SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Construction of strains

Sequence data for *P. aeruginosa* PAO1 was obtained from the *Pseudomonas* Genome Database (www.pseudomonas.com) (Stover et al., 2000; Winsor et al., 2011). Genomic DNA from PAO1 was prepared using the DNeasy Blood and Tissue Kit (Qiagen). All PCR fragments were gel purified using the Qiaex II Gel Extraction Kit (Qiagen).

All deletion mutants constructed in this study are unmarked, in-frame deletions of the genes and are produced by allelic exchange. The constructs used for allelic exchange were constructed as follows. The regions flanking the gene were produced by PCR of genomic PAO1 DNA using the UpF/UpR or DownF/DownR primer pairs (Table S2). These fragments were connected by SOE PCR and inserted into pDONRPEX18Gm using Gateway Technology (Invitrogen) to produce the deletion constructs. The deletion constructs were then mated into PAO1. Single recombination mutants were selected on VBMM agar (Vogel and Bonner, 1956) containing 100 µg/ml gentamicin or LB agar containing 100 µg/ml gentamicin and 25 µg/ml irgasan. To isolate double recombinants, strains were selected on LB without NaCl agar containing 10% sucrose and confirmed by PCR using the SeqF and SeqR primers (Table S2).

Chromosomally integrated fluorescent reporter strains were constructed using the mini-Tn7 system as previously described (Choi and Schweizer, 2006). For the mini-Tn7-compatible plasmids made in this study, plasmids containing *gfp* (pBT292) and the promoter regions (pBT244 and pBT323) were first made individually (see below) and then recombined into pUC18-miniTn7T2-Gm-GW using MultiSite Gateway Technology to create pBT331 and pBT332. For pBT292, *gfpmut3* was PCRed from pBK-miniTn7-

gfp (Lambertsen et al., 2004) using the gfp F/R primer pair and recombined into pDONR P1-P5r. For pBT244, QuikChange (Agilent Technologies) was used to introduce 65 random nucleotides into pDONR P5-P2 using the null promoter F/R primers. For pBT323, the promoter of *ibpA* was PCRed from MPAO1 using the *ibpA* promoter F/R primers and then recombined into pDONR P5-P2.

Planktonic reporter assay

Reporter strains were grown to log phase in Jensen's medium (Jensen et al., 1980) and diluted to 10^8 cells/ml in Jensen's medium containing 0 to 2 µg/ml tobramycin. Samples were tested in triplicate using a 96-well microtiter plate. GFP fluorescence and growth (via OD_{600}) for each condition was sampled every hour for 6 h using a Synergy Hybrid H1 Multi-mode Microplate Reader (Bio-Tek Instruments). To normalize the data, the GFP fluorescence was divided by the OD_{600} for each data point. This ratio was then divided by the ratio of the corresponding time point for the promoter-less GFP strain. The ratio at the zero time point was then subtracted. Finally, the resulting ratios of the triplicate samples were averaged.

Planktonic control for MBEC viability assay

Wells of a 96-well microtiter plate were inoculated with 2×10^6 CFU in 10% TSB and challenged with a checkerboard arrangement of $MnSO_4$ (0 – 20 mM) and tobramycin (0 – 0.25 µg/ml). The plates were incubated statically at 37° for 24 h. Cells were plated for viable cell counting as previously described (Harrison et al., 2010).

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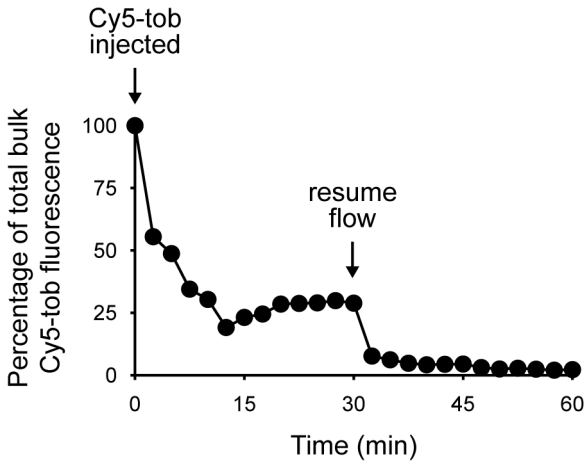
Table S1. Plasmids used in this study.

