Supplemental Information

Supplementary Materials and Methods

Chemicals and antibodies

Antibodies against PopB, PopD, PcrV, ExoS, ExoT, and maltose-binding protein (MBP) were generated in rabbits by commercial services (Covance). Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was purchased from Thermo Scientific. Anti-HA tag antibody (mouse origin) conjugated with horse-radish peroxidase was purchased form Roche Diagnostics. Anti-VSVG tag antibody (mouse origin) and valinomycin were purchased from Sigma-Aldrich. Anti-VSVG tag antibody (rabbit origin) was purchased from Bethyl Laboratories, Inc.. Mouse anti-RpoA monoclonal antibody was purchased from Neoclone. Potassium benzoate was purchased from Alfa Aesar. Amylose-resin was purchased from New England Biolabs, Inc. Protein A/G plus-agarose was purchased from Santa Crutz Biotechnology, Inc. Anti-Myc antibody (rabbit origin) was purchased from Novus Biologicals.

Strain and plasmid construction

All strains and plasmids used in this study are listed in Table S1 and Table S2, respectively. Mutations in the chromosome of *P. aeruginosa* were introduced by allelic exchange using plasmid pEXG2 (1). Plasmids were mated into *P. aeruginosa* using *E. coli* SM10 λ*pir* as a donor and cointegrates were isolated by selection on LB plates with gentamicin (30 μg/ml) and triclosan (5 μg/ml). Cointegrates were then restreaked on the same plate and an isolated colony was inoculated into 2ml of low salt LB (10g/l tryptone, 5 g/l yeast extract, 5 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$). Once the culture had reached early log (OD_{600} 0.1-0.3) 100 μl of the culture was plated on sucrose plates (10 g/l tryptone, 5 g/l yeast extract, 50 g/l sucrose) and incubated overnight at 30°C. Colonies were tested for the loss of gentamicin resistance, indicating loss of the allelic exchange vector via a second recombination event and the resultant gentamicin sensitive isolates were tested for retention of the mutation on the chromosome by PCR.
Plasmids were constructed using standard molecular biology techniques. PCR products were amplified using the primers listed in Table S3 using *Pfx*50 polymerase (Invitrogen) in the presence of 5% DMSO. Where needed, mutations were introduced by cross-over PCR (2).

Flow Cytometry

Bacteria were diluted 1:300 from overnight cultures into high salt LB-MC medium supplemented with 5mM EGTA as indicated. The bacterial cultures were grown at 37°C to mid-log phase. 1 ml of culture was removed to a microcentrifuge tube and the cells were pelleted by centrifugation at 4,000 rpm for 4 minutes, washed once in PBS-SMC (PBS with 0.1% saponin, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$) and resuspended in 500 μl of the same buffer. Cells were fixed by adding 500 μl 4% paraformaldehyde, mixed by inversion and incubated at room temperature for 20 minutes. The remaining crosslinker was quenched by the addition of 50 μl 1M Tris.Cl (pH 7.5) and the cell suspension was again mixed by inversion, incubated for 5 minutes at room temperature, and cells were pelleted at 13,000 rpm for 3 minutes, washed 1 × with PBS-SMC and 1 × with PBS-MC(containing Mg^{2+} and Ca^{2+}, as indicated for PBS-SMC but no saponin) and resuspended in PBS with 2% goat serum and 2% BSA. Blocking was performed for 30 minutes on ice at which point the bacteria were pelleted and washed twice with PBS-MC for 15 minutes on ice. 100 μl of primary antibody solution was added (anti-PcrV antibody, 1:100 dilution in blocking buffer) and the tubes were rocked for 1 hour at 4°C. Following primary antibody staining, cells were washed twice with PBS-MC for 15 minutes each on ice, followed by the addition of 100 μl of secondary antibody solution (goat-anti-rabbit-Alexa Fluor 647 conjugated antibody (Invitrogen), diluted 1:1000 in blocking buffer). The tubes were wrapped in aluminum foil and rocked at 4°C for 1 hour. The bacteria were then pelleted, washed twice with PBS-MC and resuspended in 50 μl of PBS. For each sample, 10 μl of the labeled bacteria were diluted into 1ml of PBS in a 5 ml polystyrene tube (BD Falcon) and analyzed by flow cytometry (Becton Dickinson SORP LSRFortessa, FacsDiva v6.2; 640nm coherent laser was used for excitation of Alexa Fluor 647 and emission was detected with a 670/30 bandpass filter) at the CFAR Immune Function Core Facility at Case Western Reserve University. Data were analyzed using FlowJo v7.6.4 software (Treestar, Ashland, OR). Several wash steps were tested to optimize removal of non-specifically adhered PcrV without permeabilizing the bacteria. To this end, strains PAO1F, PAO1F Δ*fleQ* Δ*exsE*, PAO1F Δ*fleQ* Δ*exsE* Δ*pscD*, as well as PAO1F Δ*pcrV2*, which had been pre-incubated with purified PcrV (50 ng/ml) for 10 minutes at room temperature, were stained using the procedure outlined above, with four different wash buffers

(instead of the PBS-SMC, which was used in the experiment depicted in Figure S2): PBS with 2% goat serum/BSA, PBS-MC with 0.1% Tritonx-100, PBS-MC with 0.1% saponin or PBS-MC with LPS (25 ug/ml). All strain/wash combinations were analyzed by flow cytometry to determine which condition resulted in the absence of non-specific staining in the PcrV-treated PAO1F Δ*pcrV2* sample (removal of non-specifically adhered PcrV) as well as lack of staining in the PAO1F Δ*fleQ* Δ*exsE* Δ*pscD* strain, which expresses high levels of PcrV, but does not secrete the protein (lack of permeabilization/stain is specific to surface-localized protein). Antibody titer: The primary anti-PcrV antibody was titered first. 8 titer points were tested while keeping the secondary antibody constant (1:250). Once the optimal primary antibody concentration was determined, the secondary antibody was titered (keeping the determined primary concentration constant (1:150).

ß-galactosidase activity assay – exoS reporter strains
Overnight cultures were diluted 1:300 into 3 ml of LB-MC medium. After 2 hours of incubation at 37 °C, 1 ml of culture was added to 1 ml of pre-warmed LB-MC medium (+ Ca^{2+} condition) and another 1 ml of culture was added to 1 ml of pre-warmed LB-MC medium with 10mM EGTA (5 mM final concentration, -Ca^{2+} condition). The cultures were then incubated for an additional 2 hours at 37 °C before being placed on ice for 10 minutes. Cells were permeabilized with chloroform and SDS and the ß-galactosidase activity was determined as described previously (3). Activities are reported as averages of three independent replicates ± standard deviation.

E. coli bacterial two-hybrid analysis
The λ cI/RNA polymerase α-subunit bacterial two-hybrid system was used to determine the interaction between PcrG and the T3SS components (4). The indicated T3SS components were fused to the C-terminus of the α subunit of RNA polymerase and PcrG was fused to the C-terminus of λ cI. Plasmids encoding the fusion proteins were transformed into the *E. coli* strain, BN469, which harbors a *lacZ* reporter gene controlled by a *lac* core promoter with adjacent λ operator site, allowing binding of the cI fusion protein. Interaction between the fusion partners leads to recruitment of RNA polymerase to the test promoter and activation of *lacZ* transcription. Overnight cultures were diluted 1:300 into 3 ml of LB medium. After 2 hours of incubation at 37 °C, 1 ml of culture was added into 1 ml of pre-warmed LB medium and another 1 ml of culture was added into 1 ml of pre-warmed LB medium with IPTG (the final concentration is noted in the figure legend). The cultures were incubated at 37 °C for an additional 2 hours. Then the cultures were put on ice for 10 minutes and *lacZ* expression was determined by β-galactosidase activity assay (3).

pscO and pcrD mutant library screens
pscO and *pcrD* ORFs were amplified by error-prone PCR with 1 mM dTTP, 1 mM dCTP, 0.1 mM dATP, 0.1 mM dGTP and *Taq* DNA polymerase (Choice *Taq* blue, Denville) and cloned into pPSV35 and pPSV39 expression vectors, respectively, which confer gentamicin resistance. The plasmids were transformed into DH5α to generate the mutant libraries, and the size of the libraries was estimated by plating a portion of the transformation mix on LB gentamicin plates. Religation was estimated by plating the empty vector control ligation transformation mix. The religation rates for *pscO* and *pcrD* libraries were 5% and 6%, respectively. The size of the *pscO* library was 78,000 transformants and the size of the *pcrD* library was 48,000 transformants. For isolating *pscO* mutants phenocopying the *pcrG*(Δ30-40,Δ60-70) mutant phenotype, plasmid DNA from the *pscO* mutant library was transformed into strain PAO1F Δ*pscO* Δ*pcrV* ΔexoS::GFP-*lacZ,* and the transformed bacteria were plated on LB-MC agar plates containing X-gal (10 g/l tryptone, 5 g/l yeast extract, 11.7 g/l NaCl, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 15 g/l agar, 100 μg/ml X-gal, 30 μg/ml gentamicin) (Figure S3A). *pscO* mutants phencopying the phenotype of *pcrG*(Δ30-40,Δ60-70) mutant formed dark blue colonies and were re-streaked on X-gal plates to confirm the dark blue phenotype. The plasmids were isolated, retransformed to confirm the mutant phenotype and sequenced to identify the mutations in the *pscO* ORF. For isolating *pcrD* mutants up-regulating effector secretion, plasmid DNA purified from the *pcrD* mutant library was transformed into strain PAO1F Δ*pcrD* Δ*exoS*::*tetR,* in which the chromosomal *exoS* gene was replaced by a tetracycline resistance gene. The transformed bacteria were plated on LB-MC agar plates containing tetracycline (10 g/l tryptone, 5 g/l yeast extract, 11.7 g/l NaCl, 10 mM $MgCl_2$, 0.5mM $CaCl_2$, 15g/l agar, 250μg/ml tetracycline). *pcrD* mutants activating effector secretion in

the presence of calcium expressed the tetracycline resistance gene and formed colonies on the LB-MC agar plates containing calcium and tetracycline (Figure S3B). Tetracycline resistant clones were re-streaked on tetracycline plates. The plasmid DNA of the tetracycline resistant mutants was purified and re-transformed into strain PAO1F Δ*pcrD* Δ*exoS*::GFP-*lacZ* to assay effector secretion control and confirm the mutant phenotype. The mutant plasmids were then sequenced to identify the mutations in the *pcrD* ORF. After the mutations in *pscO* and *pcrD* were identified, new expression plasmids with the mutations were artificially constructed using synthetic primers encoding the identified mutations, but using a different codon to confirm that the mutant phenotype is linked to the nature of the amino acid substitution, and transformed into *P. aeruginosa* to confirm the phenotype.

Measuring ATP levels

Overnight cultures of *P. aeruginosa* were diluted 1:300 into 6 ml of fresh LB-MC medium and incubated at 37 °C for 3 hours to mid-log phase. Then 4 ml of culture were divided in two microcentrifuge tubes and spun down, the supernatants were removed and one cell pellet was resuspended in 2 ml pre-warmed LB-MC medium without potassium benzoate, the other in 2 ml pre-warmed medium with 12.5 mM potassium benzoate. The cultures were incubated at 37 °C for an additional 30 minutes. Sample preparation for determination of ATP levels was based on the method described by Bakker and Mangerich (5). 500 µl of culture normalized to the same OD_{600} were mixed with 500 µl of cold 12% $HClO_4$ in 5 mM Na_2HPO_4 and incubated on ice for 30 minutes. The samples were then centrifuged at 5,000 rpm, 4°C for 10 minutes, and 500 µl of supernatant was mixed with 300 µl of cold neutralizing buffer (2 M KOH/0.3 M MOPS). The samples were frozen at -20°C, then thawed and centrifuged at 10,000 rpm, 4°C for 5 minutes. The ATP content in the supernatant was measured using a luciferase-based ATP determination kit (Molecular probes, Invitrogen detection technologies). The ATP contents were compared and presented as percentage of the ATP level in wild type *P. aeruginosa* strain without potassium benzoate treatment. The results presented in Figure S6 represent the average of at least three independent experiments ±SD.

