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Supplementary Information for

Entropically-driven aggregation of bacteria by host polymers promotes antibiotic tolerance in *Pseudomonas aeruginosa*

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Supplementary Materials and Methods.

Chemicals and strains. Polyethylene glycol (MW 2,000 and 10,000 kDa), porcine gastric mucin, HMW DNA (salmon sperm, D1626), DNase I, actin (rabbit muscle), alginate (sodium salt), and chitosan were purchased from Sigma. Hyaluronan was purchased from Glycosan Biosystems. LMW DNA was purchased from USB Corporation. Ciprofloxacin, tobramycin, and mitomycin C were purchased from Thermo Fischer. Strains and plasmids used in these studies are listed in Table S1.

Phase diagrams. Phase diagrams were constructed as described previously (1). Briefly, bacteria were cultured overnight at 37°C with shaking in lysogeny broth (LB). Bacterial cells were then washed twice in phosphate buffered saline (PBS, pH 7.2) and enumerated by serial dilution and plating. Polymer solutions were prepared in PBS + 0.1 mM EDTA (PBSE), except F-actin, which was prepared in 1x actin polymerization buffer (see below). The indicated polymer concentrations and bacterial numbers were mixed in 1.5 ml cuvettes (BioRad) and aggregation was visually scored after 6 h at room temperature as bacterial aggregation and sedimentation occurred. Phase diagrams using F-actin were produced by dissolving 5 mg G-actin in 900 µl general actin buffer (0.2 mM $CaCl_2$, 0.2 mM ATP in 5 mM Tris-HCl, pH 8.0). One hundred µl of 10x actin polymerization buffer (500 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP in 100 mM Tris, pH 7.5) was then added to induce polymerization at room temperature. ATP was omitted to construct a phase diagram using G-actin. For the phase diagram using filamentous Pf phage, phage strain Pf4 was purified from *P. aeruginosa* PAO1 and enumerated by plaque forming units as described (2). To avoid the replication of Pf4 during these experiments, *P. aeruginosa ΔpilA* was used, as this strain lacks type IV pili, the cell surface receptor Pf4 uses to infect PAO1 (3).

Antibiotic treatment. Bacteria were grown overnight (~16 h) on a shaker at 37°C in 3 ml LB broth. Overnight cultures were diluted 1:1 with fresh LB broth and incubated for 20 minutes at 37°C with shaking. For PEG-induced depletion aggregation, 100 µl (~10^8 CFUs) were added to 1 ml LB or LB PEG solutions. LB was diluted 4:6 with distilled water or 50% PEG (w/vol) prepared in distilled water to ensure that nutrient concentrations were the same in dispersed and aggregated conditions. For mucin/DNA-induced depletion aggregation, 1 ml bacteria (~10^9 CFU/ml) were added 1:1 to a mixture of mucin (8% w/vol) and DNA (4 mg/ml). Bacteria were incubated at 37°C on a roller (60 rpm) for 1 h prior to treatment with the indicated antibiotics. Bacteria were then diluted 10x with PBS, passed through a 22-gauge needle multiple times to break apart any remaining aggregates, and enumerated by serial dilution and plating. We noted that CFU enumeration of dispersed and aggregated cultures produced comparable estimates of bacterial numbers (Figure S5F). Thus, using CFUs to estimate bacterial numbers in these experiments is appropriate.

Mitomycin C treatment. For mitomycin C-dependent activation of the *recA* transcriptional reporter, wild-type *P. aeruginosa* PAO1 carrying $recA_p$::GFP were grown in 1 ml LB broth to an OD_{600} of ~0.3 and exposed to the indicated concentrations of mitomycin C for one hour. After this time, 200 µl of each culture were placed into a 96-well plate and the fluorescence of GFP was measured on a BMG Clariostar plate reader (480+/-8 nm excitation; 522+/-20 nm emission). Pre-induction of the SOS response by Mitomycin C and subsequent exposure to antibiotics was performed as described (4) with modifications. Briefly, overnight cultures of wild type, Δ*recA*, or LexA* *P. aeruginosa* PAO1 were diluted 1:1000 in 3ml fresh LB broth and incubated at 37°C for one hour with shaking. A sub-inhibitory concentration of mitomycin C (0.25 µg/ml) or PBS was then added to the cultures. After one hour, one milliliter aliquots of the cultures were pelleted by centrifugation and washed with PBS to remove residual mitomycin C. Washed pellets were then re-suspended in one milliliter LB broth and exposed to either 0.3 µg/ml ciprofloxacin or 1 µg/ml tobramycin for the indicated times. Bacteria were then enumerated by serial dilution and plating.

