An Anti-Persister Strategy for the Treatment of Chronic *Pseudomonas aeruginosa* Infections Supplemental Material

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Running Head: An Anti-Persister Strategy for *Pseudomonas* Infections

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The effect of the potentiator on tobramycin (TOB) MICs: MICs were determined for TOB vs. four strains of *Pseudomonas aeruginosa* in the presence of increasing concentrations of fumarate (Table S1). As anticipated, fumarate had no effect on the observed MICs since MICs measure the minimum concentration of a compound required to inhibit growth of the bacteria. Fumarate appears to enhance the killing of *P. aeruginosa* persisters, which by definition are not growing, through a proton motive force (PMF) mechanism that increases aminoglycoside uptake and potentially cellular respiration, and therefore it does not potentiate the activity of the aminoglycoside on growing bacteria.

Materials and methods

Media preparation: Tryptic soy broth (TSB) media (VWR International, Cat.# 101320-636) was prepared per manufacturer instructions and sterilized for 20 min using autoclaving.

Variation of pH in the PSP time kill potentiation assay: These experiments followed the general steps described in the *Planktonic stationary phase (PSP) time kill potentiation assay* Methods section of the main manuscript. More specifically, all steps up to the preparation of each combination of TOB and fumarate to be tested were as described previously. To determine the effect of media at different pH values, 1X M9 media was prepared at three different pH levels, 5.6, 7, and 8.5, and then the pH was measured and adjusted as needed utilizing 0.25 N hydrochloric acid and 2N sodium hydroxide. Next, each TOB and fumarate concentration was prepared at 2X of the final desired concentration in 0.5 mL of media. 0.5mL of the 2X fumarate plus TOB combination was mixed with 0.5mL of the re-suspended cells for a final 1X 1mL volume in an Eppendorf tube. All combinations of concentrations and pH levels were incubated for 4 h at 37°C at 200 r.p.m. After 4 h, cells were centrifuged at 5,000 x g for 5 min and washed

by re-suspending each pellet in 1mL of 1X PBS. The cells were centrifuged again at 5,000 x g for 5 min and re-suspended in 1X M9 media. Aliquots were taken from each combination and each pH level and serially diluted in a 96-well plate (#3370; Costar). 5μL aliquots were plated for each dilution on LB-agar plates. The plates were incubated overnight at 37°C and enumerated the next day.

Artificial sputum medium (ASM) preparation: Artificial sputum media (ASM) (1, 2) was prepared as follows: 5g mucin from pig stomach mucosa, 4g DNA (Sigma), 5.9mg diethylene triamine pentaacetic acid (DTPA) (Sigma), 5g NaCl, 2.2g KCl, 5mL of egg yolk emulsion (VWR International). ASM- media was as described above, while for ASM+ media 5g amino acids was added per 1L (pH 7.0) of media. A culture of the laboratory strain PAO1 was grown overnight in rich tryptic soy broth (TSB) media and an inoculum equivalent to OD_{600} of 0.05 of the overnight culture was added to 1mL of artificial sputum medium in 24-well cell culture coated plates. Plates were incubated for 16 h at 37°C with gentle shaking. Then, each possible combination of TOB (0, 8, or 128 μg/mL) plus fumarate (0mM or 15mM) in either ASM+ or ASM- media, respectively, was applied to a plate. The plates were then incubated for 6 h and the cells from each well were centrifuged at maximum speed for 10 min (until there is a noticeable pellet). The pellets were washed twice in 1mL of 1X PBS and re-suspended in 1X PBS. These were then serially diluted, spot plated on LB-agar plates, and enumerated for surviving CFUs on the following day.

The log phase/growth experiments: For each experiment, bacterial strains were first streaked out on LB agar plates and incubated at 37ºC overnight. The following day colonies were inoculated in 10ml LB media and incubated at 37ºC overnight with shaking (220 rpm). The cells were collected by centrifugation (5000 x g for 15min) and washed with 5mL PBS prior to re-

suspending to an OD_{620} nm of 0.1 into 10mL M9 media supplemented with 15mM fumarate (Sigma; F1506), succinate (Sigma S2378) or pyruvate (Acros 113-24-6), respectively. To aerate efficiently, the cultures were prepared in 50ml tubes and incubated at 37ºC with shaking at 220 rpm for 24h. Culture turbidities were recorded by OD at 0, 5 and 24 h post-inoculation as a measure of bacterial growth.

References

1. Diraviam Dinesh S. 2010. Artificial Sputum Medium.
2. Sriramulu DD, Lunsdorf H, Lam JS, Romling U. 2005. Microcolony formation: a novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. J Med Microbiol 54:667-76.

Table S1: MICs[a] for TOB in the Presence of Increasing Concentrations of Fumarate

***P. aeruginosa* Strain**	**TOB**	**TOB + 7.5mM Fumarate**	**TOB + 15mM Fumarate**	**TOB + 30mM Fumarate**
PAO1	0.8	0.8	0.8	0.8
PA14	0.8	0.8	0.8	0.8
14003 (mucoid)	0.8	0.8	0.8	0.8
14001 (non-mucoid)	0.4-0.8	0.4-0.8	0.8	0.8

[a] MICs are in μg/ml.