PLASMIDS	Relevant characteristics	Source
pTNS2	T7 transposase expression vector	(Choi and Schweizer, 2006)
pBK-mini-Tn7-*rrnBP1-gfpAGA*	miniTn7 transposon with a short half-life allele of *gfp* driven by the *rrnBP1* promoter; Ap^r, Gm^r	(Lambertsen et al., 2004)
pBK-mini-Tn7-*gfp*	miniTn7 transposon with *gfp* driven by the A1/04/03 promoter; Ap^r, Gm^r	(Lambertsen et al., 2004)
pUC18-miniTn7T2-Gm-GW	GateWay compatible miniTn7 vector with attR1 and attR2 recombination sites and *ccdB*; Ap^r, Gm^r, Cm^r	(Zhao et al., in press)
pDONR221 P1-P5r	GateWay compatible vector with attP1 and attP5r recombination sites and *ccdB*; Kn^r and Cm^r	Invitrogen
pDONR221 P5-P2	GateWay compatible vector with attP5 and attP2 recombination sites and *ccdB*; Kn^r and Cm^r	Invitrogen
pBT292	A GateWay compatible plasmid containing *gfpmut3* flanked by attR5 and attL1 recombination sites; Kn^r	This study
pBT244	A GateWay compatible plasmid containing a random sequence of 65 nts flanked by attL2 and attL5 recombination sites; Kn^r	This study
pBT323	A GateWay compatible plasmid containing the *ibpA* promoter flanked by attL2 and attL5 recombination sites; Kn^r	This study
pBT331	miniTn7 transposon with *gfp* driven by the *ibpA* promoter; Ap^r, Gm^r	This study
pBT332	miniTn7 transposon with a promoterless *gfp*; Ap^r, Gm^r	This study
pDONRPEX18Gm	A pEX18-based, GateWay-compatible suicide vector with attP1 and attP2 recombination sites and *ccdB*; Gm^r and Cm^r	Joe J. Harrison
pBT341	pDONRPEX18Gm with *ndvB* deletion construct; Gm^r	This study
pBT342	pDONRPEX18Gm with *algD* deletion construct; Gm^r	This study
pBT343	pDONRPEX18Gm with *arr* deletion construct; Gm^r	This study
pJJH228	pDONRPEX18Gm with *phoQ* deletion construct; Gm^r	This study
pJJH255	pDONRPEX18Gm with *brlR* deletion construct; Gm^r	This study
pJP362	pDONRPEX18Gm with PA0615 – PA0629 deletion construct; Gm^r	This study
pJJH279	pDONRPEX18Gm with *wbpL* deletion construct; Gm^r	Pradeep R. Singh
pEX18Gm::Δ*pelF*	pEX18Gm with *pelF* deletion construct; Gm^r	(Colvin et al., 2011)