***In vitro* biofilm formation assay.** The crystal violet biofilm assay was used to quantify *in vitro* biofilm formation, as described (2).

8-oxoG measurements. Levels of 8-oxoG were quantified using the OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs) following the manufacturer's instructions.

Growth curves. Bacteria were grown overnight (~16 h) on a shaker at 37°C in 3 ml LB broth. Cultures were then diluted 1:1 with fresh LB and incubated at 37˚C with shaking for an additional 20 minutes. About 10^8 CFUs in 20uL were added

to a test tube containing 1mL of diluted LB (4:6 LB: distilled water) or HMW PEG (4:6 LB: 50% HMW PEG in distilled water). Cultures were incubated on a roller (60 rpm) at 37°C. Twenty µL aliquots were removed at the indicated times and bacteria were enumerated by serial dilution and plating on LB agar.

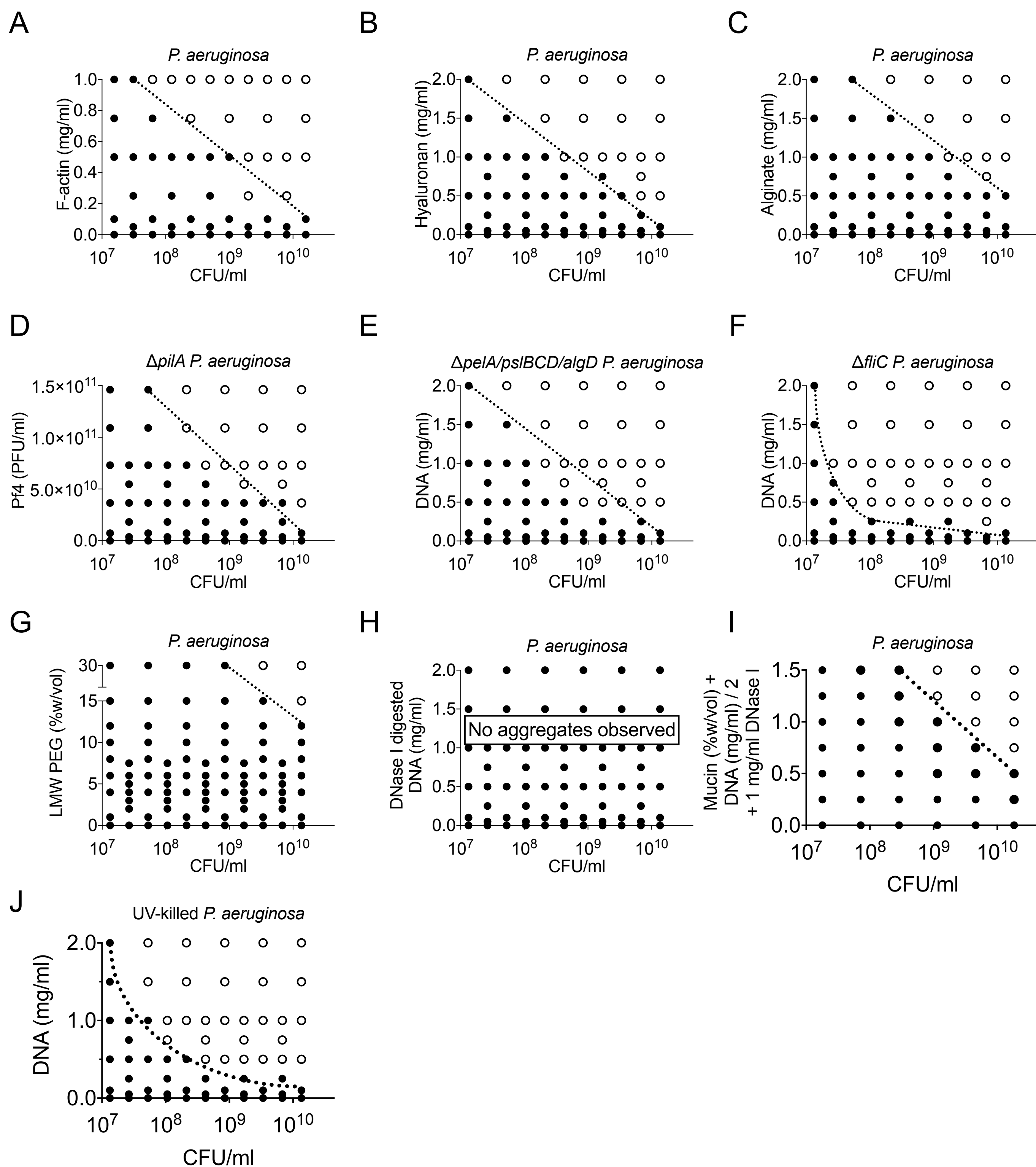