Protein secretion assay

Overnight cultures were diluted 1:300 into 6 ml of fresh LB-MC medium and incubated at 37 °C for 3 hours (mid-log phase, OD_{600}~0.4). Then 4 ml of culture were divided in two microcentrifuge tubes, the bacteria were pelleted and supernatants removed. One cell pellet was resuspended in 2 ml pre-warmed LB-MC medium (+Ca^{2+}), the other in 2 ml pre-warmed LB-MC with 5mM EGTA (-Ca^{2+}). The cultures were then incubated at 37 °C for an additional 30 minutes and subsequently placed on ice for 10 minutes. Bacteria from 1 ml of each culture were pelleted and 0.5 ml of the supernatant was transferred to a fresh microcentrifuge tube, the remaining supernatant was discarded. Supernatant proteins were precipitated with trichloroacetic acid (TCA, 10% final conc.), washed with acetone and dried. Cell pellets and TCA precipitated supernatant samples were resuspended in 1x SDS sample buffer and normalized to an OD_{600} of 10. Proteins of interest were detected by Western blot using a horseradish peroxidase conjugated secondary antibody and a chemiluminescent detection system, either by exposing the blots to film or using a GE ImageQuant LAS4000 digital imaging system. Band intensities were quantitated using ImageJ software (6).Secretion efficiency was calculated as: 100*[supernatant protein]/[total protein]. For the secretion assays with PMF inhibitors (potassium benzoate and valinomycin+200 mM KCl), the inhibitors were added for the 30-minute incubation after pelleting the bacteria and resuspending them in LB-MC.

Site-specific cysteine crosslinking

Overnight cultures were diluted 1:100 into 200 ml of fresh LB-MC medium and incubated at 37 °C for 3 hours (OD_{600}~0.5-0.6). After normalizing the OD_{600} of the cultures, the bacteria were pelleted by centrifugation and resuspended in the crosslinking buffer [1x PBS (1.06 mM KH_2PO_4, 155.17 mM NaCl, 2.97 mM Na_2HPO_4, pH 7.4), 1 mM PMSF and 0.5 mM Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)], both freshly prepared. For cultures grown in the presence of calcium, the crosslinking buffer was supplemented with 0.5 mM $CaCl_2$. The cell suspensions were incubated at room temperature in dark for 30 minutes. The bacteria were then pelleted, washed once in MBP-purification buffer [20 mM Tris pH 7.5, 100 mM NaCl, 10mM ß-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40] and resuspended in 1 ml MBP-purification buffer before being lysed by sonication.

Amylose resin purification
Crosslinked cell suspensions in MBP purification buffer were sonicated 4 times with 30 second interval at power level 3.5 with Misonix sonicator 3000. The cell lysate was centrifuged at 13,200 rpm 4°C for 10 minutes to remove cell debris. 20 μl of cell lysate were removed to a fresh tube and mixed with 20 μl of 4x SDS sample buffer and 40 μl of water as input control. 900 μl of cell lysate were transferred to a fresh tube and mixed with amylose-resins washed 3 times with MBP purification buffer. The mixtures were incubated at 4 °C on a rocker for 1 hour. Then the amylose-resin was pelleted by centrifugation and washed 3 times with MBP purification buffer. Bound proteins were eluted with two 450 μl washes of elution buffer (20mM Tris pH 7.5, 200 mM NaCl, 10 mM ß-mercaptoethanol, 1 mM EDTA, 1 mM PMSF, 0.5% NP-40, 10 mM maltose). The combined elution samples were precipitated with 100 μl of 100% TCA (final conc. 10%). Precipitated proteins were resuspended in 40 μl of 1x SDS sample buffer.

Immunoprecipitation
P. aeruginosa cell suspensions in MBP purification buffer were sonicated 4 times with 30 second interval at power level 3.5 with Misonix sonicator 3000. The cell lysate was centrifuged 13,200 rpm 4°C for 10 minutes to remove cell debris. 20 μl of cell lysate were removed to a fresh tube and mixed with 20 μl of 4 xSDS sample buffer and 40 μl of water as input control. 900 μl of cell lysate were transferred to a fresh tube and pre-cleared for 15 minutes at 4°C by incubating with protein A/G agarose beads washed with MBP purification buffer. The beads were removed by centrifugation at 8,000 rpm for 2 minutes and the supernatant was transferred to a new tube. α-VSV-G antibodies were added to each tube and the supernatants were incubated at 4°C on a rocker for 15 minutes. Then protein A/G agarose beads, washed with MBP purification buffer, were added to each tube and the mixtures were rocked for an additional 45 minutes at 4°C. The beads were then pelleted, washed 3 times with MBP purification buffer and resuspended in 40µl 1x SDS sample buffer. The samples were incubated at 55°C for 10 minutes to elute proteins bound to the beads, then the samples were vortexed, centrifuged, and supernatants were collected as elution fraction.

Protein overexpression and purification by Ni-NTA chromatography
His-tagged versions of *pcrG*, *pcrG*(Δ30-40,Δ60-70) and *pcrG*(Δ71-95), as well as a Myc-tagged version of *pscO* were overexpressed in *E.coli* BL21(DE3) Codon+ RP- (Stratagene). The bacteria were grown overnight in 2xYT medium with 30 μg/ml carbenicillin and subsequently diluted 1:200 into fresh 2xYT medium [50 ml of the His-pcrG cultures and 200 ml for the *pscO-myc* culture) and grown at 37°C for 2.5 hours. At this point, protein production was induced for one hour by adding IPTG to the culture [100 μM, final concentration]. The cells were pelleted and resuspended in binding buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 10 mM imidazole, 1 mM PMSF) and normalized to a final OD_{600} of 25. Resuspended cells were lysed by sonication and centrifuged at 13,200 rpm for 10 min. 20 μl of supernatant were taken and mixed with 17.5 μl of H_2O and 12.5 μl of 4x SDS sample buffer to make the 50 μl of input control samples. 900 μl of supernatant from each His-tagged PcrG lysates were mixed with 900 μl of supernatant from PscO-Myc lysate and incubated on a rocker at 4°C for 90 minutes. Mixtures of His-tagged PcrGs and PscO-Myc lysates were then incubated with Ni-NTA beads (Qiagen) on a rocker at 4°C for 1 hour to allow binding of PcrG to the nickel resin. The beads were washed with binding buffer and bound proteins were eluted with two 540μl aliquots of elution buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl, 500 mM imidazole, 1 mM PMSF). Eluted proteins were precipitated with 120 μl of 100% TCA (10% final concentration), pelleted and washed with acetone before being resuspended in 50 μl 1x SDS sample buffer. 15 μL of each sample were separated by SDS-PAGE and proteins were detected by coomassie stain or western blot.

Intracellular pH measurement
Strains PAO1F Δ*exsE ΔfleQ*, PAO1F *ΔexsE ΔfleQ pscO*(E88K) and PAO1F Δ*exsE* Δ*fleQ pcrG*(Δ30-40,Δ60-70) were transformed with plasmid pP40-pHluorin, which encodes a pH-sensitive, ratiometric GFP derivative (7). The bacteria were cultured overnight in LB-MC with 30 μg/ml gentamicin, diluted 1:200 diluted into fresh LB-MC with 30 μg/ml gentamicin and grown to mid-logarithmic phase. The bacteria were then pelleted and resuspended in 200 mM sodium phosphate buffer (pH 7) with different concentrations of potassium benzoate or valinomycin and incubated for 30 minutes before measuring the fluorescence emission of pHluorin (530 nm emission) excited at either 410 or 470 nm using a SpectraMax M2

fluorescence spectrophotometer (Molecular Devices). A standard curve was established by resuspending bacteria in 200 mM sodium phosphate buffer (pH 6, 7 and 8) in the presence of 40 mM potassium benzoate, and used to calculate the intracellular pH of the inhibitor-treated samples. Data is presented as ΔpH compared to the external pH of the medium (pH 7).

Change of membrane potential

The change in membrane potential of inhibitor-treated *P. aeruginosa* was detected using the fluorescent dye DiSC3(5) (AnaSpec, Inc.) based on the method described by Zhang et al. (8). The bacteria were cultured overnight in LB-MC, then diluted 1:200 in fresh LB-MC and grown to mid-logarithmic phase. Cells were then pelleted, washed and resuspended in 5 mM HEPES buffer, pH 7.7, to an OD_{600} of 0.1. DiSC3(5) dye (final conc. 0.4 mM) was added to the cells and the suspension was incubated at room temperature for 20 minutes, at which point 100 mM KCl (final concentration) was added. The cells were then treated with potassium benzoate or valinomycin and incubated for an additional 30 minutes. Disruption of membrane potential resulted in enhanced DiSC3(5) fluorescence, which was detected using a SpectraMax M2 fluorescence spectrophotometer (Molecular Devices, excitation 622 nm, emission 670 nm). Data is presented as fold increase in fluorescence intensity relative to wild-type bacteria without inhibitor treatment.

Protein sequence alignments

Protein sequences were aligned using the MacVector (MacVector, Inc.) software package and the ClustalW algorithm (9). Subdomains of PcrD were assigned according to the published domain structure of the *S. flexneri* homolog MxiA (10). FliJ residues involved in mediating the interaction with FlhA, identified by Ibuki et al. (11), were mapped onto the published crystal structure of FliJ (12). GenBank accession numbers for aligned proteins:

PcrG(AAG05094.1), LcrG(AAA27644.1), AcrG(AAS91818.1)

PscO(AAG05085.1), YscO(AAA27675.1), AscO(AAS91809.1), PA FliJ(AAG04494.1), ST FliJ(AAL20885.1), EC FliJ(AAC75009.1), YPTB FliJ(ACA68672.1), SsaO(AAL20340.1), SpaM(AAA83430.1), Spa13(CAC05825.1) corrected based on (13, 14), HrpO(ABQ88355.1)

PcrD(AAG05092.1), LcrD(AAA27647.1), MxiA(AAK18466.1), InvA(AAA16867.1), EscV(EKJ11194.1), SsaV(AAL20338.1), HrpI(AAA03015.1), PA FlhA(AAG04841.1), ST FlhA(AAL20829.1)