Table S2. MICs for TOB with Other *P. aeruginosa* Strains

Organism	**Strain**	**MIC (μg/ml)**
P. aeruginosa, non-mucoid	10022	8
	10039	64
	10084	128

Table S3. Results of COPD Clinical Isolate *P. aeruginosa* Strains Tested in the PSP Potentiation Assay

Strain	Year	Patient	E-test MIC[a]	Broth MIC[a]	Potentiation
16004728	2013	A (early)	1	-	5 logs
542368	2012	C (early)	0.38	0.125	3.5 logs
82346	2013	C (late)	0.064	0.125	3.5 logs
5054382	2009	D (early)	0.38	-	4 logs
5072829	2010	E (early)	1	0.25	5 logs
919193	2013	E (late)	0.016	0.25	5 logs

[a] MICs are in μg/ml.

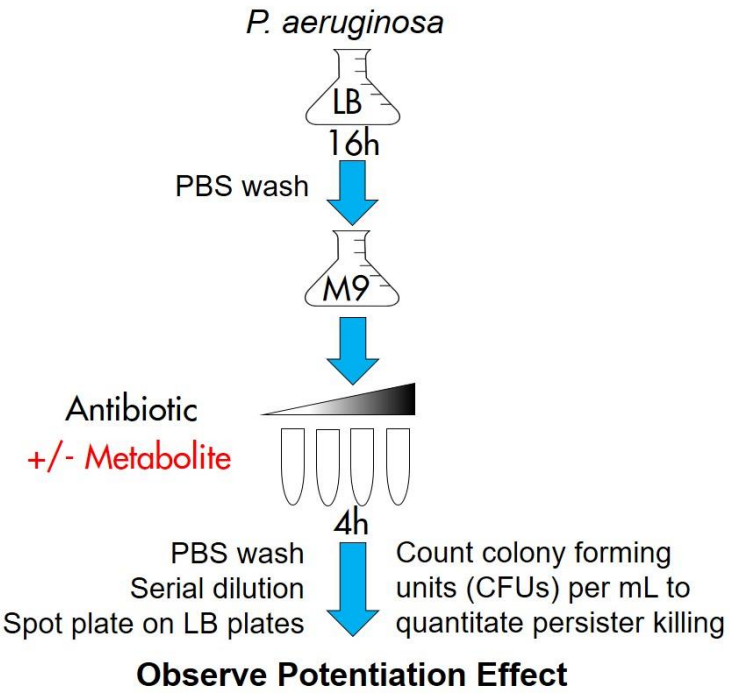

FIG S1. Workflow of PSP time kill potentiation assay. The assay involves (i) generating a culture of persisters; (ii) treating with a range of antibiotic and metabolite concentrations to identify the optimal ratio; (iii) observing the potentiation effect as enhanced persister killing.

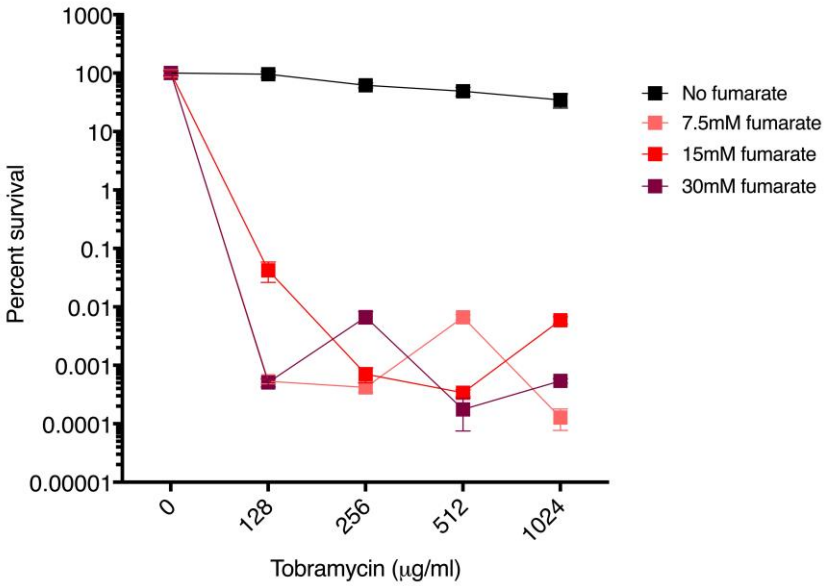

FIG S2. The potentiation effect of fumarate on TOB sensitivity is maintained at high TOB concentrations. Plot of percent survival vs. TOB concentration in the PSP assay are shown for the non-mucoid 10004 isolate at high TOB concentrations as an extension of the data presented in Fig. 2-A. Values of percent survival depict the mean ± SD (n=2).