Fig. S1. Diverse non-adsorbing polymers promote depletion aggregation of *P. aeruginosa*. Phase diagrams were generated by mixing the indicated strains and polymers in cuvettes at the indicated concentrations, and visually scoring the cultures as either dispersed (closed symbols) or aggregated (open symbols) after 6 h. Dashed lines guide the eye and indicate phase boundaries. See main text and Figures 1 and 2 for details.

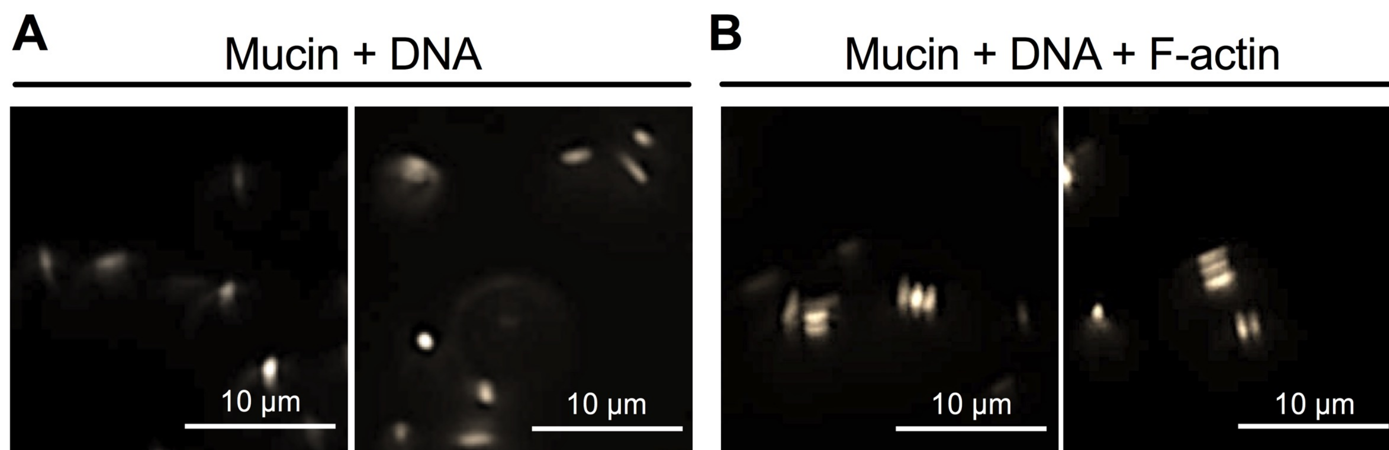

Fig. S2. Laterally-aligned aggregates characteristic of depletion-mediated aggregation were observed at low (10^5 CFU/ml) bacterial densities in physiologic polymer mixtures. Representative images showing fluorescent *P. aeruginosa* suspended in (A) mucin (8% w/vol) and DNA (4 mg/ml) or (B) mucin (8% w/vol), DNA (4 mg/ml), and F-actin (2.5 mg/ml). The addition of F-actin to the polymer mixture produced small laterally aligned aggregates.