Supplementary References

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Table S1

Strain #	genotype	reference
E. coli BN469	two-hybrid analysis strain	(15)
BL21 (DE3) CodonPlus "RP"	*E. coli* B F– *ompT hsdS*(rB– mB–) *dcm*+ Tetr *gal* l (DE3) *endA* Hte [*argU proL* Camr]	Stratagene
RP1831	PAO1F, wild type *P. aeruginosa* PAO1	(16)
RP3082	PAO1F Δ*pcrG2* Δ*exoS*::GFP-*lacZ*	(17)
RP4990	PAO1F Δ*exsE* Δ*fleQ* Δ*pcrG2*	This study
RP2645	PAO1F Δ*pcrV2* Δ*exoS*::GFP-*lacZ*	(17)
RP3335	PAO1F Δ*pcrG2* Δ*pcrV2* Δ*exoS*::GFP-*lacZ*	(17)
RP5930	PAO1F Δ*pcrG2 pcrV(F279R)* Δ*exoS*::GFP-*lacZ*	This study
RP2401	PAO1F Δ*pscO* Δ*exoS*::GFP-*lacZ*	This study
RP6683	PAO1F Δ*pscO* Δ*pcrV2* Δ*exoS*::GFP-*lacZ*	This study
RP3125	PAO1F Δ*exsE* Δ*fleQ* Δ*pscO*	This study
RP2929	PAO1F Δ*exsE* Δ*fleQ*	This study
RP8668	PAO1F Δ*exsE* Δ*fleQ pcrG(Δ30-40,Δ60-70)*	This study
RP7238	PAO1F Δ*exsE* Δ*fleQ pscO(E88K)*	This study
RP4564	PAO1F Δ*exsE* Δ*pcrG2*	This study
RP5835	PAO1F Δ*exsE* Δ*pcrG2 pcrD*-VSV-G	This study
RP5861	PAO1F Δ*exsE* Δ*pcrGV2 pcrD*-VSV-G	This study
RP5864	PAO1F Δ*exsE* Δ*pcrG2* Δ*pcr1 pcrD*-VSV-G	This study
RP8277	PAO1F Δ*exsE* Δ*pscO*	This study
RP8276	PAO1F Δ*exsE* Δ*pscO pcrD*-VSV-G	This study
RP8347	PAO1F Δ*exsE* Δ*pscO* Δ*pcrG2 pcrD*-VSV-G	This study
RP8345	PAO1F Δ*exsE* Δ*pscO* Δ*pcr1 pcrD*-VSV-G	This study
RP1868	PAO1F Δ*exoS*::GFP-*lacZ*	This study
RP8012	PAO1F *pcrD(Q626R)* Δ*exos*::GFP-*lacZ*	This study
RP8055	PAO1F *pcrD(M667T)* Δ*exoS*::GFP-*lacZ*	This study
RP8018	PAO1F *pcrD(Y587H)* Δ*exoS*::GFP-*lacZ*	This study
RP7625	PAO1F Δ*exsE pcrD*-VSV-G *pcr1*-HA	This study
RP7789	PAO1F Δ*exsE pcr1*-HA	This study
RP7650	PAO1F Δ*exsE* Δ*pcrGV2 pcrD*-VSV-G *pcr1*-HA	This study
RP7652	PAO1F Δ*exsE* Δ*pscB pcrD*-VSV-G *pcr1*-HA	This study
RP8006	PAO1F Δ*exsE pcrD(Q626R)*-VSV-G *pcr1*-HA	This study
RP2908	PAO1F Δ*exsE* Δ*fleQ* Δ*pscD*	This study
RP1865	PAO1F Δ*fleQ*	(18)
RP2422	PAO1F Δ*pcrD* Δ*exoS*::GFP-*lacZ*	This study
RP8832	PAO1F *pcrD*-VSV-G Δ*exoS*::GFP-*lacZ*	This study
RP7411	PAO1F *pcr1*-HA Δ*exoS*::GFP-*lacZ*	This study
RP6914	PAO1F ΔpcrG2 Δ*pscO* Δ*exoS*::GFP-*lacZ*	This study
RP3170	PAO1F Δ*popN* Δ*exoS*::GFP-*lacZ*	This study
RP2360	PAO1F Δ*pcr1* Δ*exoS*::GFP-*lacZ*	This study
RP2361	PAO1F Δ*pcr2* Δ*exoS*::GFP-*lacZ*	This study
RP2357	PAO1F Δ*pscB* Δ*exoS*::GFP-*lacZ*	This study

Table S2

Plasmid	Relevant features	reference
pPSV35	*colE1* origin, with gentamicin resistance gene (*gentR*), PA origin, *lacIq*, and the *lac*UV5 promoter and MCS of pUC18	(1)
pPSV37	*colE1* origin, gentR, PA origin, *oriT*, *lac*UV5 promoter, *lacIq*, stops in every reading frame preceding the MCS and T7 terminator following the MCS (relative to the *lac*UV5 promoter)	(17)
pPSV40	*colE1* origin, with gentamicin resistance gene (*gentR*), PA origin, multiple cloning site and no promoter	(1)
pEXG2	Allelic exchange vector, *colE1* origin, *oriT*, gentamycin resistance,*sacB*	(1)
pACλCI35	Two-hybrid plasmid encoding a λCI DNA binding protein, chloramphenicol resistance	(4)
pBRαLN	Two-hybrid plasmid encoding a α-subunit of RNA polymerase, carbenicillin resistance	(4)
pBRα35	Two-hybrid plasmid encoding a α-subunit of RNA polymerase, carbenicillin resistance	(4)
pMal	pPSV37 encoding a signal-sequenceless *malE* gene (codons 27-396) lacking a stop codon followed by a polylinker to create MBP fusions	(17)
pETDuet1	Co-expression vector with T7 origin, *bla* resistance	Novagen
pMal-*pcrG*	plasmid encoding an MBP-PcrG fusion protein under control of a *lac*UV5 promoter	(17)
pMal-*pcrG(A16R)*	plasmid encoding an MBP-PcrG(A16R) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(60-95)*	plasmid encoding an MBP-PcrG(a.a. 60-95) fusion protein under control of a *lac*UV5 promoter, referred to as PcrG(Δ1-59) in text	This study
pMal-*pcrG(2-70)*	plasmid encoding an MBP-PcrG(a.a. 2-70) fusion protein under control of a *lac*UV5 promoter, referred to as PcrG(Δ71-95) in text	This study
pMal-*pcrG(Δ30-40;Δ60-70)*	plasmid encoding an MBP-PcrG(Δ30-40;Δ60-70) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(P84C)*	plasmid encoding an MBP-PcrG(P84C) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(R85E; R86E)*	plasmid encoding an MBP-PcrG(R85E; R86E) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(P84C, R85E, R86E)*	plasmid encoding an MBP-PcrG(P84C, R85E, R86E) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(Δ30-40;Δ60-70; R85E; R86E)*	plasmid encoding an MBP-PcrG(Δ30-40;Δ60-70; R85E; R86E) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(M90A; R91A; G92A)*	plasmid encoding an MBP-PcrG(M90A; R91A; G92A) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(I95E)*	plasmid encoding an MBP-PcrG(I95E) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(P84C; I95E)*	plasmid encoding an MBP-PcrG(P84C; I95E) fusion protein under control of a *lac*UV5 promoter	This study
pMal-*pcrG(L64C)*	plasmid encoding an MBP-PcrG(L64C) fusion protein under control of a *lac*UV5 promoter	This study
pBRαLN-*pcrV*	Two-hybrid plasmid encoding a α-PcrV fusion protein	This study
pBRαLN-*pscO*	Two-hybrid plasmid encoding a α-PscO fusion protein	This study
pBRαLN-*pscO(G78E)*	Two-hybrid plasmid encoding a α-PscO(G78E) fusion protein	This study
pBRαLN-*pscO(E88K)*	Two-hybrid plasmid encoding a α-PscO(E88K) fusion protein	This study
pBRαLN-*pscO(A92T)*	Two-hybrid plasmid encoding a α-PscO(A92T) fusion protein	This study
pBRαLN-*pcr1*	Two-hybrid plasmid encoding a α-Pcr1 fusion protein	This study
pBRαLN-*pscK*	Two-hybrid plasmid encoding a α-PscK fusion protein	This study
pBRαLN-*pscL*	Two-hybrid plasmid encoding a α-PscL fusion protein	This study
pBRα35-*pscN*	Two-hybrid plasmid encoding a α-PscN fusion protein	This study
pBRαLN-*pscQ*	Two-hybrid plasmid encoding a α-PscQ fusion protein	This study

pBRα35-*pcrDc*	Two-hybrid plasmid encoding a α-PcrDc (aa 304-706) fusion protein	This study
pBRα35-*pscUc*	Two-hybrid plasmid encoding a α-PscUc (aa 206-349) fusion protein	This study
pACλCI35-*pcrG*	Two-hybrid plasmid encoding a λCI-PcrG fusion protein	This study
pACλCI35-*pcrG(Δ30-40)*	Two-hybrid plasmid encoding a λCI-PcrG(Δ30-40) fusion protein	This study
pACλCI35-*pcrG(Δ60-70)*	Two-hybrid plasmid encoding a λCI-PcrG(Δ60-70) fusion protein	This study
pACλCI35-*pcrG(Δ30-40,Δ60-70)*	Two-hybrid plasmid encoding a λCI-PcrG(Δ30-40,Δ60-70)fusion protein	This study
pEXG2-*pcrV(F279R)*	allelic exchange vector designed to introduce the F279→R mutation into *pcrV*	(17)
pEXG2-ΔpcrGV2	allelic exchange vector designed to delete *pcrG* and *pcrV* starting at codon 6 of *pcrG* and ending at codon 180 of *pcrV*	(17)
pEXG2-Δ*exoS*::GFP-*lacZ*	allelic exchange vector which deletes *exoS* and inserts translationally coupled versions of GFP and *lacZ* in its place	(1)
pEXG2-Δ*pcrG*	allelic exchange vector designed to delete codons 6-88 of *pcrG*	(17)
pEXG2-*Δpcr*V	allelic exchange vector designed to delete codons 44-180 of *pcrV*	(17)
pEXG2-*pcrD*-VSV-G	allelic exchange vector designed to replace the chromosomal copy of *pcrD* tagged with 2 tandem repeats of the VSV-G tag (YTDIEMNRLGK) at the C-terminus of PcrD	This study
pEXG2-*pcr1*-HA	allelic exchange vector designed to replace the chromosomal copy of *pcr1* tagged with 2 tandem repeats of the HA tag (YPYDVPDYA) at the C-terminus of Pcr1	This study
pEXG2- Δ*popN*	allelic exchange vector designed to delete codons 3-271 of *popN*	This study
pEXG2-Δ*pcr1*	allelic exchange vector designed to delete codons 10-81 of *pcr1*	(18)
pEXG2- Δ*pcr2*	allelic exchange vector designed to delete codons 6-116 of *pcr2*	This study
pEXG2-Δ*pscB*	allelic exchange vector designed to delete codons 4-133 of *pscB*	This study
pEXG2-Δ*exsE*	allelic exchange vector designed to delete codons 3-80 of *exsE*	(1)
pEXG2-Δ*pscO*	allelic exchange vector designed to delete codons 7-148 of *pscO*	This study
pEXG2-*pcrD(Q626R)*	allelic exchange vector designed to introduce the Q626→R mutation into *pcrD*	This study
pEXG2-*pcrD(M667T)*	allelic exchange vector designed to introduce the M667→R mutation into *pcrD*	This study
pEXG2-*pcrD(Y587H)*	allelic exchange vector designed to introduce the Y587→H mutation into *pcrD*	This study
pEXG2-*popB*(D-CTES)	allelic exchange vector designed to replace the last 25 codons of *popB* with those of *popD* (replacing the C-terminal Translocator Export Signal)	This study
pPSV35-*pscO*	plasmid encoding *pscO* under control of a *lac*UV5 promoter in pPSV35	This study
pPSV35-*pscO(G78E)*	plasmid encoding *pscO(G78E)* under control of a *lac*UV5 promoter in pPSV35	This study
pPSV35-*pscO(E88K)*	plasmid encoding *pscO(E88K)* under control of a *lac*UV5 promoter in pPSV35	This study
pPSV35-*pscO(A92T)*	plasmid encoding *pscO(A92T)* under control of a *lac*UV5 promoter in pPSV35	This study
pPSV35-*pscO*-myc	plasmid encoding *pscO*-myc under control of a *lac*UV5 promoter in pPSV35	This study
pPSV35-*pscO(E88K)*-myc	plasmid encoding *pscO(E88K)*-myc under control of a *lac*UV5 promoter in pPSV35	This study
pEXG2-*pscO(E88K)*	allelic exchange vector designed to introduce the E88→K mutation into *pscO*	This study
pEXG2-*pcrG(Δ30-40, Δ60-70)*	allelic exchange vector designed to delete codons 30-40 and 60-70 in *pcrG*	This study
pEXG2-Δ*pcrD*	allelic exchange vector designed to delete codons 36-679 in *pcrD*	This study
pEXG2-Δ*pscD*	allelic exchange vector designed to delete codons 34-410 in *pscD*	This study
pP40-pHluorin	plasmid for constitutive expression of pHluorin (ratiometric GFP) under control of the lacUV5 promoter lacking the lacO operator sequence	This study
pETDuet1-*pcrG*	plasmid encoding gene to produce an N-terminally His-tagged PcrG under	(17)

	control of a T7 promoter	
pETDuet1-*pcrG*(Δ71-95)	plasmid encoding gene to produce an N-terminally His-tagged PcrG(Δ71-95) under control of a T7 promoter	This study
pETDuet1-*pcrG*(Δ30-40, Δ60-70)	plasmid encoding gene to produce an N-terminally His-tagged PcrG(Δ30-40, Δ60-70) under control of a T7 promoter	This study
pETDuet1-pscO-2xmyc	plasmid encoding gene to produce an C-terminally Myc-tagged PscO under control of a T7 promoter	This study

