Supporting information

Kinetic control of quorum sensing in *Pseudomonas aeruginosa* by multidrug efflux pumps

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Materials and Methods

Strains and Growth Conditions

All strains and plasmids constructed are listed in Table 1. Unless otherwise indicated, cells were grown in Luria Bertani (LB) broth (10 g/L Tryptone, 5 g/L Yeast Extract, and 5 g/L NaCl) at 37°C with shaking, or on LB Agar (LB broth with 15g/L agar) at 37°C. Where appropriate, media was supplemented with 200 µg/ml carbenicillin or 30 µg/ml gentamicin. Expression vectors were cloned using conventional methods using PAO1 genomic DNA as a template.

MexGHI-OpmD was deleted using the established Flp-FRT recombination [1-2]. Suicide vector pEXΔGHID was constructed using pEX18Ap [1] as the backbone. The FRT-GM-FRT cassette was amplified from pUC18-mini-Tn7T-LAC [3] using the primers SalIfrtGMfrtFWD and SalIfrtGMfrtREV, and the fragment was cloned into pEX18Ap using the SalI restriction site. We amplified and inserted 500bp directly upstream of *mexG* and the last 508bp of *opmD* from PAO1 genomic DNA using the primers GHIDupFWD, GHIDupREV, GHIDdownFWD, and GHIDdownREV and the restriction sites EcoRI, KpnI, PstI, and HindIII. The plasmid was conjugated using *E. coli* SM10 λpir into *P. aeruginosa* strains. Briefly, *P. aeruginosa* strains and SM10 harboring pEX18Ap-GM-ΔGHID were grown to OD_{600} of 1.0 and 1 ml of each culture was collected by centrifugation. Cells were resuspended and mixed in 50 µL LB media, spotted onto LB Agar plates, and incubated at 37°C for 18h. The mixture was resuspended into 10 mM $MgSO_4$ and plated onto Vogel-Bonner minimal media agar plates (0.2g/L $MgSO_4$, 2g/L citric acid, 10g/L K_2HPO_4, 3.5g/L $NaNH_4HPO_4$, and 15g/L agar) supplemented with 30 or 15 µg/ml gentamicin and 10% sucrose. Colonies that were resistant to gentamicin and sensitive to carbenicillin were analyzed by PCR to confirm deletion. The GM^R cassette was subsequently

removed by transforming pFLP2 [1] into deletion mutants and selection on LB Agar plates supplemented with 10% sucrose. Excision of GM^R was confirmed by PCR.

The pore was inserted into strains by transforming pGK-LAC-FhuAΔC/Δ4L [4] as well as the helper plasmid pTNS2 [5] into the respective parental strains. Transformants were screened for gentamicin resistance and successful insertion was confirmed by PCR and spot assays using vancomycin as described before [6]. All "Pore" strains induced with IPTG became hypersusceptible to vancomycin and other antibiotics (Table 1).

For growth curves, cells were subcultured from stationary phase 1:100 and grown to OD_{600} of 1.0. Subsequently, $5*10^4$ cells per mL were inoculated into a 96-well plate. Plates were incubated in a Spark 10M microplate reader (Tecan) for 18 hours at 37°C and the OD_{600} measured every 30 min with prior shaking. The data was imported into Microsoft Excel and normalized to the OD_{600} at time point zero. Growth rates were determined by linear regression of a plot of $LN(OD_{600})$ against time.

Sequences of RND transporters were taken from the *Pseudomonas* Genome Database [7], and the alignments were done utilizing Clustal Omega [8] and BLAST [9].

Antibiotic Susceptibility Testing

Susceptibilities to antibiotics were tested in two fold broth dilutions as previously described [4]. Stationary phase cells were subcultured 1:100 into fresh media. Cultures expressing the Pore were induced at OD_{600} of 0.3 with 2 mM IPTG. Cells were grown to OD_{600} of 1.0 and inoculated into a 96-well plate at a concentration of $5*10^4$ cells per mL. The plate was set up with 2-fold increasing concentration of antimicrobial in 100 µL of LB broth per well. Plates were incubated at 37°C for 18 hours after which time the MIC was determined. For the checkerboard

study with pyocyanin and ciprofloxacin, the OD_{600} was measured using a Spark 10M microplate reader (Tecan) and the data was visualized using OriginPro (OriginLabs).

Spot Assays

Zones of inhibition of pyocyanin and PMS were determined utilizing a filter disk assay as previously described [4]. Cells were grown in LB broth to stationary phase and subsequently subcultured 1:100 into fresh media. Once cells reached an OD_{600} of 1.0, 300µL were added to 4mL of soft agar (10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl, and 7.5 g/L Agar) and poured onto LB Agar plates. After the soft agar solidified, filter disks were added to the plates and 0.05 µmoles of pyocyanin or 0.5 µmoles of PMS were spotted on the discs. Plates were incubated at 37°C for 18 hours and zones were measured.