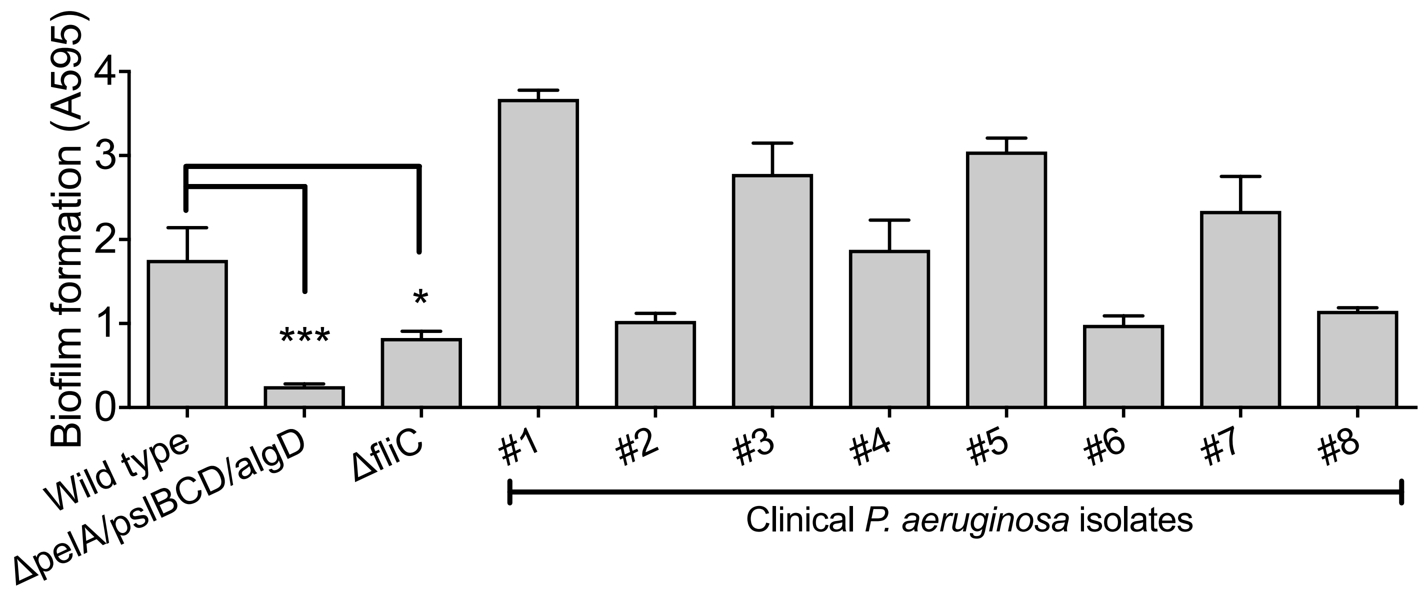

Fig S3. *In vitro* biofilm formation. The ability of the indicated *P. aeruginosa* strains to form biofilms *in vitro* was measured using the crystal violet assay (2). Results are the mean ± SD of 4 experiments, *$p<0.05$, ***$p<0.001$, relative to wild type.

