SUPPLEMENTAL MATERIAL

Conceptual Model of Biofilm Antibiotic Tolerance that Integrates Phenomena of Diffusion, Metabolism, Gene Expression, and Physiology

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Table S1. Parameter values for reaction-diffusion modeling of oxygen penetration into *P. aeruginosa* drip-flow biofilm.

Parameter	Value	Source
cell volume fraction	0.212	to match experimental biomass concentration of 63,500 g m^{-3}
biomass intrinsic density	300,000 g m^{-3}	typical bacterium
external mass transfer liquid layer thickness	10^{-2} or 10^{2} μm	to bound anticipated dimensions
biofilm relative effective diffusion coefficient	0.6	(1)
oxygen aqueous diffusion coefficient	2.11 x 10^{-4} m^2 d^{-1}	(1)
lactic acid aqueous diffusion coefficient	1.12 x 10^{-4} m^2 d^{-1}	(2)
bulk fluid oxygen concentration	6 g m^{-3}	air saturation in Bozeman, MT
maximum specific growth rate	26.2 d^{-1}	experimentally measured
yield coefficient, biomass on oxygen	1.0 g g^{-1}	typical value
yield coefficient, biomass on lactate	0.27 g g^{-1}	from elemental balances
Monod coefficient for oxygen	0.39 g m^{-3}	from data in (3)

Table S2. Analysis of ammonium production, pH change, and urea uptake in *P. aeruginosa* biofilm reactors. Ammonium production and urea uptake was determined as the difference between the concentration in the fresh medium and biofilm reactor effluent. WT – wild type; *ureA* – urease mutant; WT – no urea = urea omitted from medium. Values are given as the mean ± standard deviation.

Condition	(mM) NH_3	ΔpH	(mM) Urea
WT	1.49 ± 0.14	-0.09 ± 0.12	0.65 ± 0.34
urea	1.56 ± 0.34	-0.28 ± 0.33	0.23 ± 0.32
WT – no urea	1.51 ± 0.03	-0.01 ± 0.01	0

Table S3. Biofilm composition. Values are given as the mean ± standard deviation.

Condition	($\mu g\ cm^{-2}$) Protein	($\mu g\ cm^{-2}$) Carbohydrate	($\mu g\ cm^{-2}$) DNA
Untreated	362 ± 141	184 ± 20	13.9 ± 1.5
CIP-treated	83.4 ± 37.4	109 ± 16	10.7 ± 2.5

Table S4. Calculated oxygen requirement for Psl extracellular polysaccharide biosynthesis from various substrates in *P. aeruginosa*.

Substrate	(C-mole PSL/mole O_2) Yield
Alanine	3.96
Arginine	2.54
Aspartate	7.26
Cysteine	0.00
Glutamate	3.62
Glutamine	3.47
Glycine	3.21
Histidine	2.54
Isoleucine	1.54
Leucine	1.54
Lysine	0.87
Methionine	0.00
Phenylalanine	1.30
Proline	2.08
Serine	2.61
Threonine	2.15
Tryptophan	1.50
Tyrosine	1.76
Valine	1.02
Glucose	12.57
Lactate	2.43

Table S5. Mutant strains used in this study. UW strains are from the University of Washington transposon collection (4).