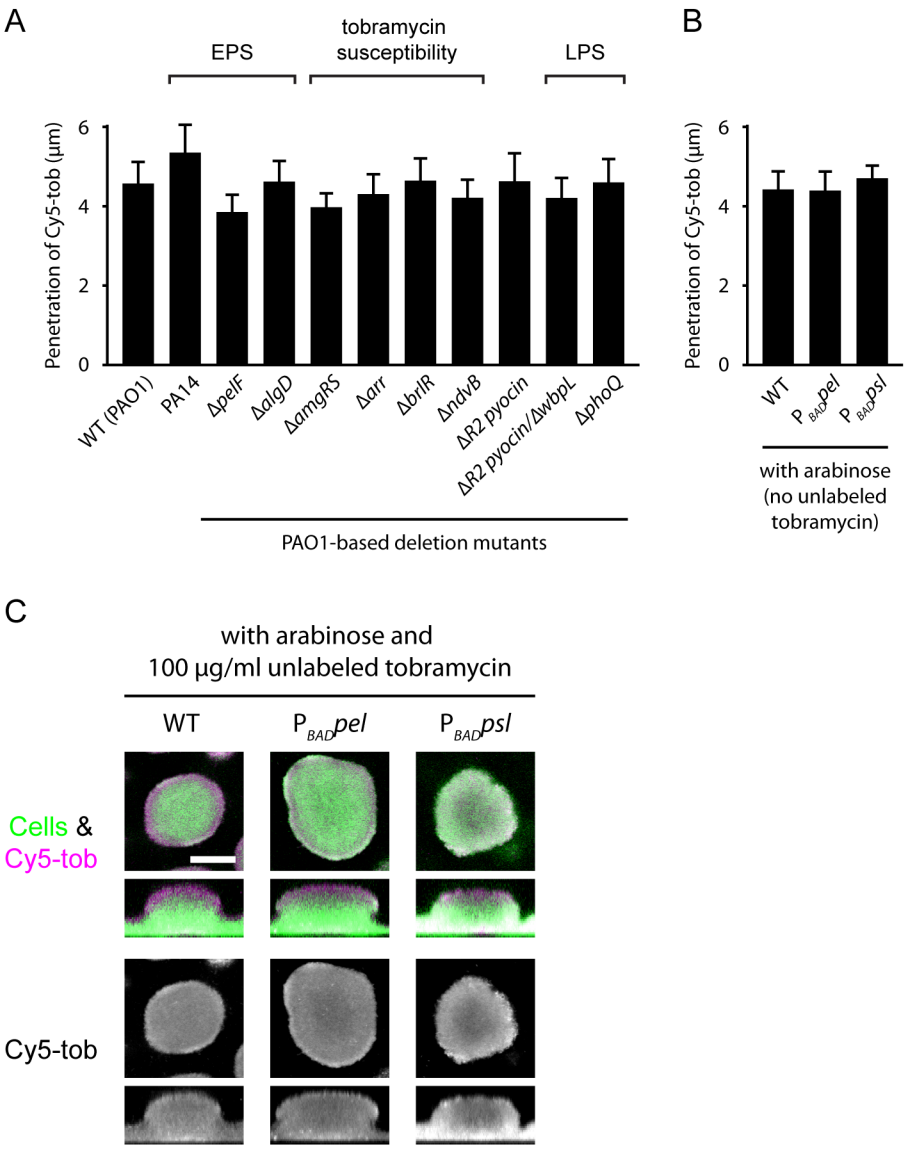
Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Kn, kanamycin

Table S2. Oligonucleotides used in this study.