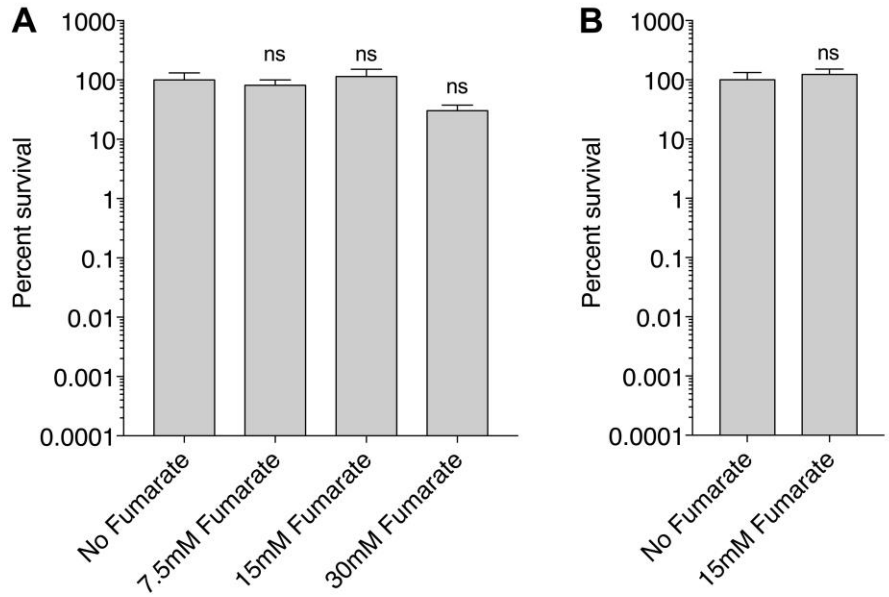

FIG S3. Fumarate alone has no effect on persister killing. Bar graphs of percent survival vs. fumarate concentration in the PSP assay are shown in (A) for the non-mucoid 10004 isolate and in (B) for the mucoid 10028 isolate at the 0 TOB concentration. Values of percent survival depict the mean ± SD (n=2). This analysis of the data presented in Fig. 2 shows that differences in the fumarate alone data points are not statistically significant in a one-way ANOVA with Dunnett's multiple comparisons test (*, $P < 0.05$).

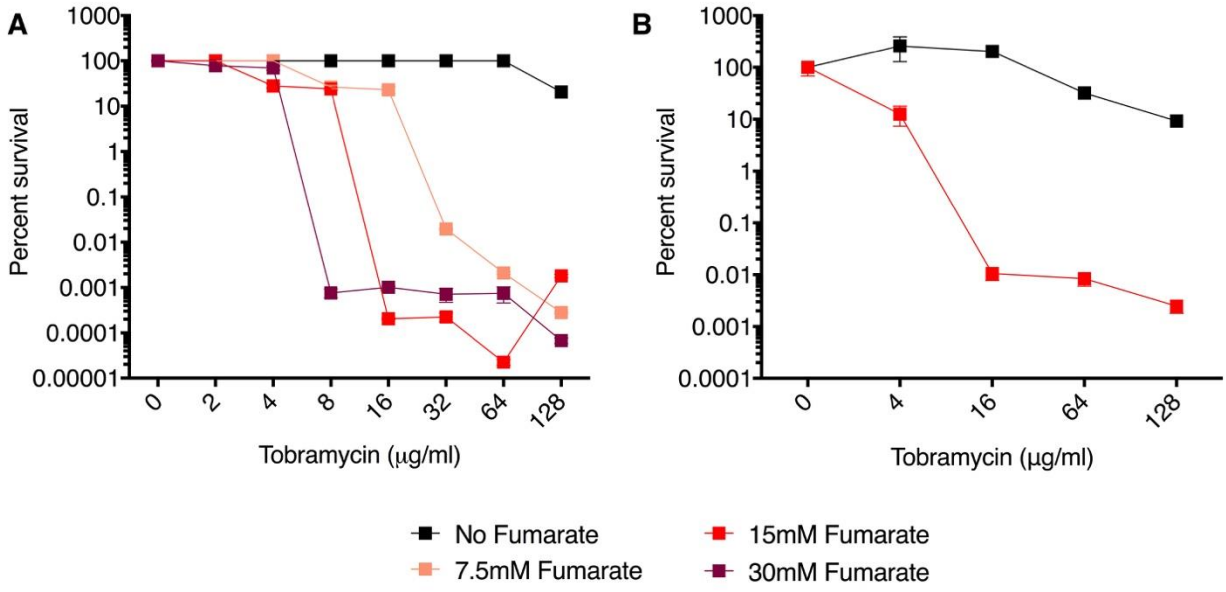

FIG S4. Potentiation effect of fumarate on TOB in *P. aeruginosa* laboratory strains. Plots of percent survival vs. TOB concentration in the PSP assay are shown in (A) for PAO1 and in (B) for PA14. Values of percent survival depict the mean ± SD (n=2).