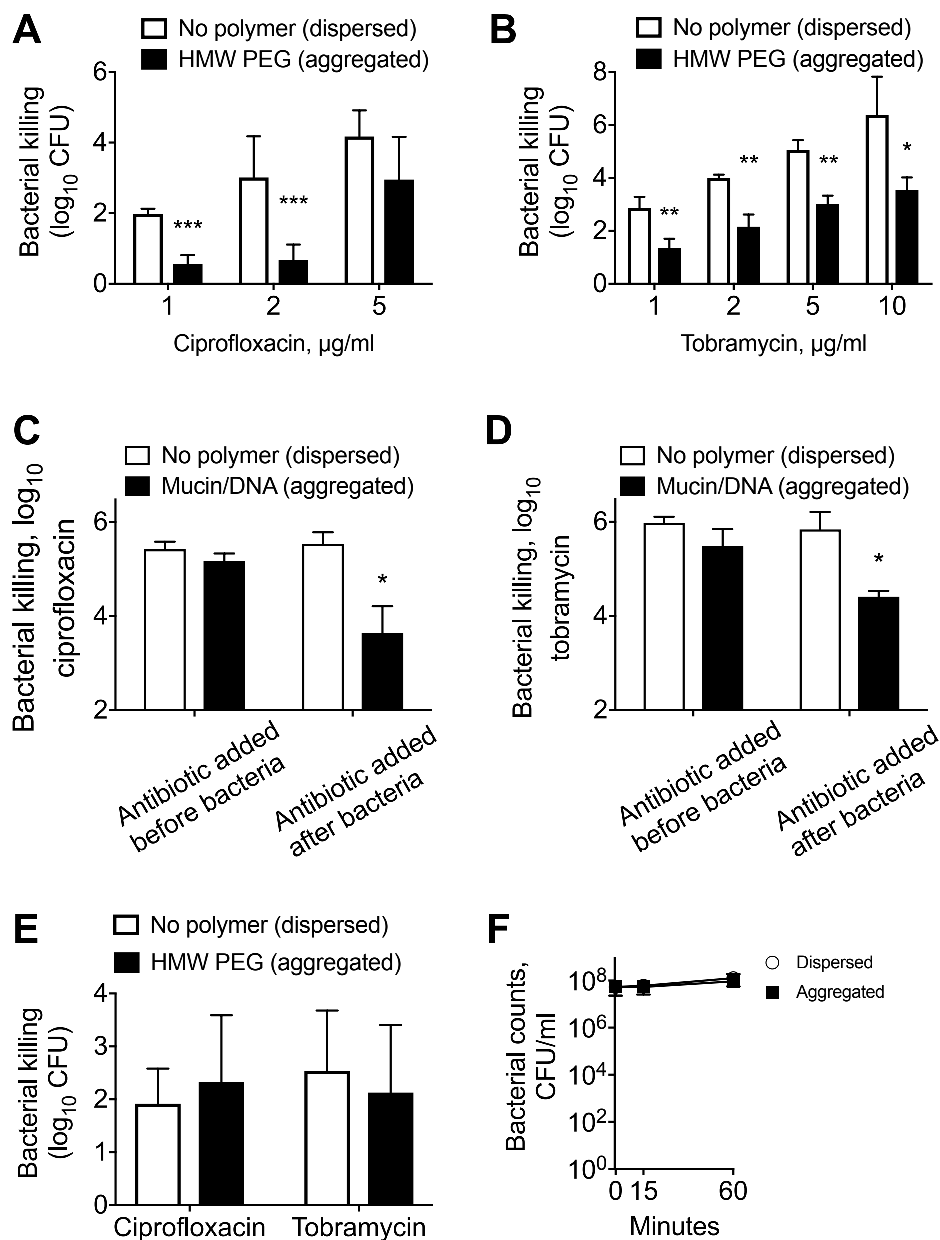

Fig. S4. Depletion aggregation promotes antibiotic tolerance. (A and B) Depletion aggregation of ~10^8 CFU/ml *P. aeruginosa* was induced with HMW PEG for 1 h, then killing after a 1-h exposure to the indicated antibiotic was measured. Killing is represented as the $\log_{10}$ reduction of viable cells recovered after antibiotic treatment, as compared to untreated controls. Results are the mean ± SD of three experiments, *$p<0.05$ relative to growth medium. (C and D) A mixture of mucin (8%w/vol) and DNA (4 mg/ml) was used to promote depletion aggregation. Antibiotics were added either 15 minutes before the addition of bacteria or 15 minutes after bacteria were added (and depletion aggregation was induced). Killing is represented as the $\log_{10}$ reduction of viable cells recovered from bacteria treated with antibiotics compared to untreated controls. Results are the mean ± SD of three experiments, *$p<0.05$ relative to cultures where antibiotics were added to polymers before the bacteria. (E) Depletion aggregation of ~10^5 CFU/ml *P. aeruginosa* was induced with HMW PEG for 1 h, then killing by the indicated antibiotic was measured after 1 h. Killing is represented as the $\log_{10}$ reduction of viable cells recovered from bacteria treated with antibiotics compared to untreated controls. Results are the mean ± SD of three experiments. (F) Comparison of CFUs recovered from dispersed and aggregated (HMW PEG) cultures of *P. aeruginosa* with no antibiotic treatment. CFUs were enumerated after serial dilution. Results are the mean ± SD of three experiments.