PRIMERS	Sequence
OBT339 (gfp F)	GGGGACAACTTTTGTATACAAAGTTGTTTCGAAAATTAATACGACTCACTATAGGGAG ACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATATCCA TGCGTAAAGGAGAAGAACTTTTCACTG
OBT340 (gfp R)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGTTACTTGTACAGCTCGTCCATGCC
OBT383 (ibpA promoter F)	GGGGACAACTTTGTATACAAAAGTTGCGGGCGAGGGAAAAAGCGTTGCTCATTG
OBT384 (ibpA promoter R)	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAAGGTCGGGATTGCGCGTTCCAT
OBT296 (null promoter F)	ATGCCAACTTTGTATACAAAAGTTGCGTCACCTTTTCGCACTCCGCGAAAGTTCGTAC CCGCGGACGGCTCTACCAGAACCAGCCTACCC
OBT297 (null promoter R)	GGGTAGGCTGGTTCTGGTAGAGCCGTCCGCGGGTACGAACTTTCGCGGAGTGCGAA AAGGTGACGCAACTTTTGTATACAAAGTTGGCAT
OBT393 (algD UpF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCGCCATGCATTCTGCAACTAGTG
OBT394 (algD UpR)	CTACCAGCAGATGCCCTCGGCGATGCTGATTCGCATCGCATTCAC
OBT395 (algD DownF)	GCCGAGGGCATCTGCTGGTAG
OBT396 (algD DownR)	GGGGACCACTTTGTACAAGAAAGCTGGGTACCTCGTCGGACATCTCGACGATG
OBT399 (algD SeqF)	AGCCCTTGTGGCGAATAG
OBT400 (algD SeqR)	GCTTGTGCCACTCGCTC
OBT401 (arr UpF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGGCCGAACGGGCAACAAGTTGTAAC
OBT402 (arr UpR)	CTAGCTCGCCGCTACGCGCGGCCTGCCGATCTGCATAG
OBT403 (arr DownF)	CGCGTAGCGGCGAGCTAG
OBT404 (arr DownR)	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCGCCGCGAAACCCTTTATACTG
OBT407 (arr SeqF)	CTGGAAGCCGGAAAGATC
OBT408 (arr SeqR)	GCGATCCGCTGAAACTG
JJH1169 (brlR UpF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGTCATCTCGCTCACCGAGG
JJH1170 (brlR UpR)	GGCAAGCGCTGATTCAGTAGATGGGCAGTTGGCCGATGGTGAGC
JJH1171 (brlR DownF)	CCCATCTACTGAATCAGCGC
JJH1172 (brlR DownR)	GGGGACCACTTTGTACAAGAAAGCTGGGTACTACCTGGTGACCAAGCTG
OBT385 (ndvB UpF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACATGGTCTCGCGGGTGACGAAATC
OBT386 (ndvB UpR)	GACCTCAACCGCCGATCTGCTCGAGCCCGATCTTGCGTGAAGACAT
OBT387 (ndvB DownF)	GAGCAGATCGGCGGTTGAGGTC
OBT388 (ndvB DownR)	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGGCGATGGCACCCTTGTGCTC
OBT391 (ndvB SeqF)	GCGCGAGAAGTTGTAGTC
OBT392 (ndvB SeqR)	TTCGACCACCGCCTTG
JJH1083 (phoQ UpF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCAAGTCCTTCCCGATCCTG
JJH1084 (phoQ UpR)	GTCTCAGACTGTAGCGAAACGTATGCGGATGCGCAGGGAACGGATC
JJH1085 (phoQ DownF)	CGCATACGTTTCGCTACAGTC
JJH1086 (phoQ DownR)	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAATTCGAAGTCCTCGCGC
JJH1187 (R2 pyocin SeqF)	GTCCCATGCACAGCAGCG
JJH1188 (R2 pyocin SeqR)	GATAATGGCGGGCAACCAGG
JJH1203 (wbpL UpF)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCTTCGGCCGTTTGCTCAAG
JJH1204 (wbpL UpR)	CCAAGGAACCCGCCTTGTATCCTACGACAACTAGACACGCGATCATC
JJH1205 (wbpL DownF)	GTAGGATACAAGGCGGGTTC
JJH1206 (wbpL DownR)	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCTCGGAGATGTCGGTTTG
JJH1257 (wbpL SeqF)	GCTGAAGCTTGTAATGTGC
JJH1258 (wbpL SeqR)	CGACACAGCTCCTTCAC

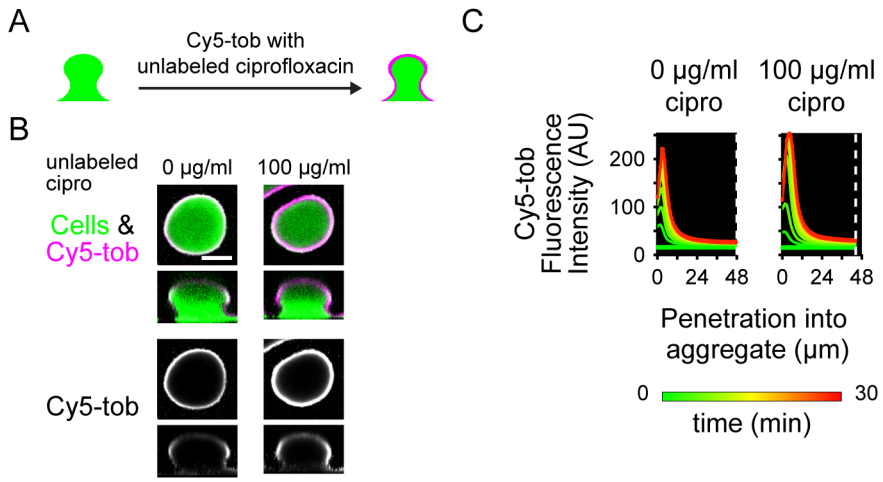


Supplemental Figure S1. Cy5-tobramycin fluorescence in the bulk fluid. Biofilms of a GFP-expressing PAO1 strain were treated statically with 20 µg/ml Cy5-tobramycin for 30 min. Confocal laser scanning micrographs were acquired in the bulk fluid above the biofilm every 2.5 min during the static incubation and a subsequent 30-min wash. Average fluorescence for each time point was determined and divided by the peak fluorescence. A representative graph showing the remaining fluorescence versus time is shown. Time of Cy5-tobramycin injection and resumption of flow are marked by arrows.