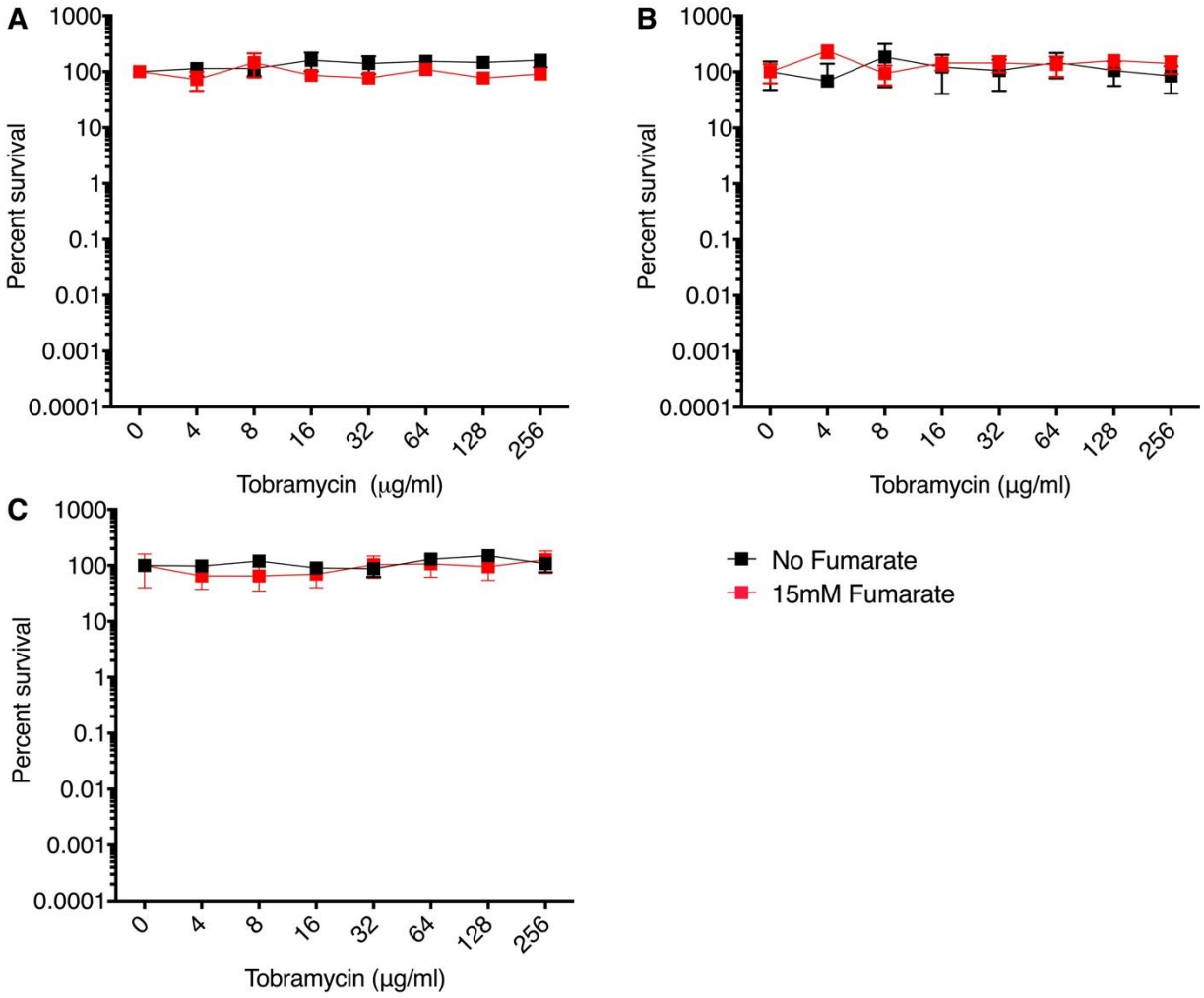

FIG S5. Potentiation effect of fumarate on TOB not observed for three TOB-resistant *P. aeruginosa* strains. A plot of percent survival vs. TOB concentration in the PSP assay is shown in (A) for strain 10022, (B) for 10039, and (C) for 10084. Values of percent survival depict the mean ± SD (n=2).