Table S3

Primer name	Sequence (5' to 3')	description
GfcI-5SsP	AAAAAaatattGAACGAATACACCGAAGACACCCT	5' and 3' primers to make cIλ-PcrG fusion protein
GfcI-3Asc	AAAAAggcgcgccTCAGATCAACAAGCCACGCATCGGCGT	
AlpO-5Not	ATATAgcggccgcaAGCCTGGCTCTGCTGTTGCGCGT	5' and 3' primers to make αLN-PscO fusion protein
AlpO-3Bam	TATATggatccTCAGCTTGAGCATGGCCAGGT	
AlpK-5Not	ATATAgcggccgcaCCATTGACGGCCTACCAGTTGCGCTT	5' and 3' primers to make αLN-PscK fusion protein
AlpK-3Bam	TATTTggatccCTAGTTCAACAAATGGAAGCAT	
AlpL-5Not	ATATAgcggccgcaCTTCCATTTGTTGAACTAGA	5' and 3' primers to make αLN-PscL fusion protein
AlpL-3Bam	TATATggatccTCAACCGGCGTCCCCTTCCTCCT	
AlpN-5RV	ATATAgatatcaCCCGCGCCTCTCTCTCCTCTCA	5' and 3' primers to make α35-PscN fusion protein
AlpN-3Asc	TATATggcgcgccTCATGCGCAGAGGCTCCGCAACT	
AlpQ-5Not	ATATAgcggccgcaAACGGTGCGGACCTCGACCT	5' and 3' primers to make αLN-PscQ fusion protein
AlpQ-3Bam	TATATggatccTCATGCTTCGTCGTGTCCGA	
pcrD-C5Not	AAAAAgcggccgcaACCTTCCTGGCTCTCGCGCTGCT	5' and 3' primers to make α35-PcrDc(aa304-706) fusion protein
pcrD-3Asc	AAAAAggcgcgccTCACAACACGATCCTGCCAAGCGGCT	
pscU-C5Not	AAAAAgcggccgcaGAACGCCACCAGCACTACAAGCAGTT	5' and 3' primers to make α35-PscUc(aa206-349) fusion protein
pscU-3Asc	AAAAAggcgcgccTCAGGGCGTATCCGTCTGCTGGCTTT	
VAlp-5Not	AAAAAgcggccgcCGAAGTCAGAAACCTTAATGCCGCTCG CGA	5' and 3' primers to make αLN-PcrV fusion protein
VAlp-3Bam	AAAAAggatccCGGCTGGTTCATGGATACCTCTA	
GM2-70-5	AAAAAggtaccAACGAATACACCGAAGACACCCT	5' and 3' primers to make MBP-PcrG(2-70) fusion protein
GM2-70-3	AAAAAaagcttTCACATGCGCCGCAGTTCGGCCA	
GM60-95-5	AAAAAggtaccGAAGAGGAGCTGCTGGCCGAA	5' Primer to make MBP-PcrG(60-95) with pcrG3H
MBP-pcrG2-5B	AAAAAGGATCCAACGAATACACCGAAGACACCCT	5' primer to make MBP-PcrG fusion protein with pcrG3H
pcrG3H	AAAAAaagcttTTCCTCAGATCAACAAGCCACGCA	3' primer to make MBP-PcrG fusion protein
PcrG-P84C3H	AAAAAAAGCTTTCAGATCAACAAGCCACGCATCGGCGT CGGACGCCGACACCGGGTACCCTGCTCGCCCT	3' primer to make MBP-PcrG(P84C) with MBP-pcrG2-5B
pcrG(PRR84CEE)-3H	AAAAAAAGCTTCAGATCAACAAGCCACGCATCGGCGTC GGCTCTTCACACCGGGTACCCTGCTCGCCCT	3' primer for PcrG (P84C, R85E, R86E)
Gd30-40-5-2	CAGCAGCTCGCCGGCGTCCGCCAGGCGGCCGCGTTCC TCGCTG	Internal primers for deleting PcrG a.a. 30-40
Gd30-40-3-1	CAGCGAGGAACGCGGCCGCCTGGCGGACGCCGGCGA GCTGCTG	
pcrGd60-70-5-2	CTGCGTCGGCTGGGAACTGCGGGCGGCTCGCGCCAGC TCTCG	Internal primers for deleting PcrG a.a. 60-70
pcrGd60-70-3-1	CGAGAGCTGGCGCGAGCCGCCCGCAGTTCCCAGCCGA CGCAG	
PcrG(MRG90AAA)	AAAAAaagcttTCAGATCAACAATGCGGCAGCCGGCGTCG GACGCCGCGGCCGGGT	Primer to introduce M90A, R91A, G92A mutations in *pcrG*
PcrG(I95E)	AAAAAaagcttTCATTCCAACAAGCCACGCATCGGCGTCG G	Primer to introduce I95E mutation in *pcrG*
pcrDCVG2-5-2	CTCGATGTCCGTGTACACTTTTCCTAATCTATTCATTTCA ATATCTGTATACAACACGATCCTGCCAAGCGGCTG	Primers to make 5' flank for introducing 2xVSV-G tag to the C-terminus of PcrD
pcrR-5-1	AAAAAgaattcAAGGACGTGGTGCAGCTCACCGAGT	

pcrDCVG2-3-1 pcrR3H	ATGAATAGATTAGGAAAAGTGTACACGGACATCGAGAT GAACAGGTTGGGCAAATGAGCGCCGATCCGCTGATTCC CTG AAAAAaagcttCGTTGCCGGAGCCTGTCAGGCACGGT	Primers to make 3' flank for introducing 2xVSV-G tag to the C-terminus of PcrD
pscO5X pscO3H	AAAAAtctagaGGAGGTCTGCGCATGAGCCTGGCTCTGCT GTT AAAAAaagcttTCAGCTTGAGCATGGCCAGGTCT	5' and 3' primers to clone *pscO*
PscO-G78E-5-1 PscO-G78E-3-2	AGGCCGGCTTCCTTTTCCCGCAGCAGTTCTACCTGCTG CTGCCAGGCTTCCA TGGAAGCCTGGCAGCAGCAGGTAGAACTGCTGCGGGA AAAGGAAGCCGGCCT	Paired with pscO5X to make 5' flank for introducing G78→E mutation in *pscO* Paired with pscO3H to make 3' flank for introducing G78→E mutation in *pscO*
PscO-E88K-5-1	TGCGCGGCCTCGGCGCAGTCCTGCTTCAGGCCGGCTT CCTTTTCCCGCA	Paired with pscO5X to make 5' flank for introducing E88→K mutation in *pscO*
PscO-E88K-3-2	TGCGGGAAAAGGAAGCCGGCCTGAAGCAGGACTGCGC CGAGGCCGCGCA	Paired with pscO3H to make 3' flank for introducing E88→K mutation in *pscO*
PscO-A92T-5-1	TTCGAGGCGCTGCGCGGCCTCTGTGCAGTCCTGTTCCA GGCCGGCTT	Paired with pscO5X to make 5' flank for introducing A92→T mutation in *pscO*
PscO-A92T-3-2	AAGCCGGCCTGGAACAGGACTGCACAGAGGCCGCGCA GCGCCTCGAA	Paired with pscO3H to make 3' flank for introducing A92→T mutation in *pscO*
Pcr1-5R	AAAAAgaattcGGAGGCGGGGCCATGGCATACGGGCCTT CT	5' primer to clone *pcr1*
Pcr1-3H	AAAAAaagcttTCAACCCAGTCCATGCTGCTGCTCCT	3' primer to clone *pcr1*
Pcr1-HA-5-2	TGGTACATCATATGGATAAGCGTAATCTGGAACATCGTA TGGGTAACCCAGTCCATGCTGCTGCTCCTCT	Paired with pcr1-5R to make 5' flank for introducing 2xHA tag to the C-terminus of Pcr1
Pcr2-5R Pcr2-3H	AAAAAgaattcGGAGGCAGCAGCATGGACTGGGTTGAGCT GGCCGT AAAAAaagcttTCATGCGGCGAGCACTTCGCT	5' and 3' primers to clone *pcr2*
Pcr1-HA-3-1	TTCCAGATTACGCTTATCCATATGATGTACCAGACTACG CATGATGGACTGGGTTGAGCTGGCCGTC	Paired with pcr2-3H for 3' flank introducing a 2xHA tag at the Pcr1 C-terminus
1699-5-1 1699-5-2	AAAAAgaattcATGCTGGCGCTGGTCGACCAGGCGT AACTCGAGCCGCAAGCATGCTGAAGGTCAATTCAGAAG GCCCGTATGCCA	Primers to make 5' flank for deleting *pcr1*
1699-3-1 1699-3-2	TTCAGCATGCTTGCGGCTCGAGTTGAGCGCGAAGAGGA GCAGCAGCATGGA AAAAAaagcttCCGACCCGGCTCATGCGGCGAGCACT	Primers to make 3' flank for deleting *pcr1*
pcrD5-1 pcrD5-2	AAAAAtctagaGCGCTGCGCGATCTCCGCCTGGCCCTCA AACTCGAGCCGCAAGCATGCTGAATGGCAGCGGCAGC ACCATCATGAACA	Primers to make 5' flank for deleting *pcrD*
pcrD3-1	TTCAGCATGCTTGCGGCTCGAGTTACTATGCCGGGCTG CCGGTGCTGTCCTA	Paired with pcrR3H to make 3' flank for deleting *pcrD*
pcrDC5X	AAAAAtctagagACCTTCCTGGCTCTCGCGCTGCT	5' primer to amplify the cytoplasmic domain of PcrD