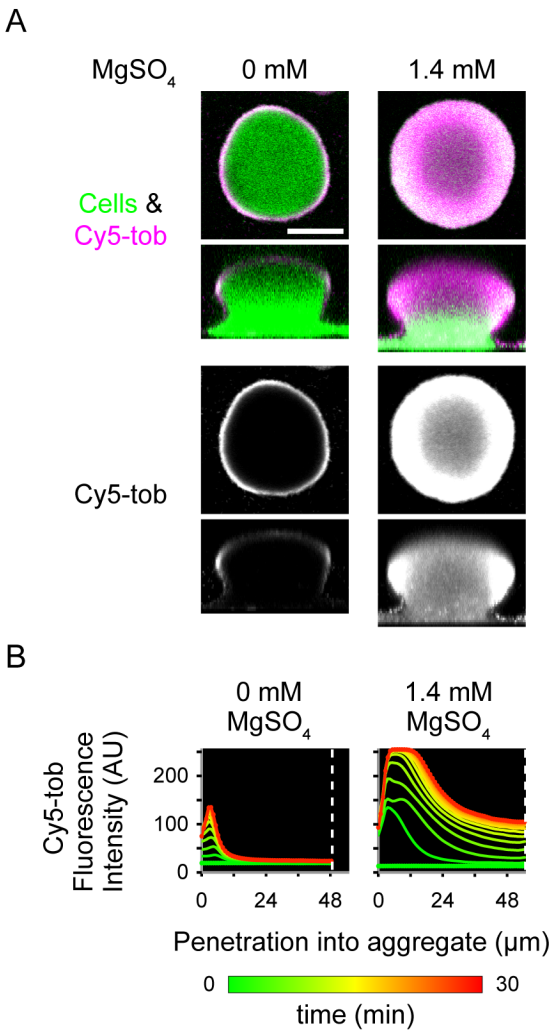


Supplemental Figure S2. Mutants of biofilm matrix components and tobramycin susceptibility do not affect the penetration of tobramycin. A and B. The average penetration of Cy5-tobramycin into biofilms. Biofilms were treated statically with 20 μg/ml Cy5-conjugated tobramycin for 30 min before imaging. The depth of penetration was determined using the z-slice containing the widest part of the biofilm. PA14, a strain that cannot produce Psl but produces a biofilm similar to wild-type PAO1, was used in place of a PAO1 Δ*psl* strain since Psl is required for biofilm formation in PAO1

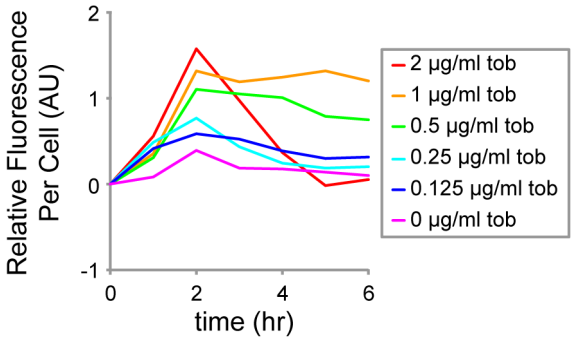
(Friedman and Kolter, 2004; Jackson et al., 2004; Matsukawa and Greenberg, 2004). The ability to produce R2 pyocin had to be removed in the Δ*wbpL* strain because *wbpL* is essential in the presence of the self-produced toxin (J. Penterman and P. R. Singh, unpublished). Error bars represent one standard deviation. C. Biofilms of GFP-expressing wild type PAO1, PAO1 over-producing Pel (P_{BAD}*pel*), and PAO1 over-producing Psl (P_{BAD}*psl*) were grown in the presence of 0.02% arabinose. Biofilms were treated statically with 20 μg/ml Cy5-conjugated tobramycin and 100 μg/ml unlabeled tobramycin for 30 min before imaging. Representative micrographs are displayed as in Figure 1. Bar, 50 μm.