Quantification of Pyocyanin

Extracellular pyocyanin was quantified using previously published methods with the following modifications [10]. Cultures were grown in LB broth supplemented with 50 mM HEPES-KOH buffer pH 7.0 to stationary phase. A 1 mL aliquot was taken and cells were removed through ultracentrifugation at 100,000xg at 4°C. 500 µL of chloroform was added to the supernatant and samples were vortexed for 1 min. The two phases were separated by centrifugation and the blue organic phase was transferred to a new tube. Chloroform was evaporated under nitrogen and samples were resuspended in 250 µL LB broth with 50 mM HEPES-KOH pH 7.0. The absorbance was read at 690 nm and the concentration of pyocyanin was calculated using the extinction coefficient (4,310 M^{-1} cm^{-1}).

Fluorescent dye uptake assay

Kinetic measurements of Hoechst33342 uptake into cells were done using a Spark 10M microplate reader (Tecan) as previously described[4]. Cells were subcultured 1:100 from stationary phase into fresh LB supplemented with 200 µg/mL carbenicillin at 37°C with shaking. Cells were induced at OD_{600} of 0.3 with 2 mM IPTG and subsequently grown to OD_{600} of 1.0. A low binding F-bottom 96-well plate (Greiner Bio-One, inc) was set up with 2-fold dilutions of Hoechst33342 in 100 µL HMG buffer (50mM HEPES-KOH pH 7.0, 1mM $MgSO_4$, and 0.4% Glucose). Cells were harvested using centrifugation and washed twice with HMG buffer. 100 µL of the cell suspension in HMG buffer at OD_{600} of 1.0 were injected into each well using the microplate reader and fluorescence emission was measured at 460 nm (excitation at 355 nm) every 20 seconds for 10 minutes with shaking.

The uptake data was normalized to the emission of Hoechst33342 without cells and subsequently fit to an exponential equation in the form of $y = A_1 + A_2 (1-\exp(-k_2*t))$ using the fittype function in MATLAB. From these fit parameters, initial rates and steady state concentrations of HT uptake were calculated and plotted against the total Hoechst33342 concentration.

Protein Purification and Crosslinking

For chemical crosslinking assays, PΔ3 cells expressing pMexGHI-flag were harvested by centrifugation. Pellets were washed twice in PBS buffer (8g/L NaCl, 0.2g/L KCl, 1.44g/L Na_2HPO_4, 0.24g/L KH_2PO_4 at pH 7.4) and, subsequently, 4 mM DSP (dithiobis(succinimidyl propionate)) (Thermo Scientific) or DMSO was added to samples and controls, respectively. Cells were incubated for 2 hours at 37°C while rotating. The reaction was quenched with 100

mM Tris-HCl pH 8.0. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 100 µg/ml DNAse I, and 100 µg/ml Lysozyme. The lysis mixture was incubated on ice for 30 min. EDTA was added to the samples to a final concentration of 5 mM and cells were incubated for an additional 30 min on ice. Lysis was completed by sonication and unbroken cells were separated from broken cells by centrifugation. Membrane fractions were isolated by ultracentrifugation at 100,000xg at 4°C for 1 hour. Pellets were resuspended in 50 mM Tris-HCl, 150 mM NaCl, 5 mM Imidazole, 1 mM PMSF, and 5% Triton X-100 at pH 8.0 and incubated at 4°C overnight while rotating. Soluble compounds were separated from insoluble ones by centrifugation at 100,000xg at 4°C for 1 hour. His-tagged proteins were purified with His•Bind Resin (Novagen) charged with 50 mM $CuSO_4$. Samples were eluted from the column with 20 mM Tris-HCl, 500 mM NaCl, 1 mM PMSF, 0.2% Triton X-100, and 500 mM Imidazole at pH 8.0. Samples were subsequently analyzed by 12% SDS-PAGE and immunoblotting onto polyvinylidene fluoride membranes (Santa Crux Biotechnology) with Anti-His (Invitrogen) or Anti-FLAG (Agilent) primary antibodies followed by secondary Anti-Mouse antibody (Sigma) conjugated with alkaline phosphatase. The membranes were developed with 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Expression of plasmids was checked from cells grown to stationary phase. Briefly, cells were collected by centrifugation and incubated on ice in 10 mM Tris-HCl, 5 mM EDTA, and 100 µg/ml Lysozyme at pH 8.0 for one 1 hour. The lysis mixture was sonicated and membrane fractions were collected by ultracentrifugation at 100,000xg for 1 hour at 4°C. Membrane pellets were resuspended in 10 mM Tris-HCl, 150mM NaCl, and 1mM PMSF and the protein concentration was determined using the Bradford Protein Assay (Bio-Rad). Samples were boiled

and 22.5 µg of total membranes per sample were loaded onto a 12% SDS-PAGE and subsequently analyzed by immunoblotting as described above.