PcrD3H	AAAAAaagcttTCACAACACGATCCTGCCAAGCGGCT	3' primer to clone *pcrD*
PcrD(Y587H)-5-2	GTAGCGCTTGAGGCTGCTGCGGATATGCTCGGTGAGCT GCACCACGTCCTT	Internal primers to introduce Y587→H mutation in *pcrD*
PcrD(Y587H)-3-1	AAGGACGTGGTGCAGCTCACCGAGCATATCCGCAGCAG CCTCAAGCGCTAC	
PcrD(Q626R)-5-2	AGGGCCAGGTAGCTGCCGGCGCTGGTTCGGCGGATGC CACCGCGGATCTGCT	Internal primers to introduce Q626→R mutation in *pcrD*
PcrD(Q626R)-3-1	AGCAGATCCGCGGTGGCATCCGCCGAACCAGCGCCGG CAGCTACCTGGCCCT	
PcrD(M667T)-5-2	TTGCGCACGTAGCGGCGGATATCGGTGGAAACGATCAG CACCGGCCGGT	Internal primers to introduce M667→T mutation in *pcrD*
PcrD(M667T)-3-1	ACCGGCCGGTGCTGATCGTTTCCACCGATATCCGCCGC TACGTGCGCAA	
pscO-5-2	AAAAAaagcttTGATGGAGCGTGCCGGGCAAT	Primers to make 3' flank for deleting *pscO*
pscO-5-1	TTCAGCATGCTTGCGGCTCGAGTTCAGCAGAGCCAGGC TCATGCGCAGA	
pscO-3-2	AACTCGAGCCGCAAGCATGCTGAAACCCGCCACGAGAC CTGGCCAT	Primers to make 5' flank for deleting *pscO*
pscO-3-1	AAAAAgaattcCGGAGACCGCCGGCGCTTGCCGTGCTA	
PscO-2xmyc-1	AGTTTCTGCTCGCCCAAATCTTCTTCAGAAATCAACTTTT GTTCGCTTGAGCATGGCCAGGTCTCGTG	Internal primers to put 2xMyc tag to the C-terminus of PscO
PscO-2xmyc-2	AAGATTTGGGCGAGCAGAAACTGATCTCGGAGGAGGAC CTGTGATGCTCAAGCTGAACGCCGTGG	
pcrGA16R-3-1	TGCGGGCGACCGTCCAGGCCcgaGAACTGGCGATTCGC GA	Paired with pcrG3H to make 3' flank for introducing A16R mutation in *pcrG*
pcrGA16R-5-2	TCGCGAATCGCCAGTTCTCGGGCCTGGACGGTCGCCC GCA	Paired with pcrG-5-1 to make 5' flank for introducing A16R mutation in *pcrG*
PscB-5R	AAAAAgaattcGGAGGCTGAATCATGGATCATCTGTT	5' primer to clone *pscB*
PscB-3H	AAAAAaagcttTCAGGGACGCCACACCGGAGCCT	3' primer to clone *pscB*
pscB5-1	AAAAAtctagaCTGCGAGTCAAGTCCGAATTCTGGT	Primers to make 5' flank for deleting *pscB*
pscB5-2	AACTCGAGCCGCAAGCATGCTGAAATGATCCATGATTC CTCGGTA	
pscB3-1	TTCAGCATGCTTGCGGCTCGAGTTCAGGCTCCGGTGTG GCGTCCCTGAT	Primers to make 3' flank for deleting *pscB*
pscB3-2	AAAAAaagcttCGGAAGGGTCGGCACGCCAGCCGAA	
1700-5-1	AAAAAgaattcGAGCGCCGACCTGGACAGTCAGT	Primers to make 5' flank for deleting *pcr2*
1700-5-2	AACTCGAGCCGCAAGCATGCTGAACTCAACCCAGTCCA TGCTGCTGCTCCT	
1700-3-1	TTCAGCATGCTTGCGGCTCGAGTTCGCAGCGAAGTGCT CGCCGCATGA	Primers to make 3' flank for deleting *pcr2*
1700-3-2	AAAAAaagcttGCGTCGGTTTCAACGTCATCGATT	
popNn-5R	AAAAgaattcTGGCTTGTTGATCTGAGGAATCACGATGGA CATCCTCCAGAGTT	5' and 3' primers to clone *popN*
popN-3H	AAAAAaagcttGCCGGTCAATTCAGAAGGCCCGT	
popNn5-1	AAAAAgaattcGCGAGCAGCGCCAGGCTCTGGTCGGGAT	Primers to make 5' flank for deleting *popN*
popNn5-2	AACTCGAGCCGCAAGCATGCTGAAGTCCATCGTGGCTG TGGTTCCTGGTC	
popNn3-1	TTCAGCATGCTTGCGGCTCGAGTTCTCTGGCAGGTGCT GGTGACAGGGGA	Primers to make 3' flank for deleting *popN*
popNn3-2	AAAAAaagcttGGTGCCGCTGTCCTCGAAATCCAGTT	
pcr1-5Not	AAAAAgcggccgcaGCATACGGGCCTTCTGAATTGAC	5' and 3' primers to make αLN-Pcr1 fusion protein
pcr1-3Asc	AAAAAggcgcgccTCAACCCAGTCCATGCTGCTGCTC	
pscD5-1	AAAAAtctagaCCGTGCGCCGCTCGGTGCGGCTGTTCCTC AT	Primers to make 5' flank for deleting *pscD*
pscD5-2	AACTCGAGCCGCAAGCATGCTGAAGAGCAACACCAGGT	

	CGGCCTGCAACGGA	
pscD3-1 pscE3H	TTCAGCATGCTTGCGGCTCGAGTTCATCATCAATCTCAA AGGGGAGGT AAAAAaagcttTTAGTCCTCTGTGAGCTGCGAAA	Primers to make 3' flank for deleting *pscD*
pHluorin-Tn-5Bam pHluorin-3H	AAAAAggatccGTGCTTGACACTTTATGCTTCCGGCTCGTA TAATGTGTGGAAACATCAGGAGAAGGCAACCATCATGA GTAAAGGAGAAGAACTT AAAAAaagcttTTATTTGTATAGTTCATCCATGCCATG	Primers to clone *pHluorin*
pcrG5-Bam	AAAAAggatccgAACGAATACACCGAAGA	5' primer to clone *pcrG* into pETDuet1
pscO5-Nde C2myc-3Kpn	AAAAAcatatgAGCCTGGCTCTGCTGTTGCGCGT AAAAAggtaccTCACAGGTCCTCCTCCGAGATCAGTTTCT GCTCGCCCAAATCTT	Primers to clone *pscO-myc* into pETDuet1
popD-5-1 BDC25-5-2 BDC25-3-1 popB-3-2	AAAAAgaattcGTGGTCAGCTTCGGCGGCTCAGCGGT AGGCCTGGTTATGGCTCTGGGTGTACTGCTGGATGAGC TGCAGGACGTCCTTGATTTCCTGGAATGCTTCCAGCAT ACCCAGAGCCATAACCAGGCCTGGCGTGCGGCGGCCG GAGTGGTCTGAGGAGACGTCACATGATC AAAAAaagcttGATCACCGCCATGGCGATCATCAGCGT	Primers to clone *popB*(D-CTES) into pEXG2

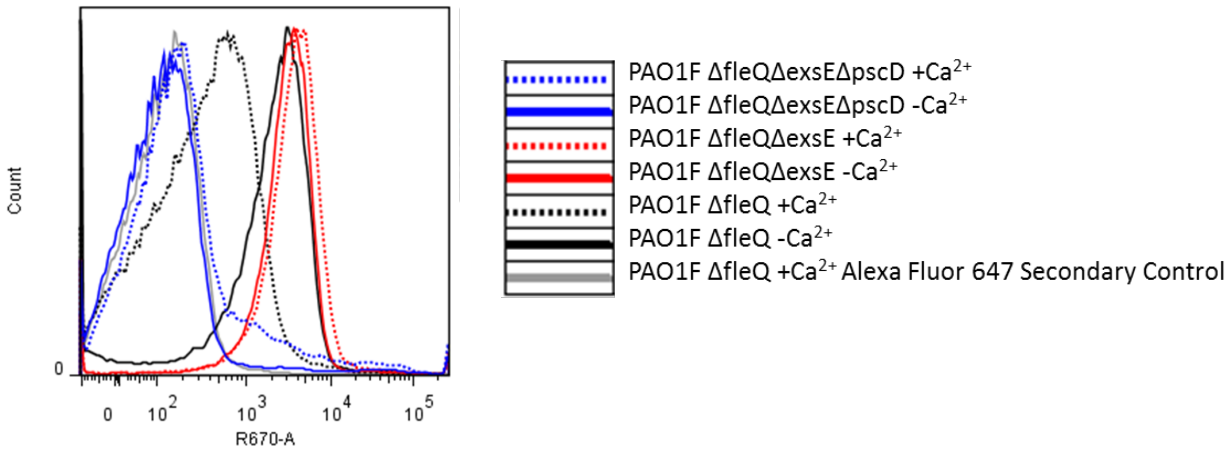


Figure S1. The *P. aeruginosa* strain with an *exsE* deletion assembles more T3SS apparatus at bacterial surface than wild-type bacteria in the presence of calcium (effector secretion off) and has equal number of the apparatus with/without calcium in the medium. Related to Figure 1 of the manuscript. Assembled T3SS were quantified by staining bacteria grown under the indicated culture condition with an affinity purified antibody directed against the needle-tip protein PcrV and an Alexa-fluor 647 conjugated secondary antibody. Stained bacteria were then analyzed by flow cytometry. All the *P. aeruginosa* strains tested are in *ΔfleQ* background to prevent any non-specific secretion of PcrV through the flagellar secretion system. The *P. aeruginosa* strain lacking the *pscD* gene, which encodes an essential inner membrane component of the T3SS, served as a T3SS null control. EGTA was added to remove Ca^{2+} from the culture medium. 10,000 single cell events were analyzed for each strain and condition and the peak height was normalized during the overlay for clarity.

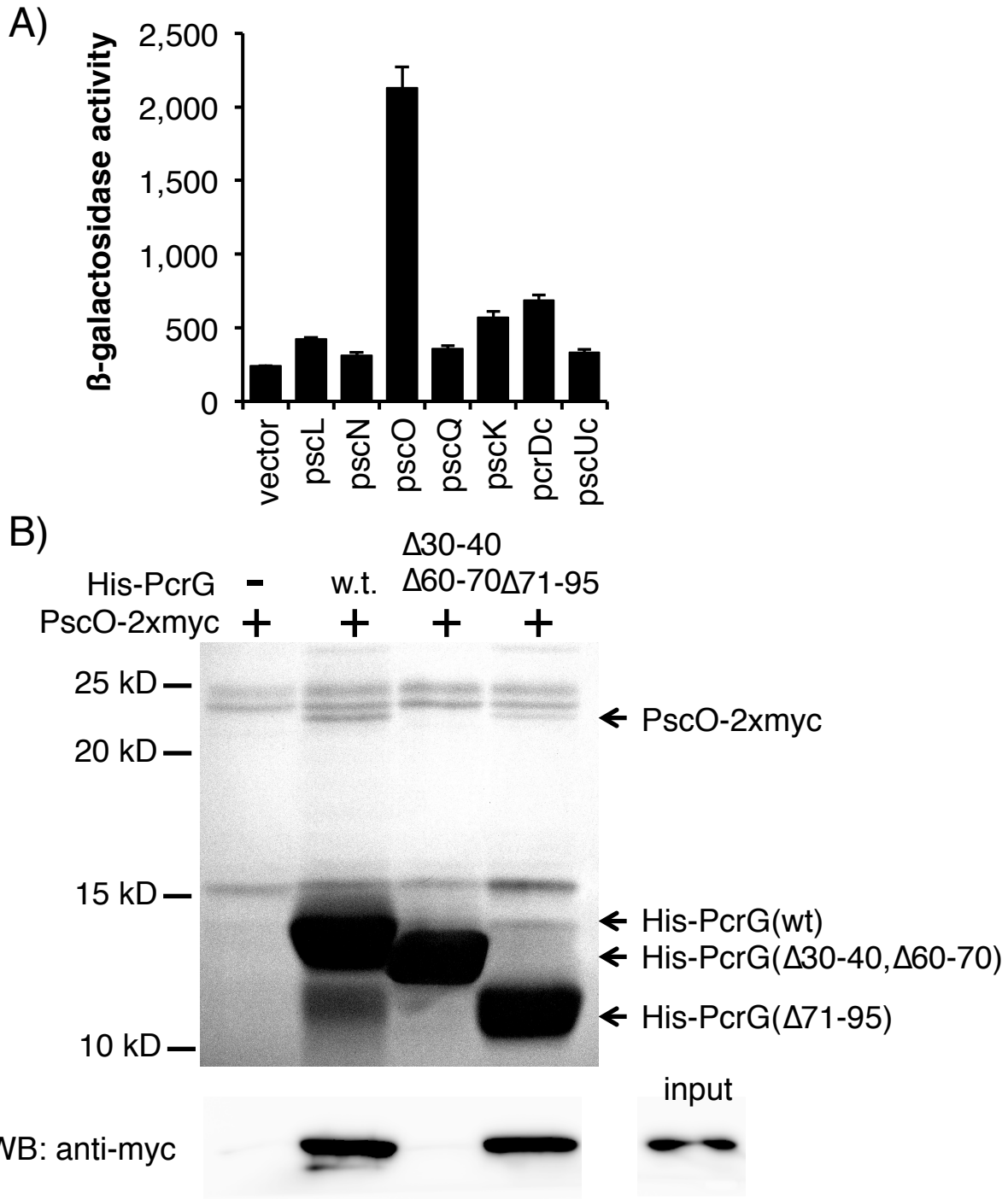


Figure S2. PcrG and PscO interact. Related to Figure 2 of the manuscript. A) Bacterial two-hybrid screen of PcrG-interaction with cytoplasmic apparatus components. *pcrG* was expressed as a fusion to cI and the indicated apparatus component genes were fused to the alpha subunit gene of RNA polymerase. Expression was induced by adding IPTG to 50μM. B) The lysate of an *E. coli* strain expressing a Myc-tagged version of PscO was mixed with lysates of *E. coli* expressing His-tagged versions of PcrG, PcrG(Δ30-40, Δ60-70) or PcrG(Δ71-95). After 90 minutes of incubation, the His-tagged PcrG was purified by cobalt-affinity chromatography and separated on a 15% Gel and visualized by Coomassie blue staining. Co-purified PscO is indicated by an arrow. Co-purification of PscO was corroborated by anti-Myc western blot. The input sample was run on the same gel with intervening bands cropped out of the exposure. The input sample represents 8μl of the 950μl lysate mixture, the output samples represents 16μl of the 40μl elution fraction.