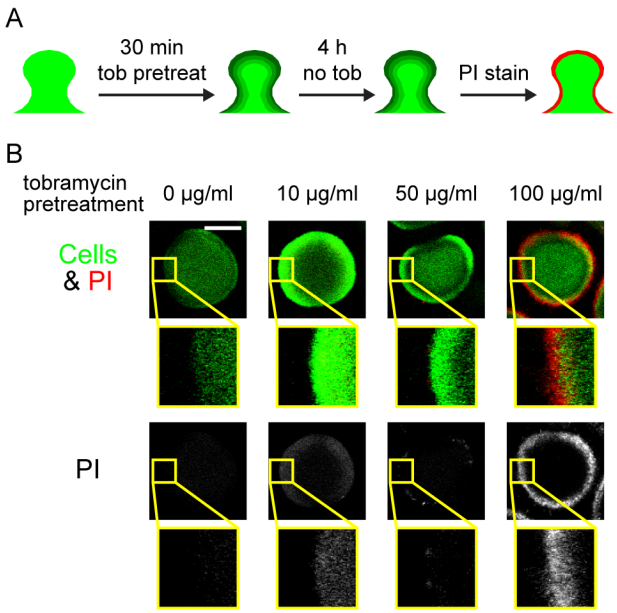
Supplemental Figure S3. The limited penetration tobramycin is not affected by ciprofloxacin treatment. Biofilms of a GFP-expressing PAO1 strain were treated statically with 20 µg/ml Cy5-tobramycin and 0 (control) or 100 µg/ml ciprofloxacin concurrently. Confocal laser scanning micrographs were acquired every 2.5 min during the 30-min static incubation. Representative micrographs are displayed as in Figure 1. Bar, 50 µm. B. Graphs, as described in Figure 2, representing the Cy5-tobramycin penetration kinetics.



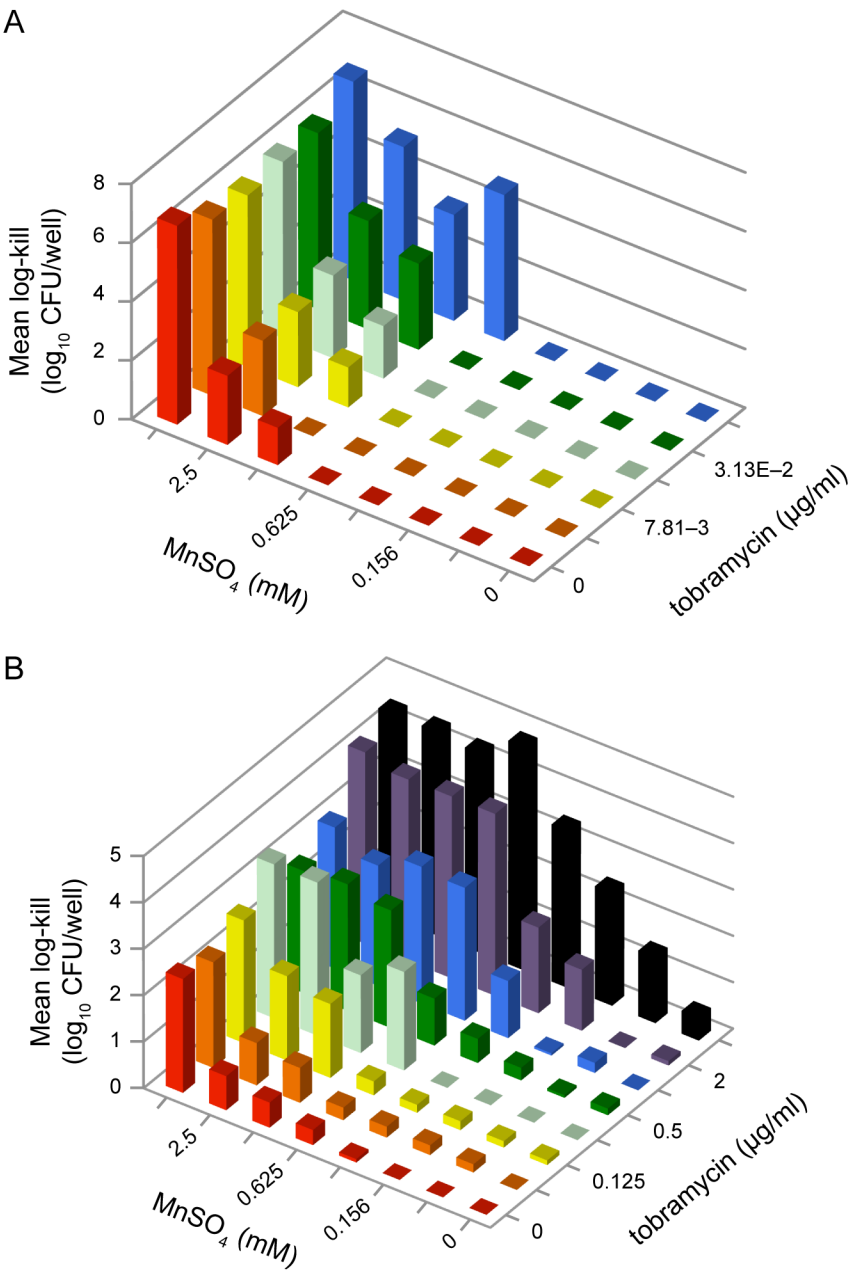
Supplemental Figure S4. High concentrations of magnesium allow tobramycin to penetrate the biofilm. Biofilms of a GFP-expressing PAO1 strain were treated statically with 20 μg/ml Cy5-tobramycin and 0 or 1.4 mM $MgSO_4$ concurrently. Confocal laser scanning micrographs were acquired every 2.5 min during the 30-min static incubation. A. Representative micrographs are displayed as in Figure 1. Bar, 50 μm. B. Graphs, as described in Figure 2, representing the Cy5-tobramycin penetration kinetics.