MexG was purified from PAO1 cells harboring pMexG or plasmids with the corresponding mutation in MexG. Whole cell pellets were washed in 10 mM Tris-HCl and resuspended in 50 mM Tris-HCl, 1 mM $MgCl_2$, 100 µg/ml DNAse I, and 100 µg/ml Lysozyme at pH 8.0. After 30 min of incubation on ice, EDTA was added to final concentration of 5 mM and samples were incubated for an additional 30 min. Samples were sonicated until they became clear and unbroken cells were separated via centrifugation. Membranes were collected by ultracentrifugation at 100,000xg at 4°C for 1 hour, resuspended in 50 mM Tris-HCl, 150 mM NaCl, 5 mM Imidazole, 1 mM PMSF, and 2% N-Dodecyl β-D-maltoside (DDM) at pH 8.0, and incubated overnight at 4°C with rotation. MexG was purified with His•Bind Resin (Novagen) and eluted in 20 mM Tris-HCl, 500 mM NaCl, 1 mM PMSF, 0.03% DDM, and 500 mM Imidazole at pH 8.0. Purified MexG samples were dialyzed twice using a Pur-A-Lyzer Dialysis Kit (Sigma-Aldrich) in 50 mM Tris-HCl, 500 mM NaCl, 1 mM PMSF, and 0.03% DDM at pH 8.0. The concentration of MexG was estimated by 16% SDS-PAGE analysis with a BSA (Sigma) standard and the Quantity One software (Bio-Rad).

Fluorescence Binding Assay

Fluorescence binding assays were carried out with 1 µM purified MexG in 50 mM Tris-HCl, 500 mM NaCl, 1 mM PMSF, and 0.03% DDM. We used a RF-5301PC Spectrofluorophotometer (Shimadzu) and measured emission spectra from 300 nm to 550 nm with excitation at 290 nm. Readings were done in fast mode and excitation and emission slits set to 5. All experiments were carried out at 25°C in triplicates. Pyocyanin (Sigma) was dissolved in

ethanol and titrated into the MexG sample. We measured the binding by monitoring the emission at 330 nm.

Analysis of the fluorescence quenching was done assuming a 1 to 1 binding model as described earlier[11-12]. The following sets of equations were used for the fitting:

$$K_a = \frac{[PL]}{[P]_{free} * [L]_{free}} \quad (1)$$

where

$$[P]_{free} = [P]_{total} - [PL] \quad (2)$$

and

$$[L]_{free} = [L]_{total} - [PL] \quad (3)$$

Substituting Eq. 2 and Eq. 3 into Eq. 1 yields:

$$K_a = \frac{[PL]}{([P]_{total} - [PL]) * ([L]_{total} - [PL])} \quad (4)$$

Solving for [PL] gives:

$$[PL] = \frac{-\sqrt{K_a^2[L]_t^2 - 2K_a^2[L]_t[P]_t + K_a^2[P]_t^2 + 2K_a[L]_t + 2K_a[P]_t + 1} + K_a[L]_t + K_a[P]_t + 1}{2K_a} \quad (5)$$

$$F = F_0 - [PL] * \Delta F \quad (6)$$

where $[P]_{free}$ and $[L]_{free}$ are the free protein and ligand concentrations; [PL] is the concentration of the protein ligand complex; K_a is the association constant; F_0 and F are the initial fluorescence and the observed fluorescence after addition of ligand, respectively; and ΔF is the change of fluorescence upon addition of the ligand.

Fluorescence data were first corrected for dilution and inner filter effects as described earlier [12], and subsequently fitted to equation 4 and 5 using OriginPro (OriginLabs).

References for methods

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Table S1. Strains and Plasmids

Plasmids	Description	Source
pBSPII (SK-)	Cb^r; broad-host-range cloning vector	[13]
pMexEF-OprN	Cb^r; pBSPII *mexEF-oprN*; Bla^r; expresses MexEF-$OprN^{His}$	This study
pMexHI-OpmD	Cb^r; pBSPII *mexHI-opmD*; Bla^r; expresses MexHI-$OpmD^{His}$	This study
pMexGHI-OpmD	Cb^r; pBSPII *mexGHI-opmD*; Bla^r; expresses MexGHI-$OpmD^{His}$	This study
pMexG	Cb^r; pBSPII *mexG*; Bla^r; expresses $MexG^{His}$	This study
pMexGHI	Cb^r; pBSPII *mexGHI*; Bla^r; expresses $MexGHI^{His}$	This study
pMexGHI-flag	pmexGHI with additional FLAG tag on N-terminus of *mexG*	This study
pMexGH-flag	pMexGH with additional FLAG tag on N-terminus of *mexG*	This study
pMexG-HAWA	Cb^r; pBSPII *mexG*; Bla^r; expresses MexG-$HAWA^{His}$	This study
pEX18Ap	Ap^r; $oriT^+$ $sacB^+$, gene replacement vector	[1]
pUC18-mini-Tn7T-LAC	Gm^r; mini-Tn7T based suicide vector	[3]
pEX18Ap-Gm	pEX18Ap with FRT-Gm^r-FRT fragment from pUC18-mini-Tn7T-LAC	This study
pEXΔGHID	pEX18Ap-Gm with upstream and downstream fragment of *mexGHI-opmD* flanking FRT-Gm^r-FRT	This study
pFLP2	Cb^r; $sacB^+$ Plasmid carrying FLP recombinase	[1]
pGK-LAC-FhuAΔC/Δ4L	pUC18-mini-Tn*7*T-Gm^r-$lacI^q$-pLAC vector containing *fhuA* Δ*C/*Δ*4L* gene	[4]
pTNS2	Helper plasmid containing transposase complex	[5]