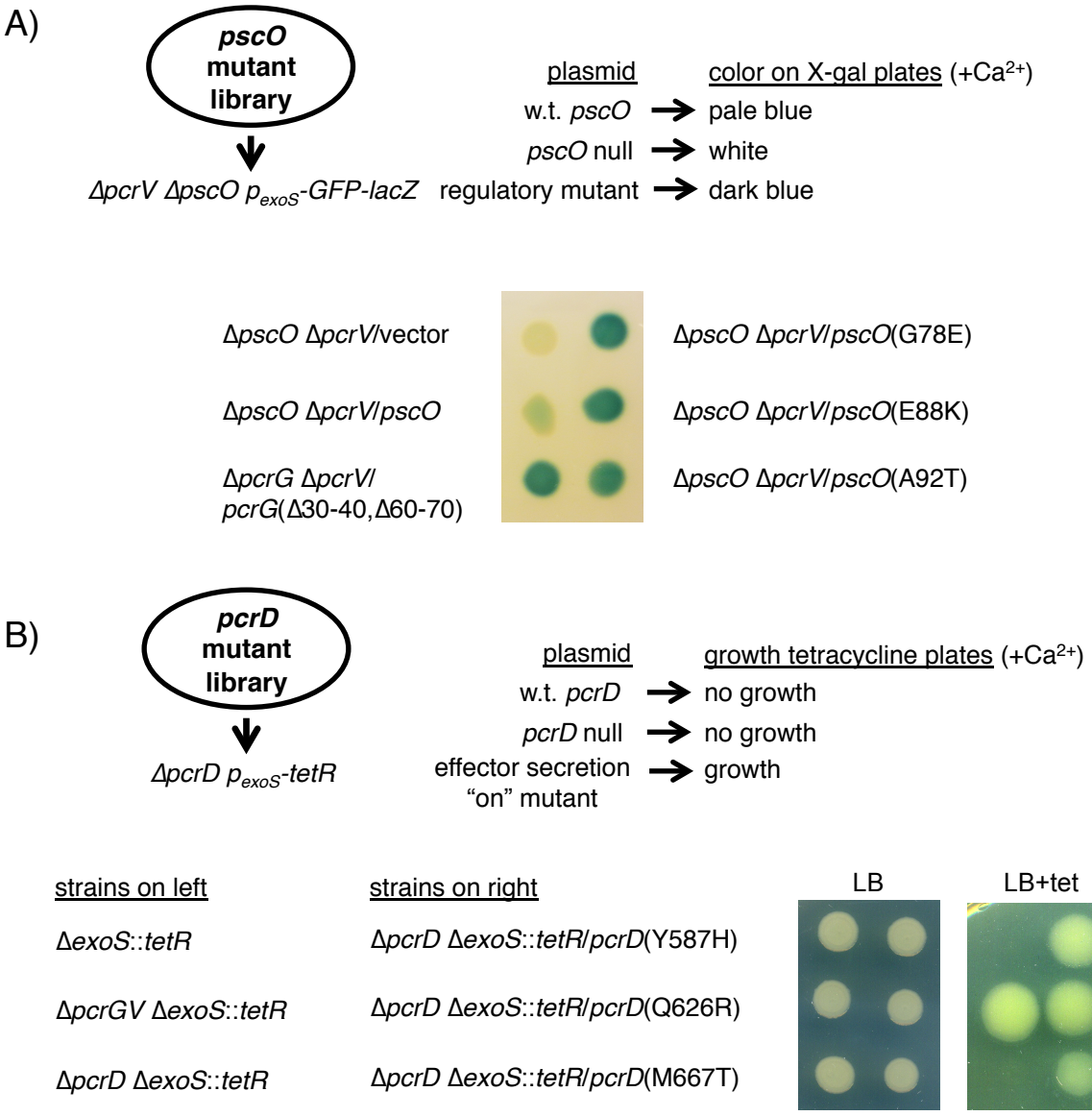
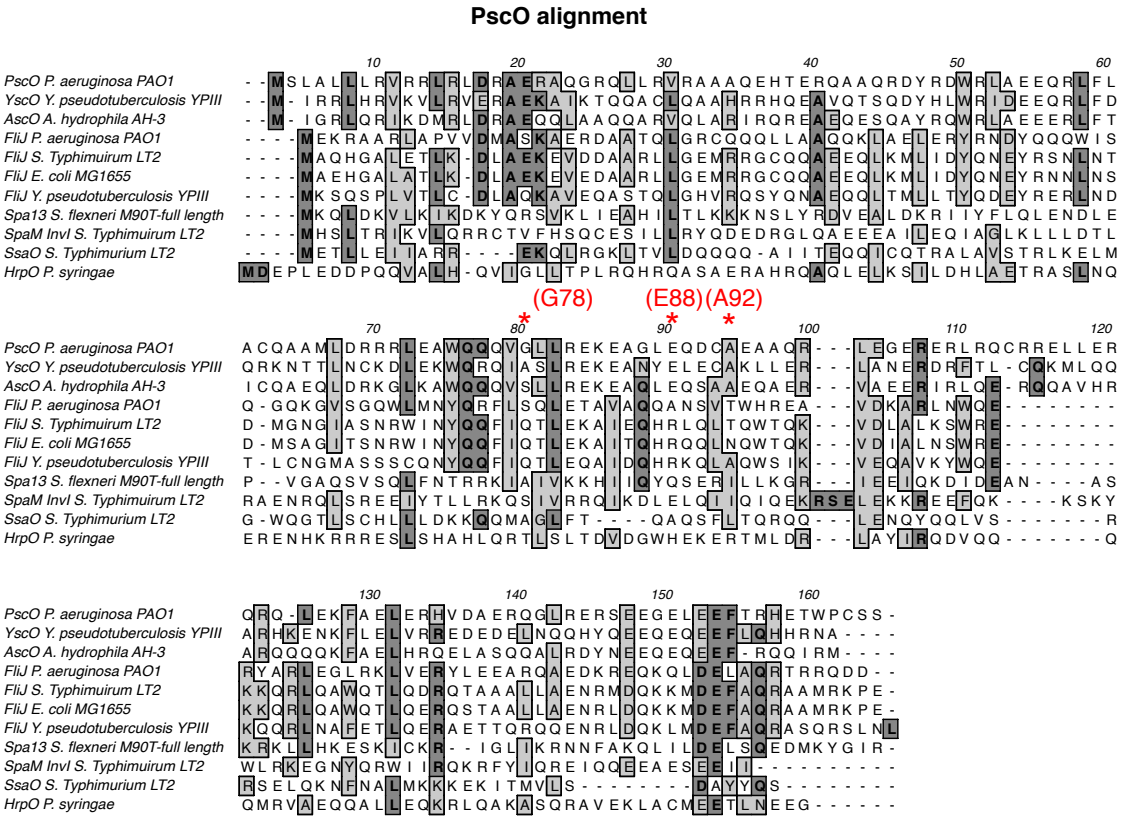


Figure S3. Genetic screens and selections. Related to Figures 4 and 7 of the manuscript. A) PCR mutagenesis was used to generate a plasmid-based library of *pscO* mutant alleles. To identify mutants of *pscO* that up-regulate effector secretion in a strain lacking *pcrV*, we complemented a *pscO* null mutant with the plasmid library. Since PscO is an essential component of the apparatus, null mutations result in white/very pale blue colonies on X-gal plates. A fully functional copy of *pscO* results in an intermediate blue color, whereas mutations that result in up-regulation of effector secretion result in a dark blue colony color. 2.5μl spots of overnight cultures representing each mutant class on an X-Gal plate are depicted below the schematic. The relevant genotype is noted as well. B) A *pcrD* mutant library was generated using PCR mutagenesis. To identify mutants in *pcrD* that up-regulate effector secretion, the library was transformed into strain PAO1F Δ*pcrD* Δ*exoS*::tetR and transformants were plated on tetracycline plates to identify *pcrD* mutants that result in up-regulation of effector secretion and concomitant up-regulation of *exoS* expression. Growth in the presence and absence of tetracycline is illustrated for each mutant class.

A)



B)

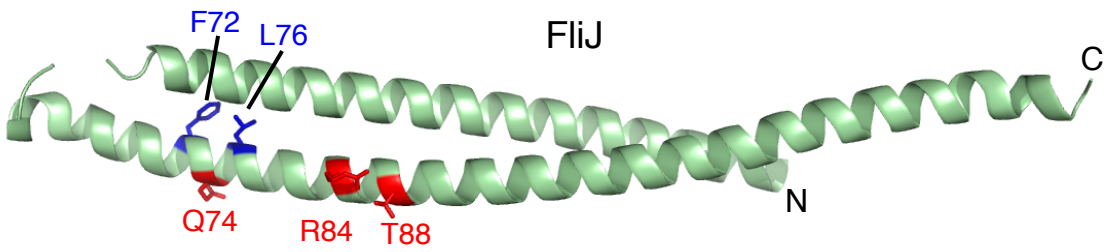


Figure S4. Alignment of PscO with homologous proteins. Related to Figure 4 of the manuscript. A) PscO of *P. aeruginosa* PAO1, as well as its homologs were aligned using ClustalW. Residues that, when mutated, result in an up-regulation of secretion activity are indicated with a red asterisk. B) Based on the alignment, residues that correspond to amino acids changed in our regulatory PscO mutant proteins were mapped onto the crystal structure of *S.* Typhimurium FliJ [PDB: 3AJW, (10)], (red: PscO G78-> FLiJ Q74;PscO E88-> FliJ R84; PscO A92-> FliJ T88). Additionally, residues F72 and L76 of FliJ, which are thought to be involved in contacting FlhA (flagellar homolog of *P. aeruginosa* T3SS component PcrD) are indicated in blue (9). Accession numbers: PscO(AAG05085.1), YscO(AAA27675.1), AscO(AAS91809.1), PA FliJ(AAG04494.1), ST FliJ(AAL20885.1), EC FliJ(AAC75009.1), YPTB FliJ(ACA68672.1), SsaO(AAL20340.1), SpaM(AAA83430.1), Spa13(CAC05825.1, corrected based on (13, 14)), HrpO(ABQ88355.1)