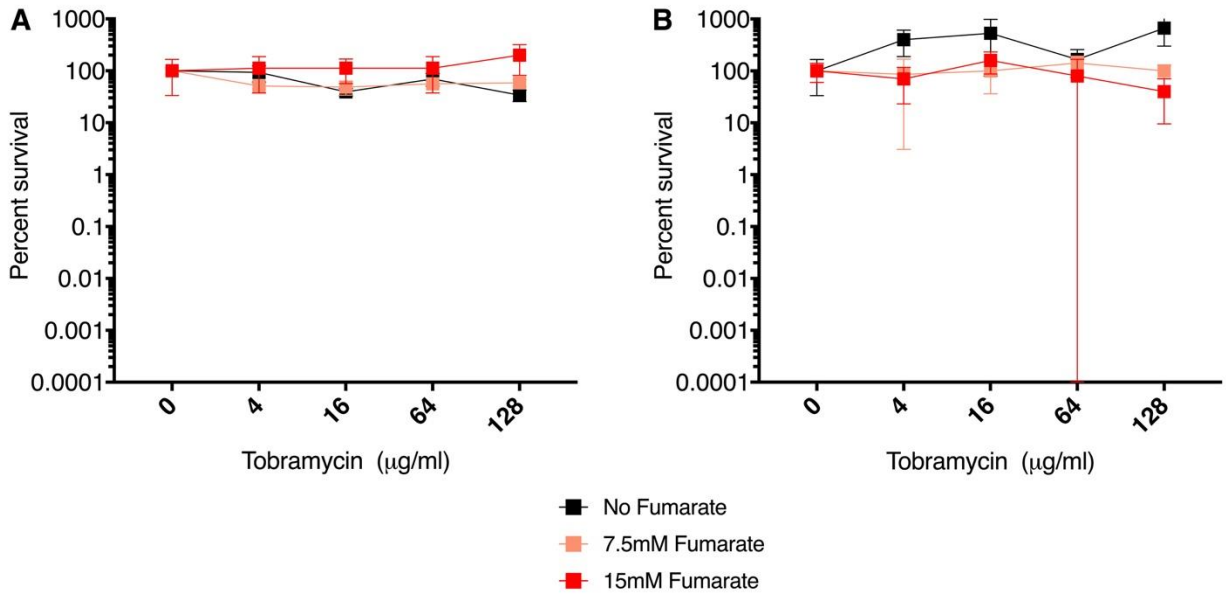

FIG S6. Potentiation effect of fumarate on TOB not observed for *P. aeruginosa* strains 10020 and 10077. Plots of percent survival vs. TOB concentration in the PSP assay are shown in (A) for 10020 and in (B) for 10077. The plots show that these particular TOB-sensitive strains are tolerant in stationary phase to TOB but are not potentiated. Values of percent survival depict the mean ± SD (n=2).