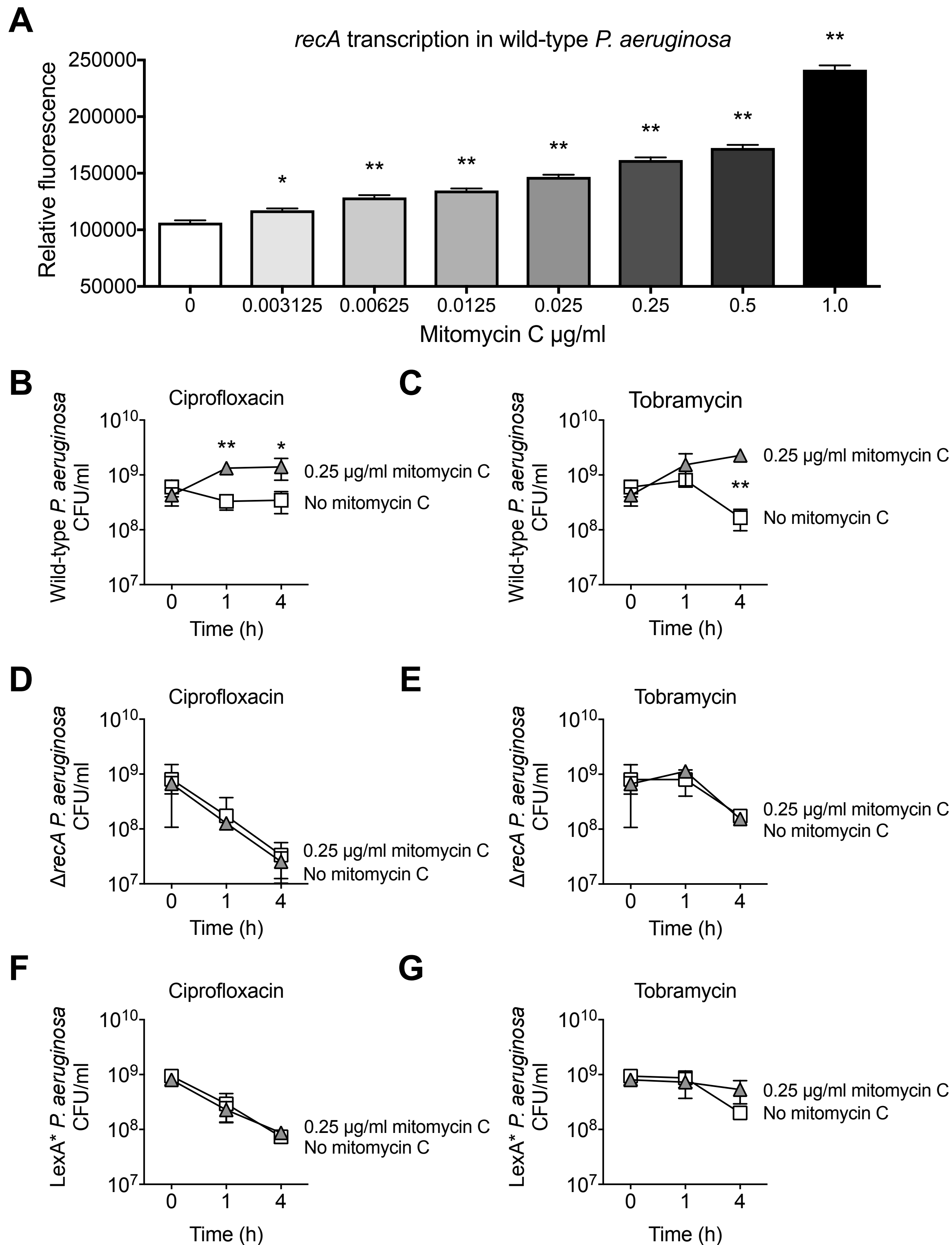

Fig. S5. Mitomycin C pre-induction of the SOS response promote antibiotic tolerance. (A) Mitomycin C-induced activation of the fluorescent transcriptional reporter $recA_p$::GFP was measured on a plate reader. Results are the mean ± SD of 3 experiments, *p<0.05; **p<0.01. (B–G) The indicated *P. aeruginosa* strains were either pre-treated or not pre-treated for 1 h with 0.25 µg/ml mitomycin C followed by exposure to ciprofloxacin (0.3 µg/ml) or tobramycin (1 µg/ml). Bacterial CFUs were enumerated at the indicated times. Results are the mean ± SD of 3 experiments, *p<0.05; **p<0.01.

Table S1. Strains and plasmids used in this study.

Strain/plasmid	Description	Source
PAO1	Wild-type *P. aeruginosa*	(5)
ΔfliC PAO1	Non-flagellated *P. aeruginosa*	(6)
ΔpelA/pslBCD/algD PAO1	Exopolysaccharide deficient	(7)
ΔrecA PAO1	Clean *recA* deletion	(8)
LexA ^{G86V} PAO1	Autoproteolytic resistant LexA	(9)
PrtR ^{S162A} PAO1	Autoproteolytic resistant PrtR	(9)
AlpR ^{S153A} PAO1	Autoproteolytic resistant AlpR	(9)
ΔsulA PAO1 (PW6038)	*sulA*-D06::ISlacZ/hah	(10)
ΔpilA	Type IV pilus-deficient	(11)
recA$_{p}$::GFP	*recA* transcription reporter	(12)

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