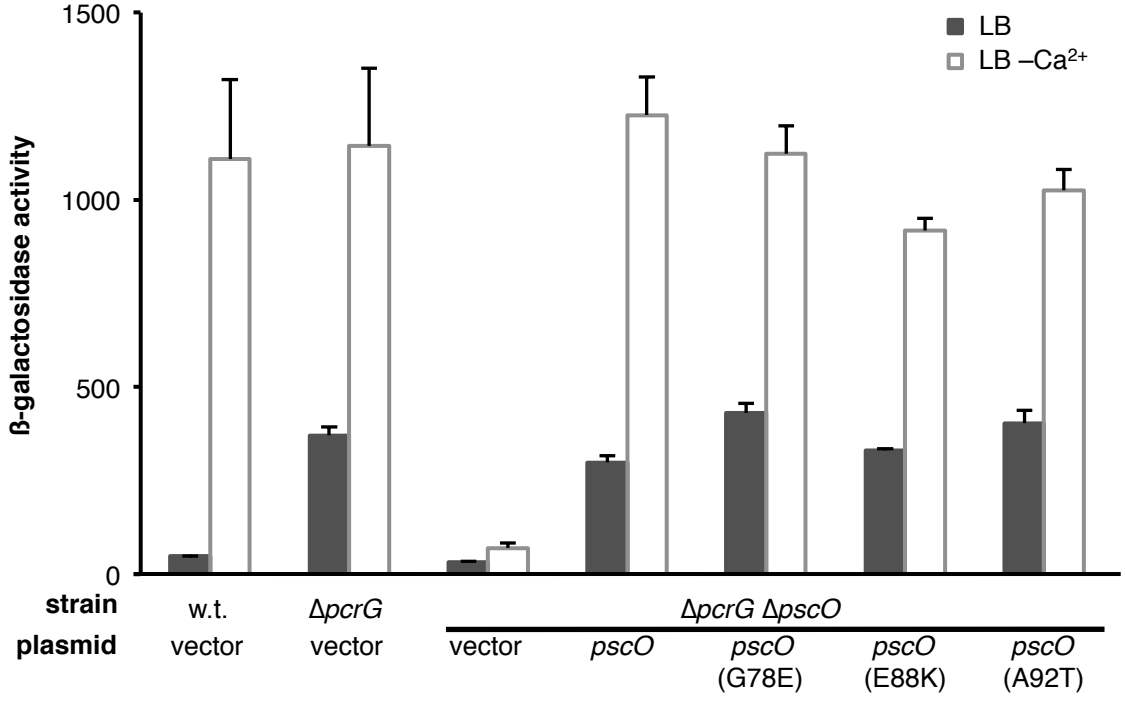


Figure S5. The PscO mutants do not enhance effector secretion in a *pcrG* deletion mutant. Related to Figure 4 of the manuscript. Triggering of effector secretion in the strains, PAO1F Δ*exoS*::GFP-*lacZ* (w.t. control), PAO1F Δ*pcrG* Δ*exoS*::GFP-*lacZ and* PAO1F Δ*pcrG* Δ*pscO* Δ*exoS*::GFP-*lacZ,* was monitored by the ß-galactosidase assay. Expression of plasmid-based wild type *pscO* and the indicated *pscO* mutants was induced through the addition of IPTG [10μM final concentration].

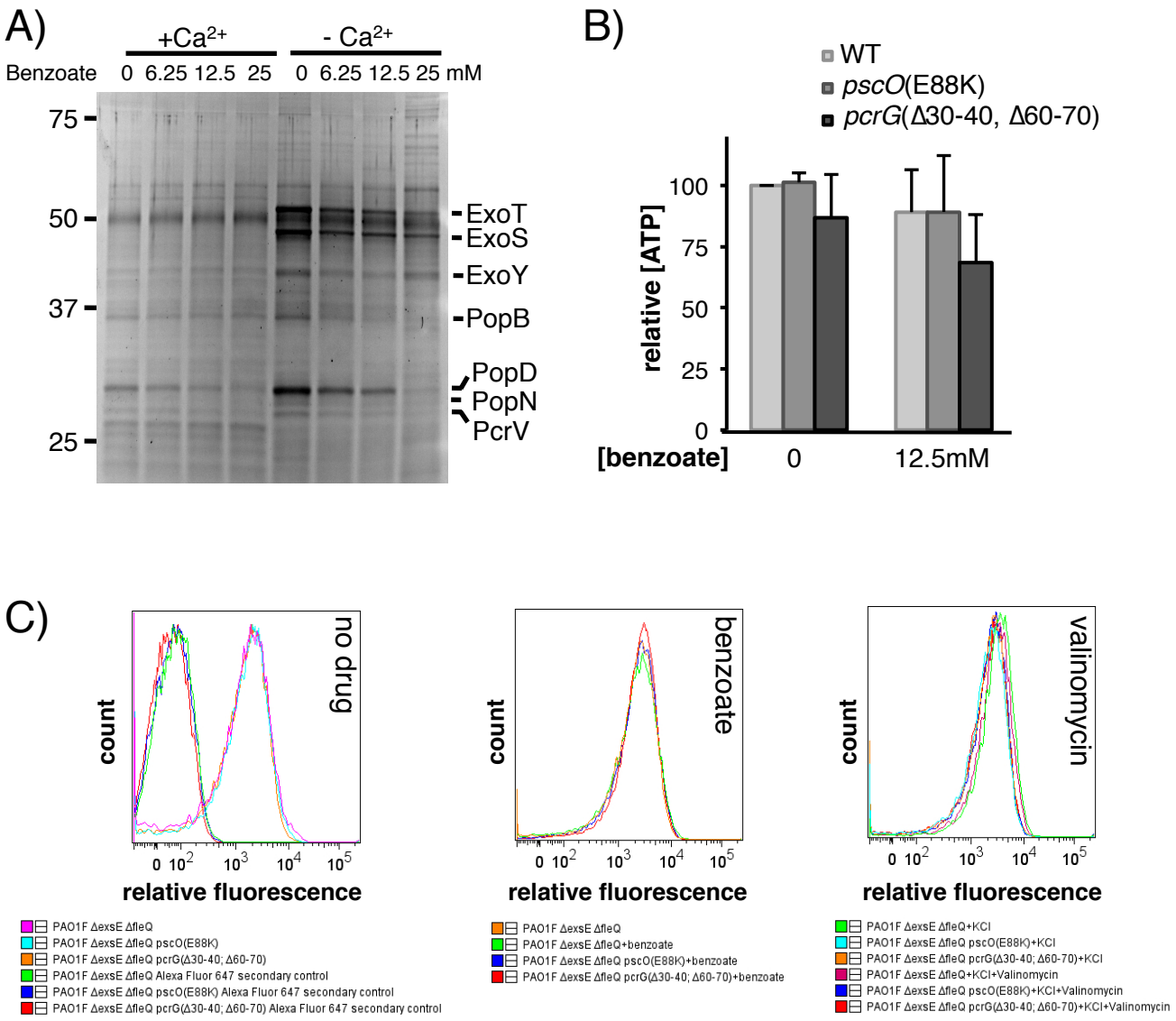


Figure S6. Collapsing pmf interferes with Type III secretion, but does not affect needle number . Related to Figure 5 of the manuscript. A) Strain PAO1F Δ*exsE* Δ*fleQ* grown in LB-MC in the presence of the indicated concentration of benzoate to collapse ΔpH. Supernatants were collected following the same protocol as indicated for the secretion assays in Fig. 5. The experiment was conducted in the presence or absence (5mM EGTA) of calcium as indicated. Supernatant proteins were separated by SDS-PAGE and stained with Sypro Ruby (Invitrogen). B) The ATP content of strain PAO1F Δ*exsE* Δ*fleQ* (WT), as well as *pcrG*(Δ30-40,Δ60-70) and *pscO*(E88K) mutant derivatives, with or without 12.5mM potassium benzoate treatment, was determined by luciferase assay. ATP levels were compared to the wild type control without benzoate treatment and represent mean of 3 independent experiments ± SD. C) Assembled T3SS were quantified by staining bacteria grown under the indicated culture condition with an affinity purified antibody directed against the needle-tip protein PcrV and an Alexa-fluor 647 conjugated secondary antibody. Stained bacteria were then analyzed by flow cytometry. All the *P. aeruginosa* strains tested are in *ΔfleQ* background to prevent any non-specific secretion of PcrV through the flagellar secretion system. Strains were grown up and treated as for the secretion assays reported in Fig. 5. 10,000 single cell events were analyzed for each strain and condition and the peak height was normalized during the overlay for clarity. The primary antibody was omitted in the secondary control samples.

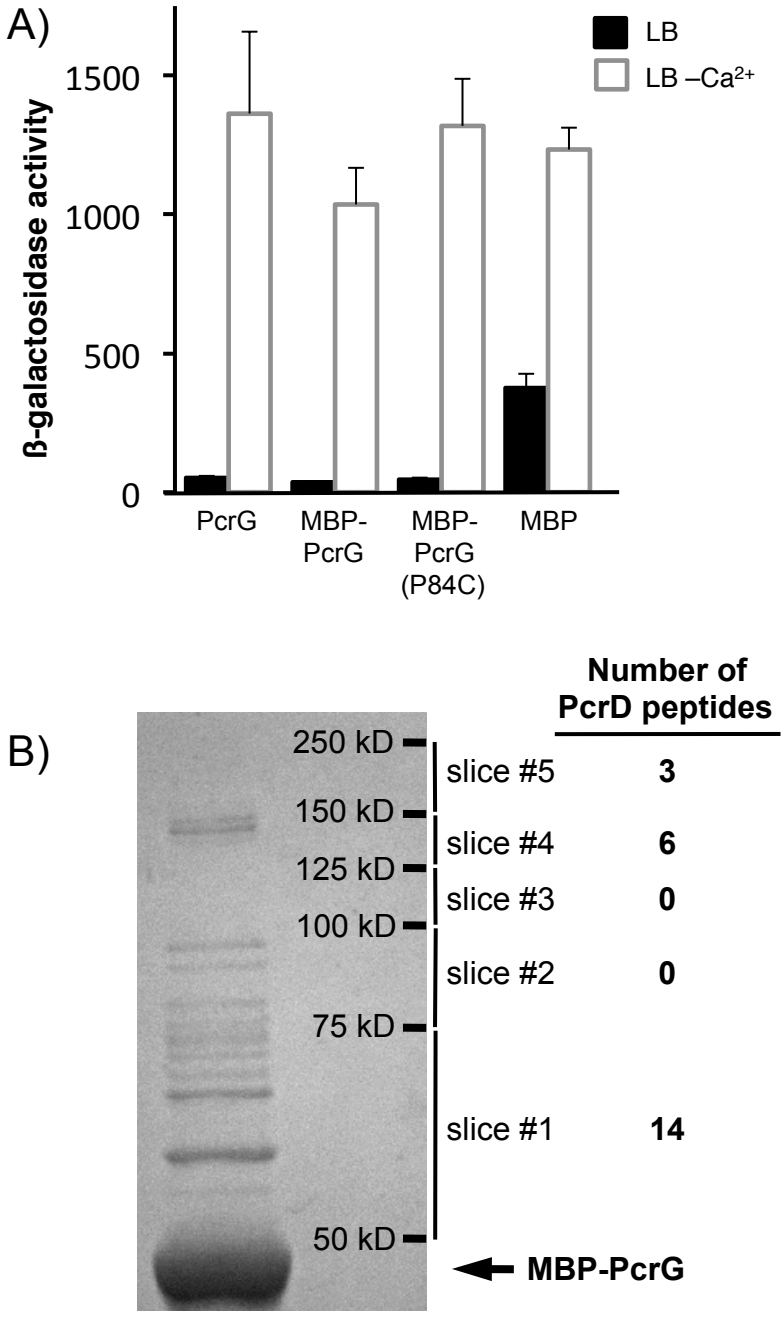


Figure S7. PcrG(P84C) is functional and can be used to precipitate crosslinked, interacting proteins. Related to Figure 6 the manuscript. A) Complementation of a Δ*pcrG* null mutant was assayed by beta-galactosidase assay using a chromosomal *exoS*-GFP*lacZ* reporter. The indicated protein was produced from a complementing plasmid and the bacteria were grown in the presence or abesence of calcium. B) Proteins interacting with MBP-PcrG(P84C) after SMCC crosslinking were purified by amylose resin affinity purification and run on a 10% SDS gel. Purified proteins were stained by Coomassie brilliant blue, subsequent to which the indicated gel slices were excised, chopped up with a razor blade and submitted for mass-spectrometric analysis. PcrD was the only T3SS apparatus component that co-purified with MBP-PcrG. The number of PcrD-derived peptides in each slice are noted to the right.