Strains	Description	Source
PAO1	Wild type	Gift from O. Lomovskaya
PAO1Δ3	PAO1 Δ*mexAB* Δ*mexCD* Δ*mexXY*	Gift from O. Lomovskaya

PAO314	PAO1 Δ*mexAB-oprM* Δ*mexCD-oprJ* Δ*mexJKL*	[14]
PAO325	PAO1 Δ*mexAB-oprM* Δ*mexCD-oprJ* Δ*mexJKL* Δ*mexXY*	[14]
PAO509 (PΔ5)	PAO1 Δ*mexAB-oprM* Δ*mexCD-oprJ* Δ*mexEF-oprN* Δ*mexJKL* Δ*mexXY*	[15]
PAO1116	PAO1 Δ*mexAB-oprM* Δ*mexCD-oprJ* Δ*mexEF-oprN* Δ*mexJKL* Δ*mexXY ΔtriABC*	[15]
GKCW111 (PAO1)	PAO1 *att*Tn*7*::mini-Tn*7*T-Gm-*lacI*^{q}-pLAC-MCS	This study
GKCW112 (PΔ3)	PAO1Δ3 *att*Tn*7*::mini-Tn*7*T- Gm^{r}-*lacI*^{q}-pLAC-MCS	This study
GKCW113 (PΔ4)	PAO325 *att*Tn*7*::mini-Tn*7*T- Gm^{r}-*lacI*^{q}-pLAC-MCS	This study
GKCW114 (PΔ6)	PAO1116 *att*Tn7::mini-Tn7T-Gm-*lacI*^{q}-pLAC-MCS	This study
GKCW119 (PAO1-Pore)	PAO1 *att*Tn*7*::mini-Tn*7*T- Gm^{r}-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study
GKCW 116 (PΔ3-Pore)	PAO1Δ3 *att*Tn*7*::mini-Tn*7*T- Gm^{r}-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study
GKCW121 (PΔ4-Pore)	PAO325 *att*Tn*7*::mini-Tn*7*T- Gm^{r}-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study
GKCW122 (PΔ6-Pore)	PAO1116 *att*Tn7::mini-Tn7T- Gm^{r}-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study
DW101 (PAO1ΔGHID)	PAO1 Δ*mexGHI-opmD*-scar	This study
DW102 (PΔ3ΔGHID)	PAO1Δ3 Δ*mexGHI-opmD*-scar	This study
DW103 (PΔ4ΔGHID)	PAO325 Δ*mexGHI-opmD*-scar	This study
DW104 (PΔ6ΔGHID)	PAO1116 Δ*mexGHI-opmD*-scar	This study
DWBN110 (PΔ6ΔGHID-Pore)	DW104 *att*Tn*7*::mini-Tn*7*T- Gm^{r}-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study
DW111 (PΔ4ΔGHID-Pore)	DW103 *att*Tn*7*::mini-Tn*7*T-Gm-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study
DW112 (PΔ5-Pore)	PAO509 *att*Tn*7*::mini-Tn*7*T-Gm-*lacI*^{q}-pLAC-*fhuA*ΔC/Δ4L	This study

Table S2. List of Primers

Primer Name	Sequence (5' – 3')
GHIDpbspFWD	TTAGAAGCTTAGGAGGAATTCACCATGCAGCGCTTCATCGATAAC
GHIDpbspREVhis	ATATGGATCCTCAATGGTGATGGTGATGGTGACGGTTGGCCCCGGCGG
GpbspREVhis	ATATGGATCCTCAATGATGATGATGATGATGGGCCTTCTGGTAGGTGGC
GHpbspREVhis	ATATGGATCCTCAATGATGATGATGATGATGGCGGCCGGCGACCGGCAC
GHIpbspREVhis	ATATGGATCCTCAATGATGATGATGATGATGATGATGATGATGTGCCTTGTTCACCAGCAGATCGG
HIDpbspFWDhis	TTAGAAGCTTAGGAGGAATTCACCATGCAGAAACCCGTCCTGATC
GpbspFWDflag	TTAGAAGCTTAGGAGGAATTCACCATGGACTACAAAGACGATGACGACAAGGGAGGACAGCGCTTCATCGATAACTCG
GHIDnestedFWD	ATATGGTACCTAAGCGGTCATCCGCACTAC
GHIDnestedREV	ATATAAGCTTCGATGAAGGCGAGCAA
GHIDupFWD	ATATGAATTCCGAATAACGCCAGGGGCGTTATC
GHIDupREV	ATATGGTACCGGGTCGTTCCTTGTGCTGGTC
GHIDdownFWD	ATATCTGCAGCCTGGGCGGCTTCATCGGTTTC
GHIDdownREV	ATATAAGCTTTCAACGGTTGGCCCCGGCGG
SalIfrtGMfrtFWD	ATATGTCGACCTTGCGGCCCGGACGATCGAATTGG
SalIfrtGMfrtREV	ATATGTCGACGTGAGCGCAACGCAATGCATGATCG
GdloopFWD	ATATACTAGTGCAGCCCCGGCCTGGCTGTTCAACATCGCC
GdloopREV	ATATACTAGTTGCGAACAGCTTCGCCAGTCCCGAGGC
GdHWfwd	CGCCGGTCTTGCTCGCGAAGGTGGCGACGATGAGGATGG
GdHWrev	CCATCCTCATCGTCGCCACCTTCGCGAGCAAGACCGGCG