Strain	Genotype and relevant characteristics	Source or Reference
P. aeruginosa PAO1ΔPA2231	*P. aeruginosa* with markerless *pslA* deletion	(5)
P. aeruginosa PAO1ΔPA0934ΔPA5338	*P. aeruginosa* with markerless *relAspoT* deletion	(6)
P. aeruginosa PAO1ΔPA3622	*P. aeruginosa* with markerless *rpoS* deletion	(7)
P. aeruginosa PAO1 IS*lacZ*::PA5200	*P. aeruginosa* with transposon insertion in *amgR*	UW strain PW9752
P. aeruginosa PAO1 IS*phoA*::PA3540	*P. aeruginosa* with transposon insertion in *algD*	UW strain PW6997
P. aeruginosa PAO1 IS*phoA*::PA5100	*P. aeruginosa* with transposon insertion in *hutU*	UW strain PW6916
P. aeruginosa PAO1 IS*phoA*::PA4876	*P. aeruginosa* with transposon insertion in *osmE*	UW strain PW9201
P. aeruginosa PAO1 IS*lacZ*::PA2826	*P. aeruginosa* with transposon insertion in *PA2826*	UW strain PW5733
P. aeruginosa PAO1 IS*phoA*::PA4217	*P. aeruginosa* with transposon insertion in *phzS*	UW strain PW8154
P. aeruginosa PAO1 IS*phoA*::PA4865	*P. aeruginosa* with transposon insertion in *ureA*	UW strain PW9185
P. aeruginosa PAO1 IS*phoA*::PA3064	*P. aeruginosa* with transposon insertion in *pelA*	UW strain PW6141
P. aeruginosa PAO1 IS*lacZ*::PA0927	*P. aeruginosa* with transposon insertion in *ldhA*	UW strain PW2681
P. aeruginosa PAO1 IS*lacZ*::PA0519	*P. aeruginosa* with transposon insertion in *nirS*	UW strain PW1951
P. aeruginosa PAO1 IS*lacZ*::PA2570	*P. aeruginosa* with transposon insertion in *lecA*	UW strain PW5513
P. aeruginosa PAO1 IS*phoA*::PA3361	*P. aeruginosa* with transposon insertion in *lecB*	UW strain PW6664
P. aeruginosa PAO1 IS*lacZ*::PA3478	*P. aeruginosa* with transposon insertion in *rhlB*	UW strain PW6884
P. aeruginosa PAO1 IS*phoA*::PA0524	*P. aeruginosa* with transposon insertion in *norB*	UW strain PW1962
P. aeruginosa PAO1 IS*lacZ*::PA2634	*P. aeruginosa* with transposon insertion in *aceA*	UW strain PW5404

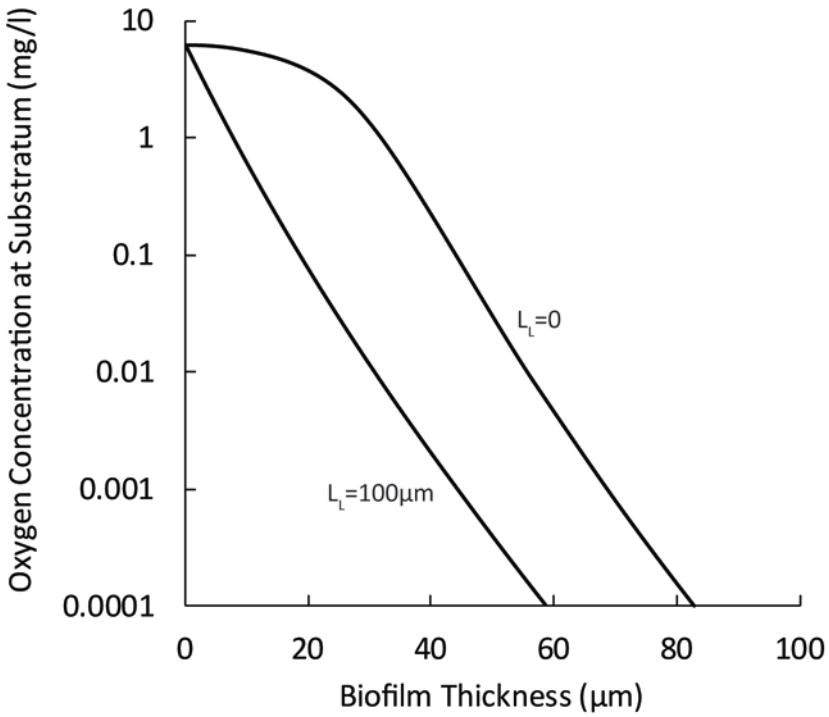

Figure S1. Reaction-diffusion modeling predicts oxygen limitation inside *P. aeruginosa* biofilms. The concentration of oxygen at the base of the biofilm decreases geometrically with increasing biofilm thickness. The parameter L_L denotes the thickness of an external mass transfer fluid layer.