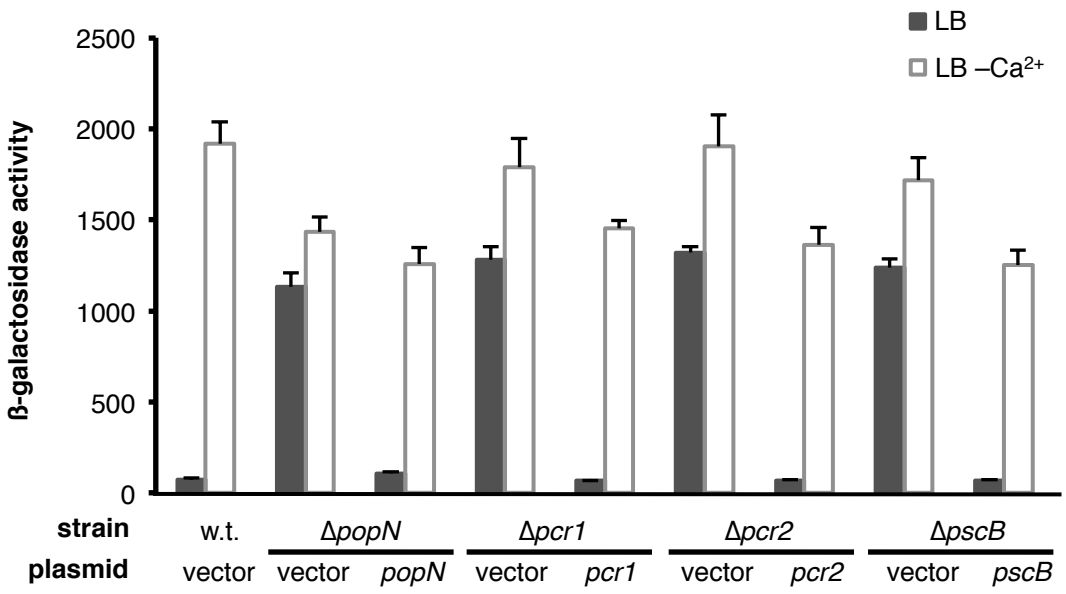


Figure S8. Control of effector secretion by the PopN complex. Related to Figures 6 and 7 of the manuscript. The parent strain, PAO1F Δ*exoS*::GFP-*lacZ*, or mutant derivatives deleted for components of the PopN complex (PopN: Δ*popN*; Pcr1: Δ*pcr1*; Pcr2: Δ*pcr2*; PscB: Δ*pscB*) were transformed either with a complementing plasmid or the corresponding vector control. Expression of the complementing gene was induced with 50μM IPTG (*popN*) or10μM IPTG (*pcr1*, *pcr2*, *pscB*) and control of effector secretion was monitored by ß-galactosidase assay.

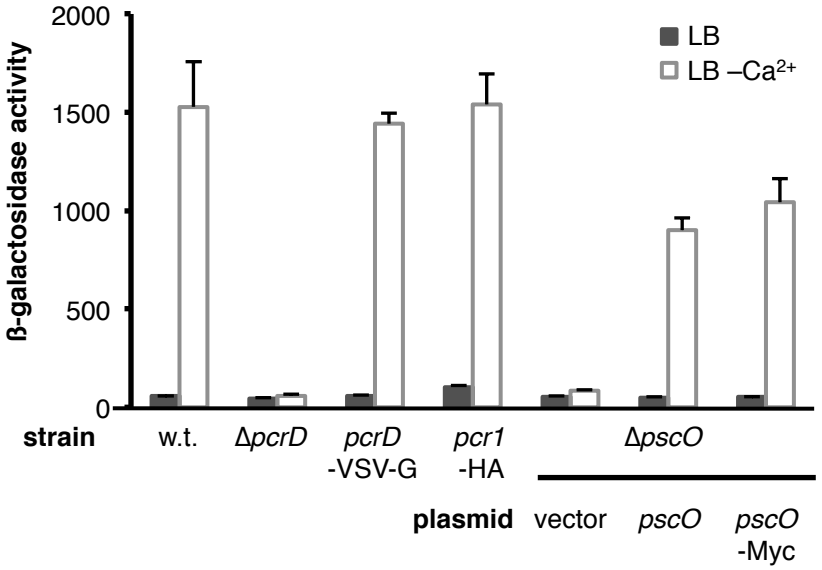


Figure S9. Tagged T3SS components are fully functional. Related to Figures 6 and 7 of the manuscript. Strains PAO1F Δ*exoS*::GFP-*lacZ*, PAO1F Δ*exoS*::GFP-*lacZ ΔpcrD*, PAO1F Δ*exoS*::GFP-*lacZ pcrD*-VSV-G, PAO1F Δ*exoS*::GFP-*lacZ pcr1*-HA or PAO1F Δ*exoS*::GFP-*lacZ ΔpscO* complemented with a plasmid-based copy of wild-type *pscO* or *pscO*-Myc were evaluated for their ability to control effector secretion by ß-galactosidase assay. Expression of the complementing *pscO* alleles was induced with 10μM IPTG.

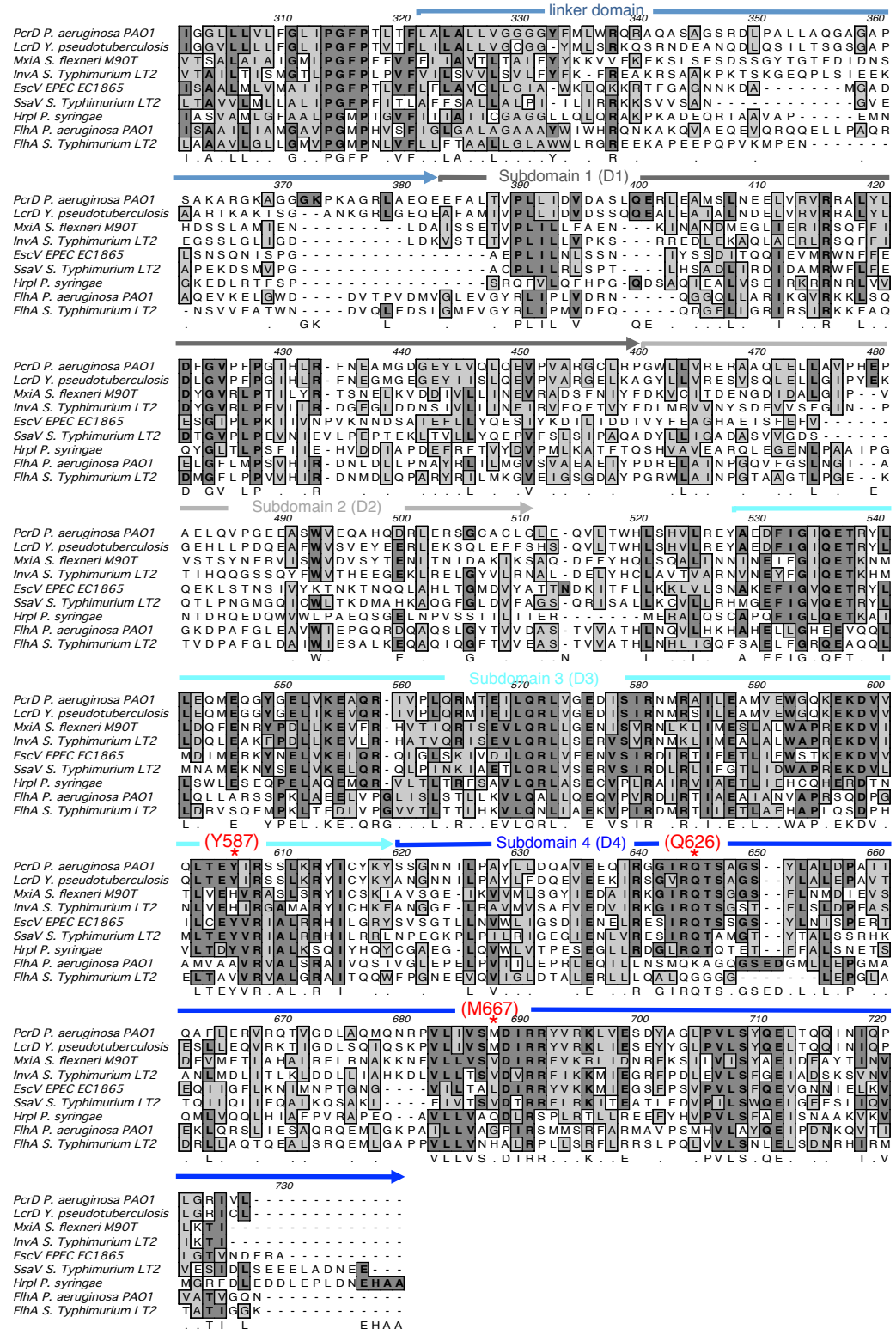


Figure S10. Alignment of the C-terminal cytoplasmic domains of PcrD and its homologs. Related to Figure 7 of the manuscript. PcrD of *P. aeruginosa* PAO1, as well as its homologs were aligned using ClustalW (see Additional Experimental Procedures for accession numbers). Identical residues are shaded dark grey, similar residues are shaded light grey. A consensus sequence is depicted below the alignment. Residues involved in effector secretion regulation are indicated with a red asterisk, as well as the residue number in PcrD. Subdomains D1-4, based on the structure of *S. flexneri* MxiA (Abrusci et al., 2013), are indicated using the same color scheme as used in the structure depicted in Figure 7B. Dark grey: identity; light grey: similarity. Accession numbers for proteins: PcrD(AAG05092.1), LcrD(AAA27647.1), MxiA(AAK18466.1), InvA(AAA16867.1), EscV(EKJ11194.1), SsaV(AAL20338.1), HrpI(AAA03015.1), PA FlhA(AAG04841.1), ST FlhA(AAL20829.1)

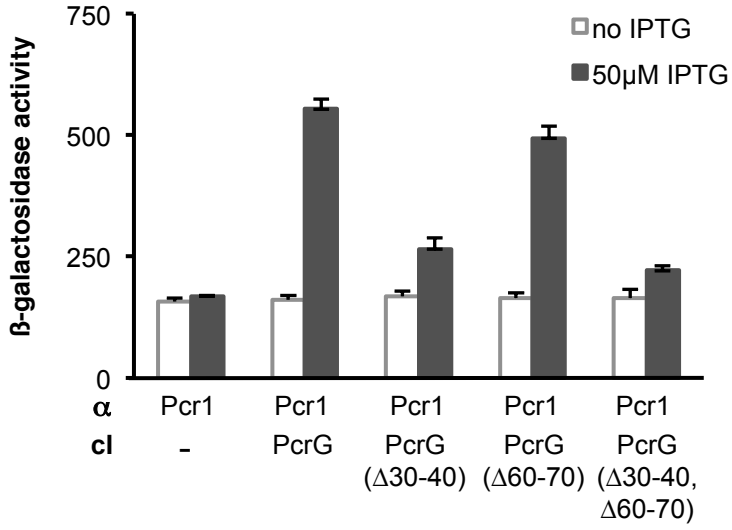


Figure S11. Two-hybrid analysis of the PcrG-Pcr1 interaction. Related to Figure 7 of the manuscript. The interaction between PcrG and Pcr1 was monitored using the *E. coli* RNA polymerase α/lambda cI, bacterial two-hybrid system. Expression of the indicated α- and cI-fusion proteins was induced by adding 50 μM IPTG to the medium. Interaction-dependent recruitment of RNA polymerase to the test promoter results in activation of *lacZ* expression, which was assayed by ß-galactosidase assay.