Supplementary Figures

Figure S1. Structures of phenazines and quorum signals. A: Showing the synthesis pathway of pyocyanin. The intermediate 5-methylphenazine-1-carboxylic acid (5-Me-PCA) was previously shown to be a substrate of MexGHI-OpmD. **B:** Oxidized and reduced form of pyocyanin. The virulence factor can act as an electron shuttle in oxygen poor environments. **C:** HHQ is a proposed substrate of MexEF-OprN (left). Structure of the synthetic phenazine PMS (right).

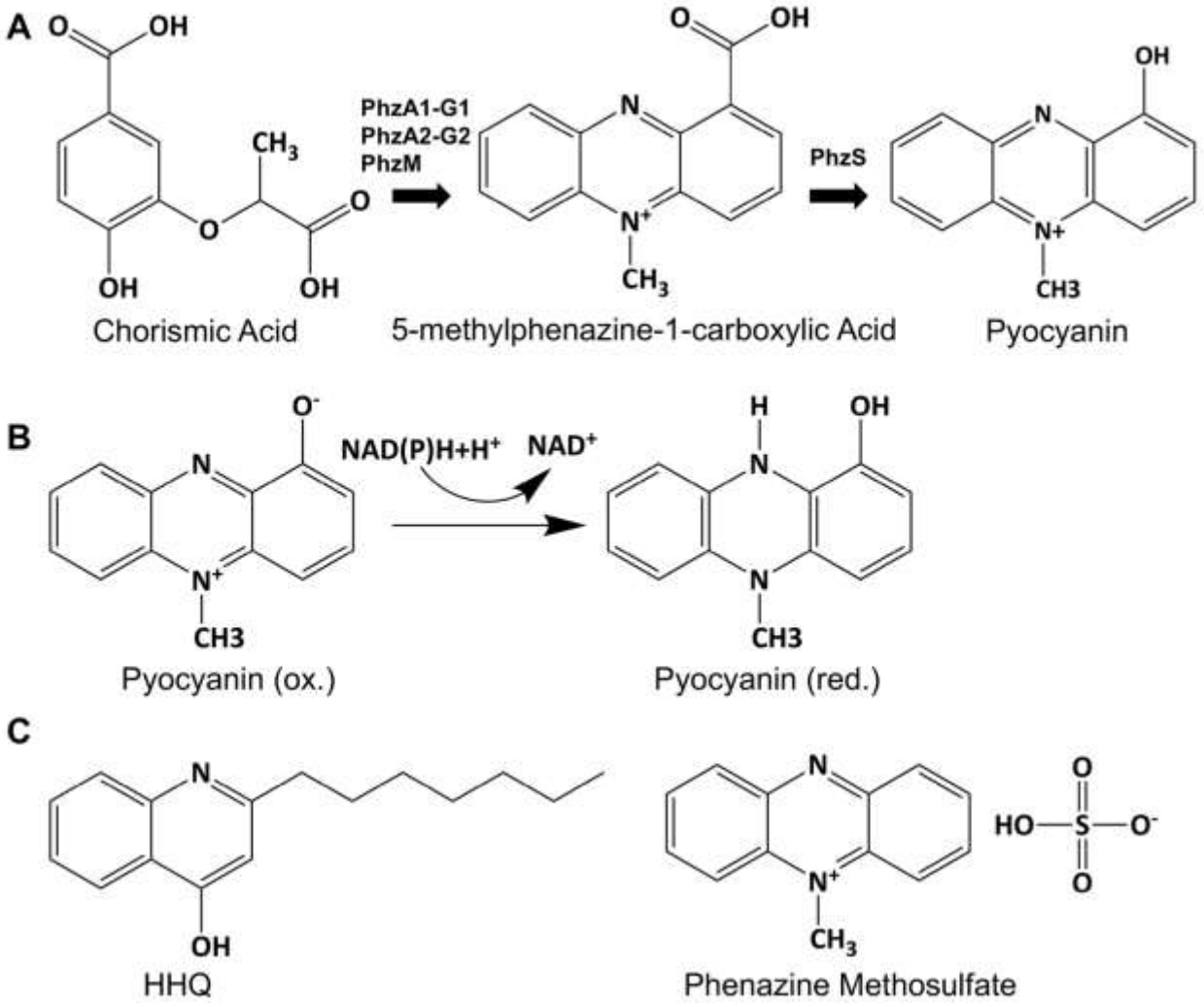

Figure S2. The effect of inducers on growth of *P. aeruginosa* cells with different efflux capacities. A. Growth curves of GKCW111 (PAO1)**,** GKCW112 (PΔ3). GKCW113 (PΔ4), GKCW114 (PΔ6) strains. Overnight cultures were diluted 1:100 into a fresh LB medium; the cells were grown for 24 hours and OD_{600} measured every 30 min. Data shown are the averages of three repeats and the error bars are SD (n=3). **B.** The same as A but cells were grown in the presence of 0.1 mM IPTG. **C.** Growth rates of indicated *P. aeruginosa* strains at increasing concentrations of IPTG. Averages are shown with SD (n=3 for strains with native barriers; n=6 for hyperporinated cells) as the error bars.