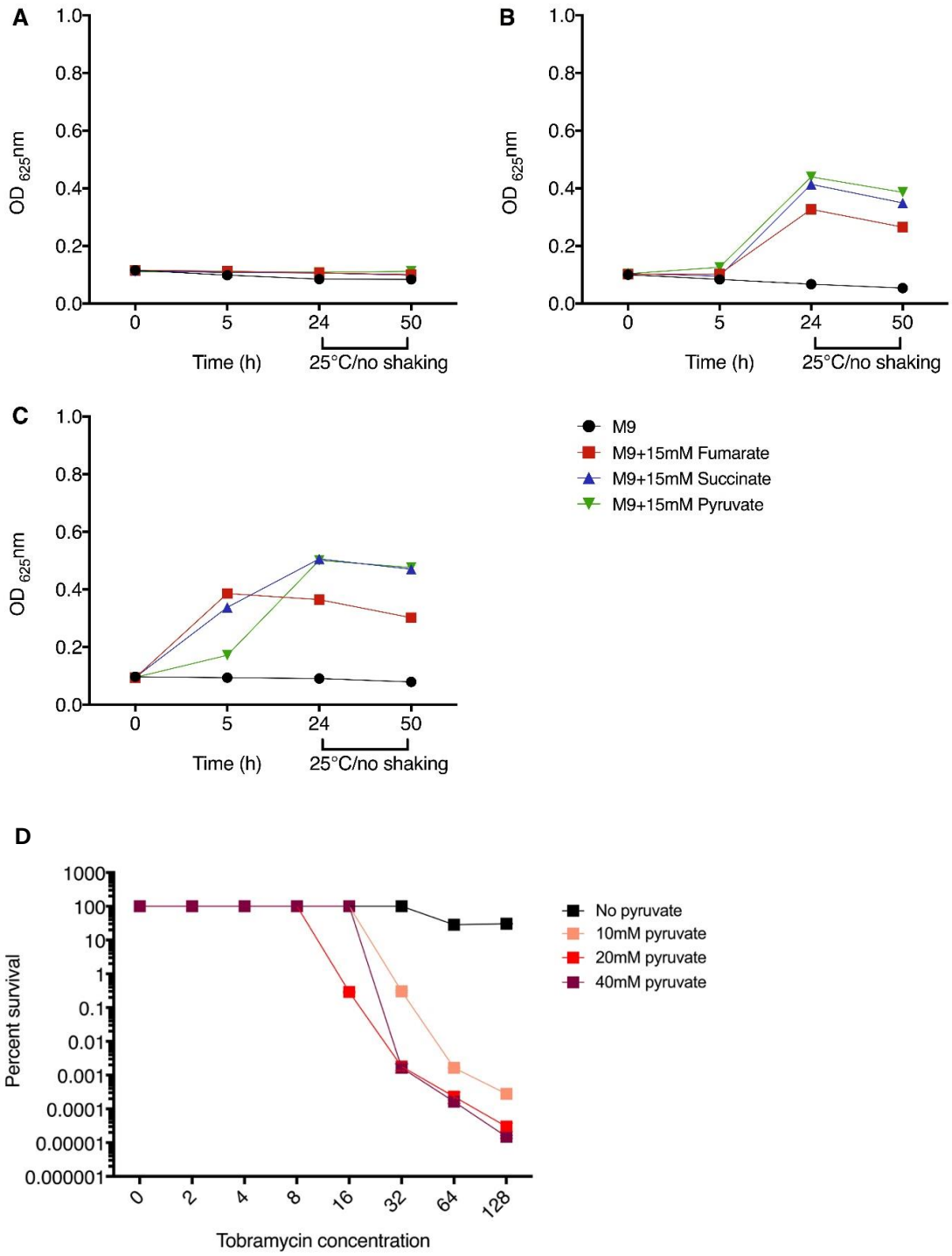

FIG S7. Growth curves for *P. aeruginosa* strains 10020 and 10077. Stationary phase cultures of 10020 and 10077 were diluted in fresh M9 media supplemented with 15mM fumarate, succinate, or pyruvate and bacterial growth was measured over 50 h. In (A) 10020 shows a growth defect over the entire 50 h; in (B) 10077 shows delayed growth for at least the first five hours of transfer to M9; and in (C) PAO1 is shown as a reference.

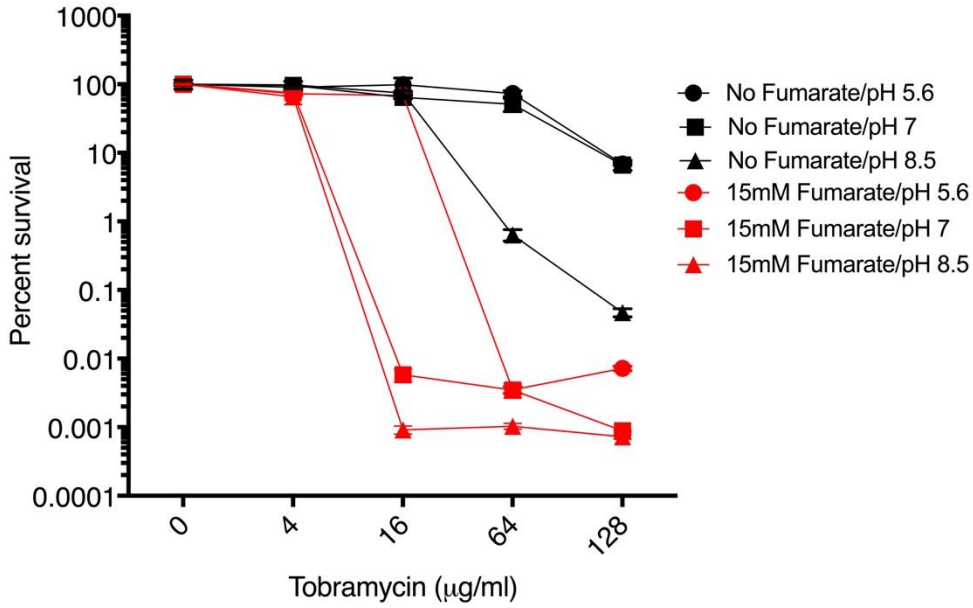

FIG S8. Potentiation effect of fumarate on TOB in *P. aeruginosa* is observed over a range of pH relevant to the CF lung. A plot of percent survival vs. TOB concentration in the PSP assay is shown for strain PA14 at pH ranging from 5.5 to 8. Values of percent survival depict the mean ± SD (n=2).

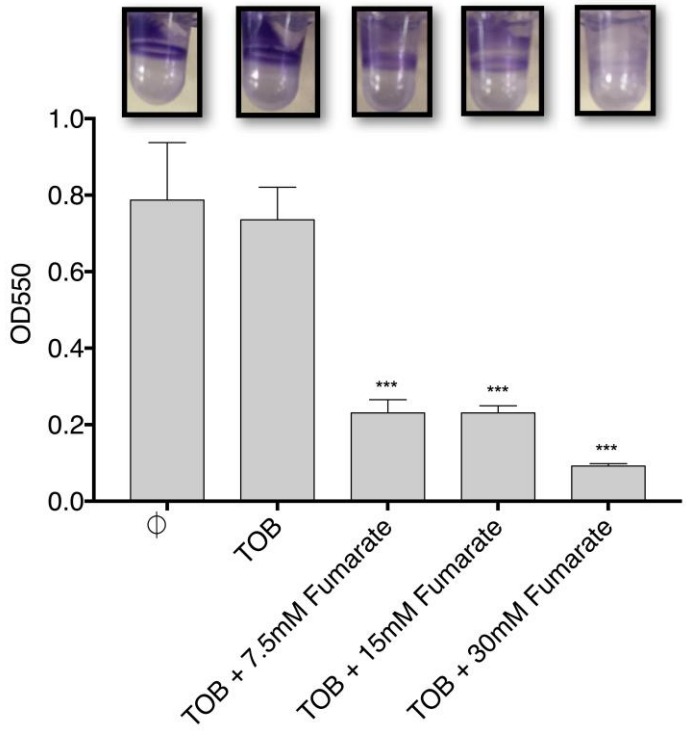

FIG S9. Potentiation observed in the 96-well plate biofilm assay for *P. aeruginosa* strain PAO1. The effect of TOB plus fumarate on the reduction of biofilm was measured using CV dye by quantifying absorbance at 550 nm. The height of each bar represents the average OD550 values of 4 replicate wells from one representative experiment, and error bars indicate standard deviations. Each experiment was carried out twice, yielding similar results. The difference in OD550 between the TOB-only control and each of the other TOB with fumarate conditions was statistically significant in a one-way ANOVA with Dunnett's multiple comparisons test (***, $P < 0.001$).