Supplemental Figure S5. *ibpA* reporter responds to tobramycin in planktonic culture. Planktonic cultures of PAO1 Tn7::P_{ibpA}*gfp* were treated with the indicated concentration of tobramycin. The GFP fluorescence and the OD_{600} of the culture were measured every hour for 6 h. The y-axis represents a normalized relative fluorescence per cell (see Supplemental Methods and Materials). Samples were treated in triplicate and the experiment was repeated three times. A representative graph is shown. The fluorescence of the cells treated with 2 µg/ml tobramycin dropped after 2 h due to planktonic cell death (as indicated by a decrease in the OD_{600}).



Supplemental Figure S6. The penetration of the biofilm by a short, high dosage of tobramycin does not greatly affect cell viability. A. Schematic of experiment. Biofilms of PAO1 were treated with 0, 10, 50, or 100 µg/ml tobramycin sulfate for 30 min followed by continuous media flow (no antibiotic treatment) for 4 h. Biofilms were then stained with propidium iodide for viability and then imaged. B. Representative micrographs are shown. In the top row, the biomass is pseudo-colored green and PI, red. The bottom row is PI alone in grayscale. Underneath each cross-sectional image is a 4.25x magnified region (inset yellow box). Bar, 50 µm.



Supplemental Figure S7. Greater synergistic killing of $MnSO_4$ and tobramycin is observed in biofilm cells in comparison to planktonic cells. Planktonic cells were grown in a 96 well plate (A) and biofilms were grown on polystyrene pegs (B). The cells were then challenged with various combinations of tobramycin and $MnSO_4$. Viable cell counts were determined by spot dilution plating. Mean log-kill was determined by

subtracting the final from the initial $\log_{10}$-transformed cell counts. At the end of the experiment, untreated growth control samples contained 8.56 ± 0.07 $\log_{10}$-transformed CFU per well for planktonic cells and 5.47 ± 0.41 $\log_{10}$-transformed CFU per peg for biofilm cells. Bars of the same color represent samples treated with the same concentration of tobramycin. Samples that did not grow or that grew (e.g. samples with mean log-kill values ≤ 0) are displayed as 0. The results represent the average of three independent trials.