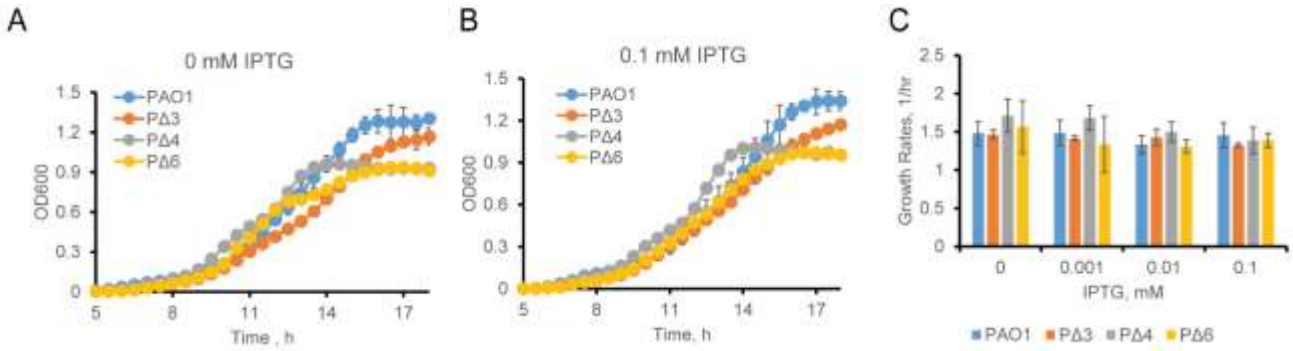

Figure S3. A real-time course of intracellular accumulation of 32 μM HT in PΔ4 (**A**) and PΔ-Pore (**C**) expressing indicated efflux pump constructs. Kinetic curves were fitted to an exponential equation and initial rates were calculated.

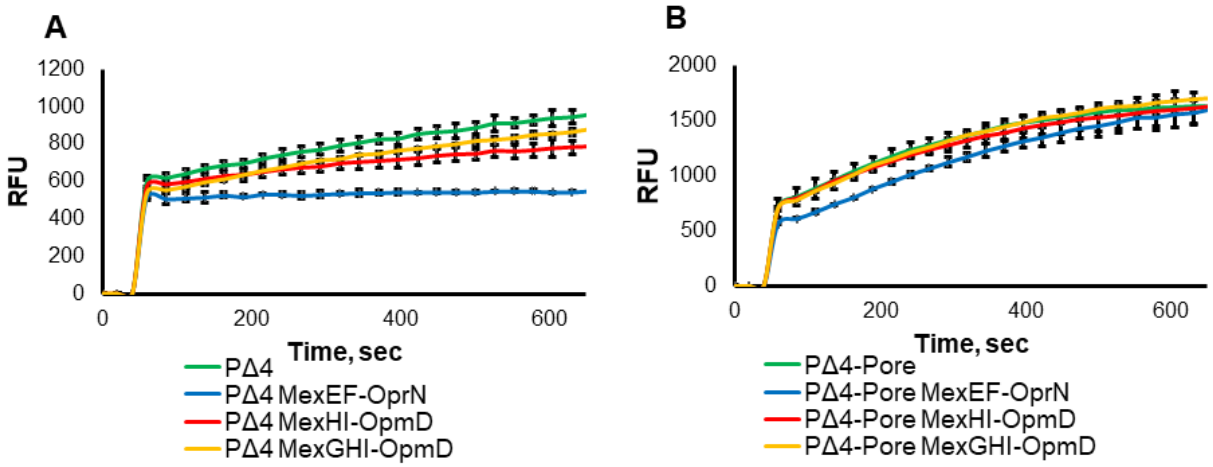

Figure S4. Growth curves of MexGHI-OpmD deletion mutants. A. Growth curves of PAO1 and PΔ3 with and without MexGHI-OpmD deletion. **B.** Same as **A**, but showing PΔ4 and PΔ6. Cells were grown for 18 hours at 37°C and the OD_{600} was determined every 30 min.