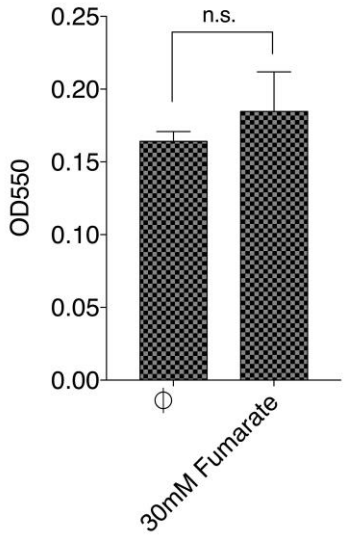

FIG S10. Fumarate alone does not reduce the biofilm in the 96-well plate assay for *P. aeruginosa* strain PA14. The effect of fumarate on the reduction of biofilm was measured using CV dye by quantifying absorbance at 550 nm. The height of each bar represents the average OD550 values of 4 replicate wells from one representative experiment, and error bars indicate standard deviations. The difference in OD550 between the null (no TOB/no fumarate) control and the 30mM fumarate well was not statistically significant in a two-tailed t-test (*, $P < 0.05$).

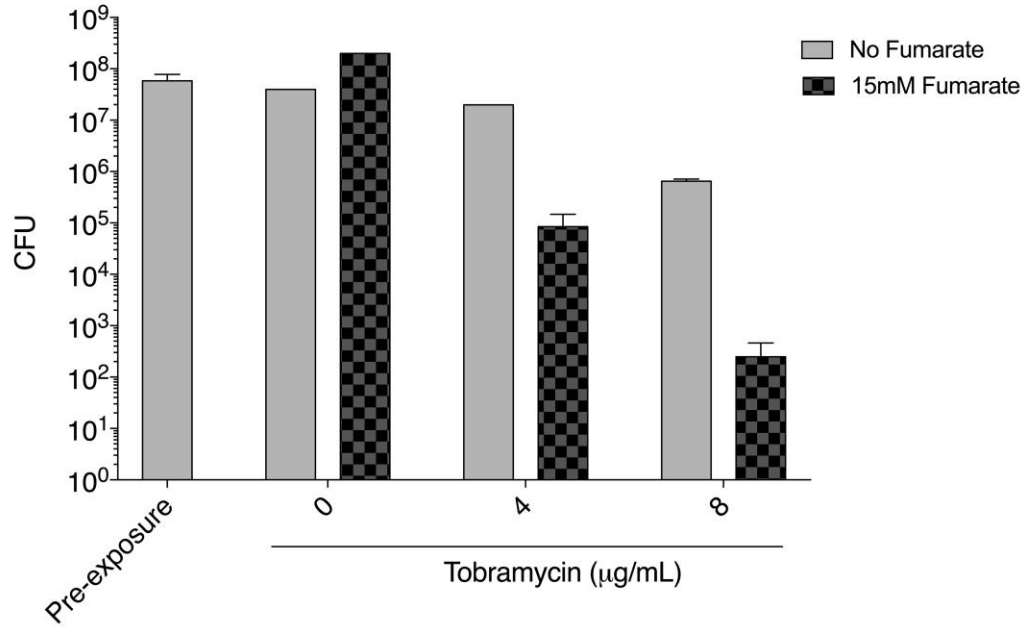

FIG S11. Pre- and post-exposure CFUs are similar in the colony biofilm models. *P. aeruginosa* strain PA14 was tested in the colony biofilm assay and the data are shown in Fig. 5B. Here the pre-exposure CFUs for the no TOB/no fumarate control are shown relative to the CFUs for the various conditions. CFU values depict the mean ± SD (n=2).