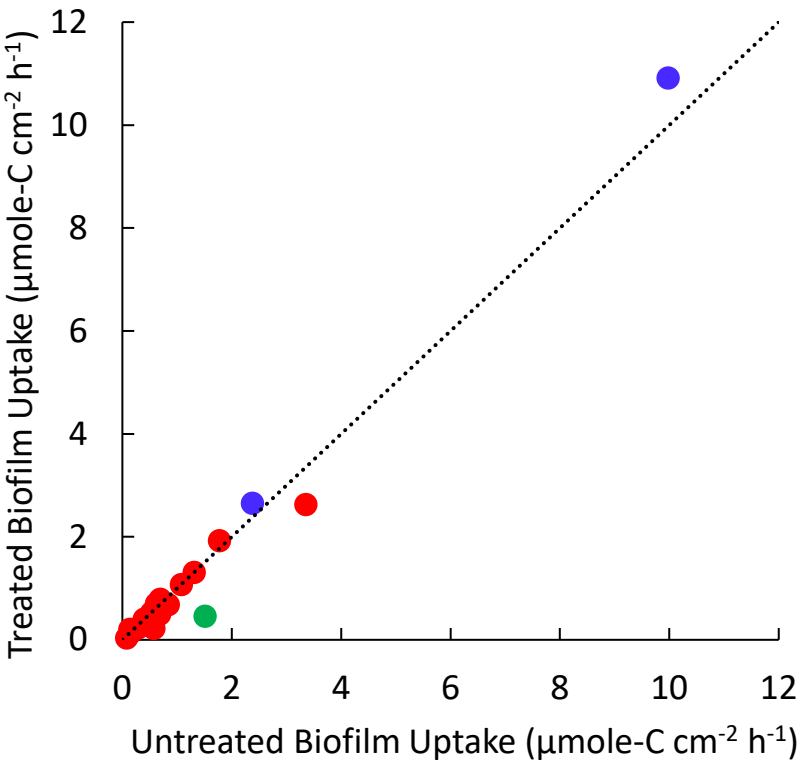

Figure S2. Continued utilization of carbon sources by *P. aeruginosa* biofilms treated with ciprofloxacin for 24 h. Colors denote amino acids (red), lactate and glucose (blue), and the excreted product acetate (green).

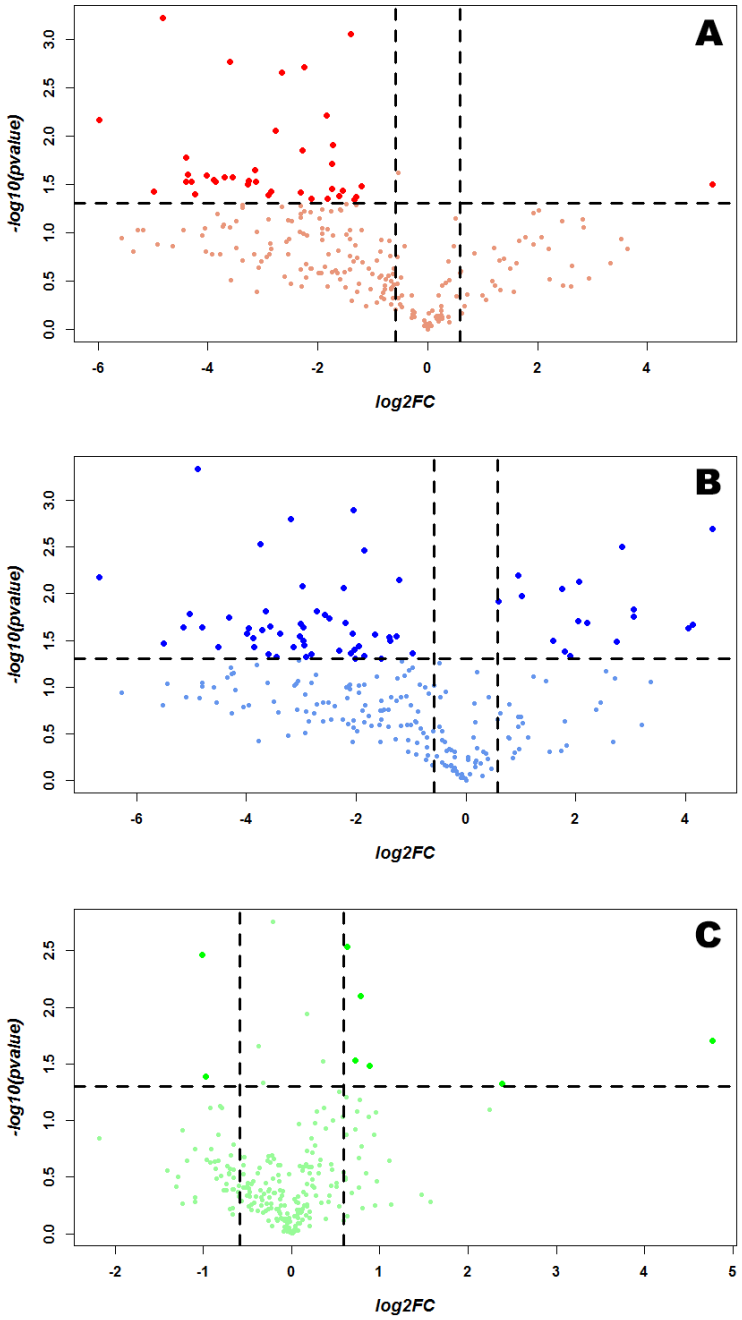

Figure S3. Volcano plot comparing metabolite concentrations between A, untreated biofilm and planktonic; B, treated biofilm and planktonic; C, treated biofilm and untreated biofilm. For each plot, comparisons were made such that a negative fold change correlates to decreased abundance in the untreated biofilm (A), treated biofilm (B), or treated biofilm (C), while a positive fold change indicates increased abundance for the same samples. Shown are metabolites with