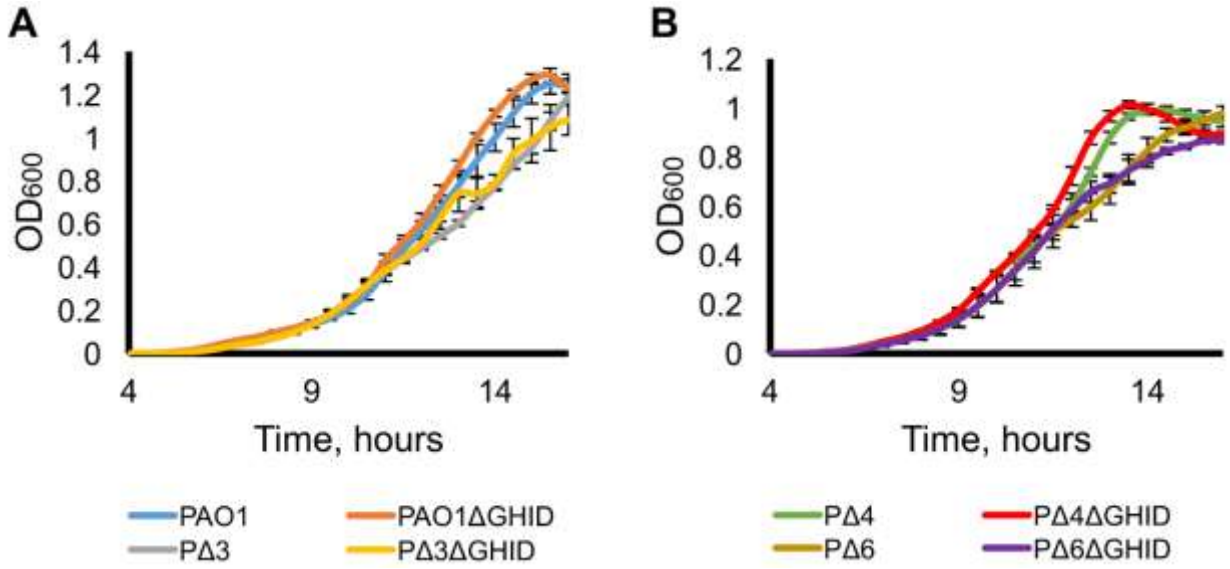

Figure S5. Checkerboard assay with pyocyanin and ciprofloxacin. Growth of the indicated strains at different combinations of pyocyanin and ciprofloxacin concentrations. The optical densities of cultures are indicated with green (high) to red (low).