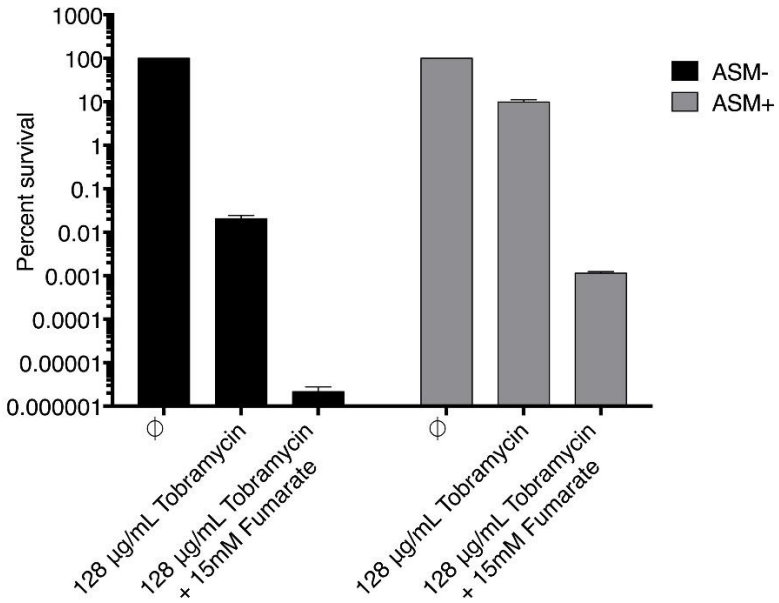

FIG S12. Potentiation observed in the presence of artificial sputum media (ASM). A plot of percent survival vs. TOB concentration in the PSP assay in the presence of ASM ± amino acids is shown for strain PAO1 strain of *P. aeruginosa*. Values of percent survival in (A) and (B) depict the mean ± SD (n=2).

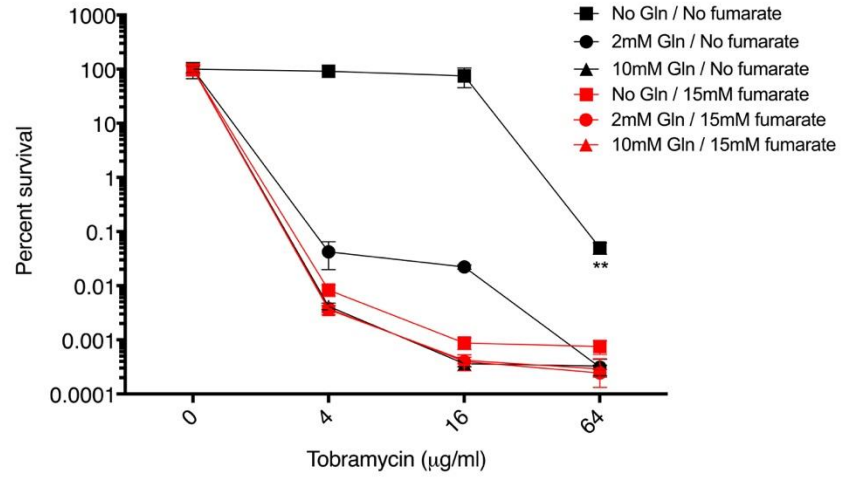

FIG S13. Potentiation effect of glutamine alone and in combination with fumarate on TOB in *P. aeruginosa*. A plot of percent survival vs. TOB concentration in the PSP assay is shown for strain PA14. **For the no glutamine/no fumarate data point, most of the pellet was accidentally aspirated which likely explains the unexpected reduction in CFUs. Values of percent survival in (A) and (B) depict the mean ± SD (n=2).

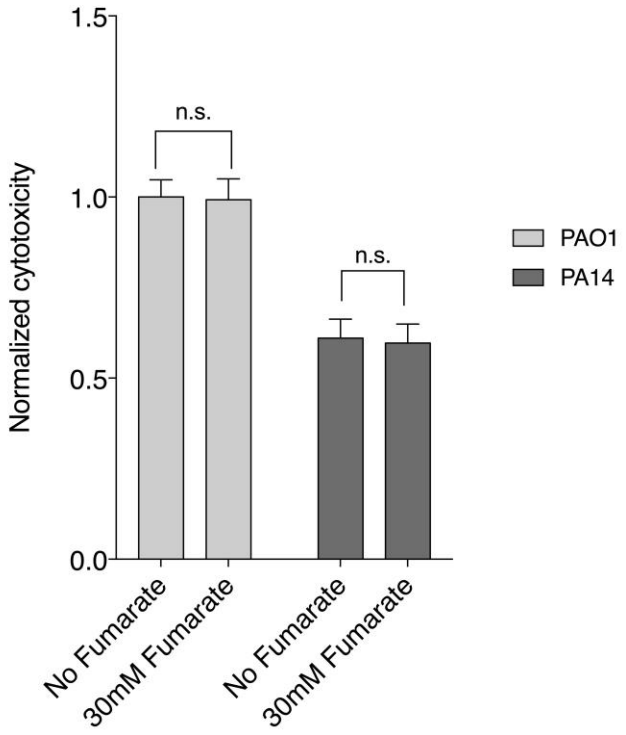

FIG S14. Fumarate alone has not effect on the cytotoxicity of *P. aeruginosa* on human epithelial cells. Cytotoxicity was measured in a colorimetric assay that quantitates the amount of lactate dehydrogenase (LDH) released into the media from damaged cells (by recording the absorbance at 490nm) as a biomarker for cellular cytotoxicity. Cytotoxicity values are normalized to the cytotoxicity of the human airway epithelial cells in response to PAO1 *P. aeruginosa* untreated with TOB or fumarate. Means ± SD (n=3) are depicted, and statistically significant differences in a two-tailed t-test are indicated (*, $P < 0.05$; n.s. = not significant).