* Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature.* 406(6799): 959-64. 2000.

5. Chuanchuen R, Murata T, Gotoh N, Schweizer HP. Substrate-dependent utilization of OprM or OpmH by the *Pseudomonas aeruginosa* MexJK efflux pump. *Antimicrob Agents Chemother.* 49(5): 2133-6. 2005.

6. Simon R, Priefer U, Pühler A. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Nature Biotechnol.* 1: 784-91. 1983.

7. Uehara T, Dinh T, Bernhardt TG. LytM-domain factors are required for daughter cell separation and rapid ampicillin-induced lysis in *Escherichia coli. J Bacteriol.* 191(16): 5094-107. 2009.

8. Uehara T, Parzych KR, Dinh T, Bernhardt TG. Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. *EMBO J.* 29(8): 1412-22. 2010.

9. Cherepanov PP and Wackernagel W. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene.* 158(1): 9-14. 1995.

10. Peters NT, Dinh T, Bernhardt TG. A fail-safe mechanism in the septal ring assembly pathway generated by the sequential recruitment of cell separation amidases. *J Bacteriol.* 193(18): 4973-83. 2011.

11. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ. ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa. Proc Natl Acad Sci USA.* 102(22): 8006-11. 2005.

12. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences:

application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene.* 212(1): 77-86. 1998.

13. Newman JR and Fuqua C. Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli araBAD* promoter and the *araC* regulator. *Gene.* 227(2): 197-203. 1999.

14. Datsenko KA and Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS.* 97(12): 6640-5. 2000.

15. Turner KH. Bistability in *Pseudomonas aeruginosa.* Dissertation, Harvard University. Ann Arbor: ProQuest/UMI. 2012.

16. Bernhardt TG and de Boer PA. SlmA, a nucleoid-associated, FtsZ binding protein required for blocking septal ring assembly over chromosomes in *E. coli. Mol Cell.* 18(5): 555-64. 2005.

17. Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP. A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods.* 2(6): 443-8. 2005.

18. Choi KH and Schweizer HP. mini-Tn7 insertion in bacteria with single *attTn7* sites: example *Pseudomonas aeruginosa. Nat Protoc.* 1(1): 153-61. 2006.

19. Baba T*, et al.* Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol.* 2:2006.0008. 2006.

20. Haldimann A and Wanner BL. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. *J Bacteriol.* 183(21): 6384-93. 2001.