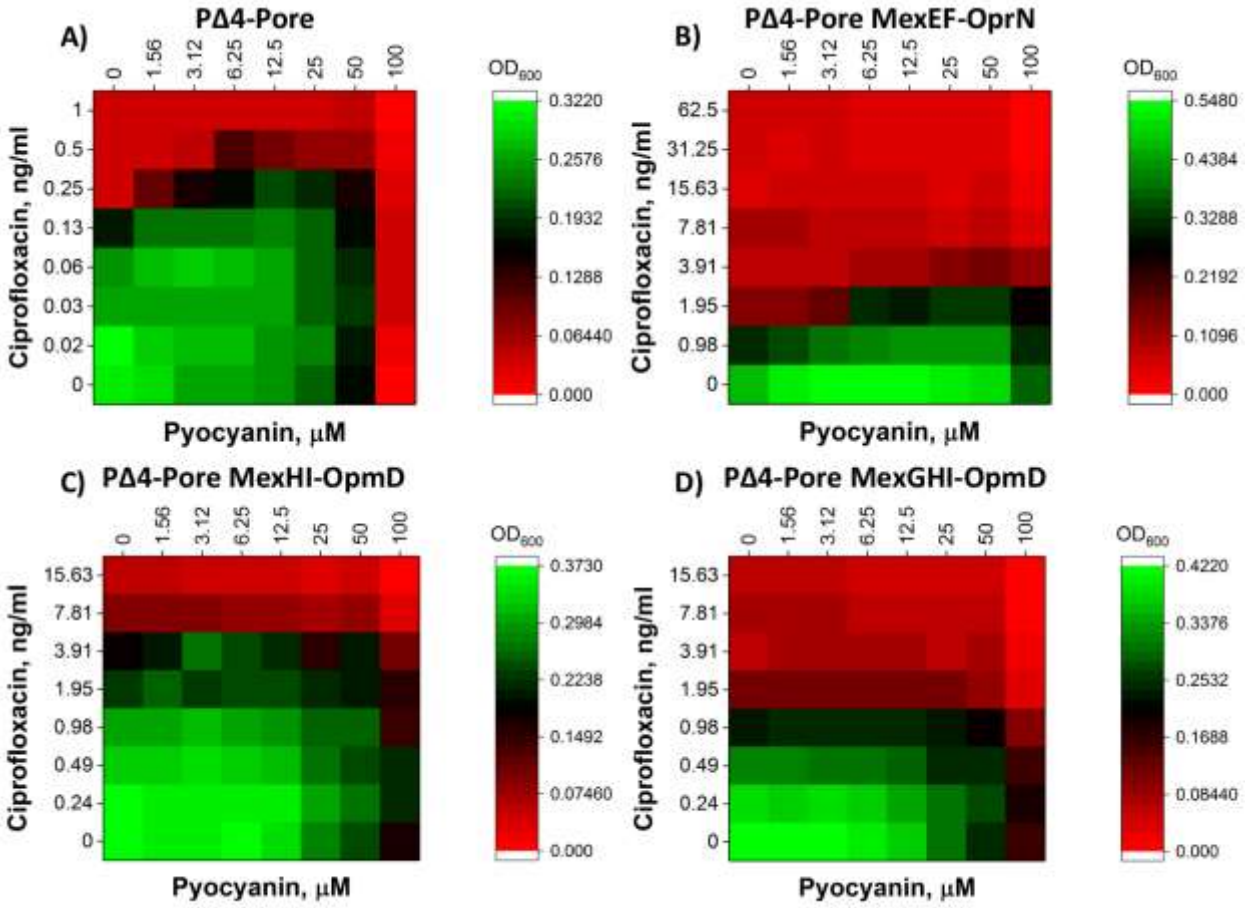

Figure S6. A: Fluorescence emission spectra of MexG (1 µM) and pyocyanin (10 µM) excited at 290 nm. **B:** Titration of indicated concentrations of pyocyanin into a sample of 1 µM MexG. Addition of pyocyanin quenches MexG fluorescence indicating a physical interaction. **C:** Effects of ethanol (solvent for pyocyanin) on fluorescence of MexG. Amounts of ethanol shown are exceeding the ones used during the experiment.

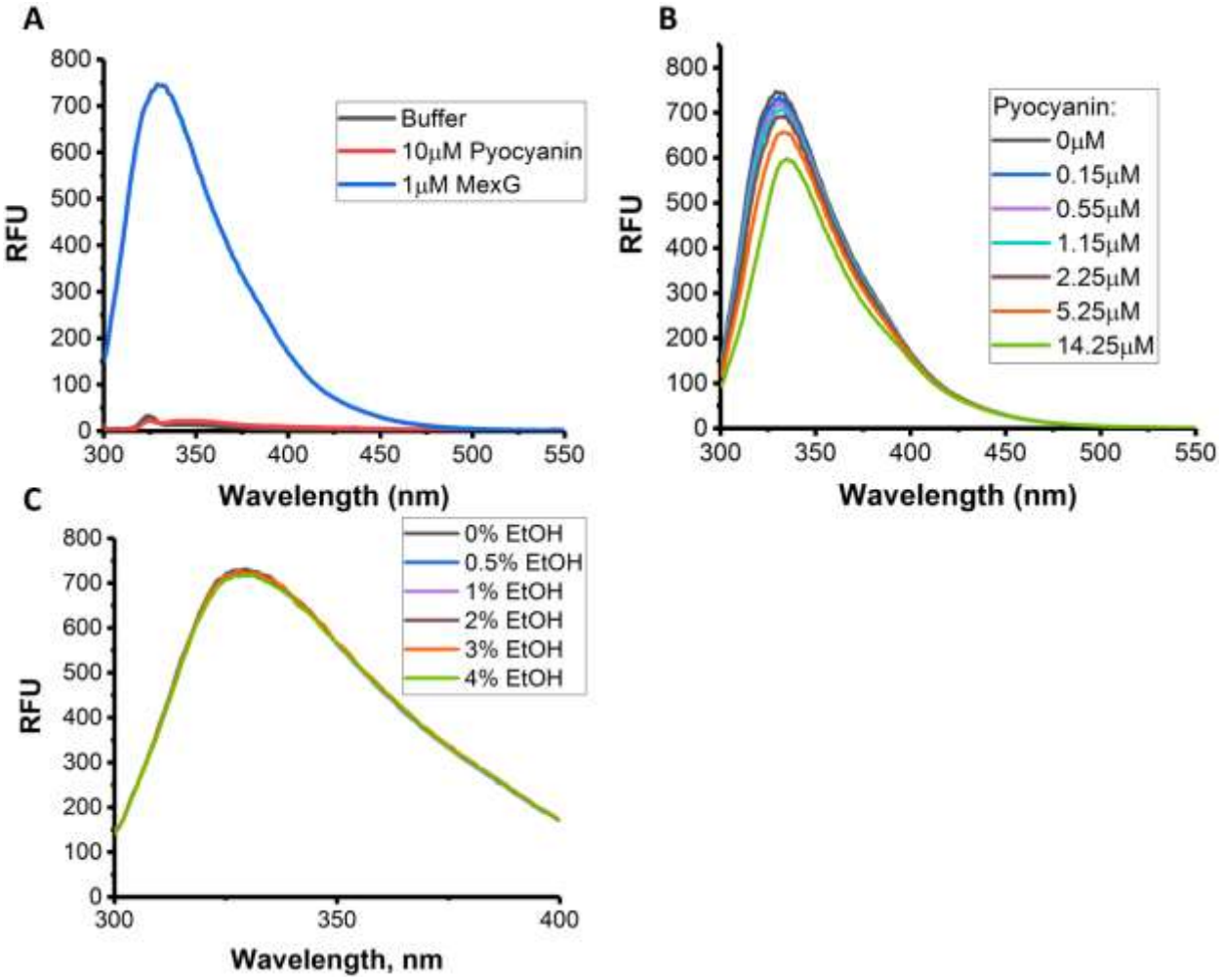

Figure S7. Fitting of pyocyanin binding curves. Fluorescence emission was measured at 330 nm (excitation at 290 nm) and corrected for dilution and inner filter effects. The data was subsequently fitted using to a 1:1 binding model using OriginPro (OriginLabs). Results are shown for MexG WT (**A**) and MexG–HAWA (**B**).