tentative identifications. Those that were significantly different (fold change > 1.5, *p*-value <0.05) between the two conditions compared are denoted by larger, brighter circles. Metabolites above the dashed horizontal line have a *p*-value < 0.05, and metabolites to the left or right side of the vertical lines have a fold change > 1.5.

Figure S4. Image of untreated *P. aeruginosa* drip-flow biofilm at 96 h. Arrow indicates the spot at which the medium dripped onto the slide.

Computation of oxygen requirement to synthesize Psl from different carbon sources

We computed the theoretical oxygen requirement to synthesize Psl from each of the carbon sources present in the artificial chronic wound exudate (ACWE) medium (i.e., lactate, glucose and the 20 amino acids). To perform this computation, we used a mathematical method called flux balance analysis (FBA) (8). This method uses linear optimization to assess the capability of a metabolic network to synthesize a metabolic product such as biomass or Psl. In FBA, the metabolic network is represented as a linear system of algebraic equations:

$$\mathbf{S} \cdot \mathbf{v} = \mathbf{0} \quad (1)$$

Where the stoichiometric matrix **S** has dimensions $n \times m$, and **v** is a $m \times 1$ vector representing the metabolic fluxes or rates of the metabolic reactions. The rows of **S** represent the mass balances of each of the n metabolites and each column represents the stoichiometry of each of the m reactions included in the network. Thus, the coefficients of a column of **S** correspond to the stoichiometric coefficients of the metabolites participating in the corresponding reaction. The mass balances represented by Eq. 1 constrain the yield at which a metabolic network can transform substrates into products. In this work, we used a *Pseudomonas aeruginosa* metabolic network model with 584 metabolites and 702 metabolic reactions (9). The metabolic network included all major known pathways of *P. aeruginosa* metabolism such as glycolysis/gluconeogenesis, TCA cycle, pentose phosphate pathway, amino acids synthesis and degradation, nucleic acid synthesis, as well as the Psl synthesis pathway.

To compute oxygen requirement to synthesize Psl from each carbon source in the ACWE, we follow a two-step procedure. First, we solved the following optimization problem to obtain the maximum Psl yield from each carbon source in the ACWE medium:

$$\begin{aligned} &\max_{\mathbf{v}} v_{Psl} \\ &\text{s.t.} \\ &\mathbf{S} \cdot \mathbf{v} = \mathbf{0} \\ &\mathbf{l} \leq \mathbf{v} \leq \mathbf{u} \\ &v_i = 1, \, i \text{ denotes one of the carbon sources} \\ &v_j = 0, j \text{ denotes all carbon sources, except } i \end{aligned} \quad (2)$$

Where v_{Psl} denotes the synthesis rate of Psl, **l** and **u** are the lower and upper bounds of the metabolic fluxes **v**. For a carbon source *i*, its uptake flux is set to 1 (in arbitrary units), while the uptake rates for the rest of the carbon sources are set to 0.

Then, we compute the minimum oxygen uptake required to attain the maximum Psl yield for each carbon source by solving the following optimization problem:

$$\begin{aligned} &\min_{\mathbf{v}} v_{oxy} \\ &\text{s.t.} \\ &\mathbf{S} \cdot \mathbf{v} = \mathbf{0} \\ &\mathbf{l} \leq \mathbf{v} \leq \mathbf{u} \\ &v_i = 1, \, i \text{ denotes one of the carbon sources} \\ &v_j = 0, j \text{ denotes all carbon sources, except } i \\ &v_{Psl} = v_{Psl,i} \end{aligned} \tag{3}$$

Where v_{oxy} denotes the oxygen uptake rate and $v_{Psl,i}$ denotes the maximum Psl yield for carbon source *i*. The oxygen requirement to synthesize Psl from each carbon source is then obtained by the ratio $v_{Psl,i} / v_{oxy}$. The results for each carbon source are provided in Table S4.

References

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