Stress Introduction Rate Alters the Benefit of AcrAB-TolC Efflux Pumps

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ABSTRACT  Stress tolerance studies are typically conducted in an all-or-none fashion. However, in realistic settings—such as in clinical or metabolic engineering applications—cells may encounter stresses at different rates. Therefore, how cells tolerate stress may depend on its rate of appearance. To address this, we studied how the rate of stress introduction affects bacterial stress tolerance by focusing on a key stress response mechanism. Efflux pumps, such as AcrAB-TolC of Escherichia coli, are membrane transporters well known for the ability to export a wide variety of substrates, including antibiotics, signaling molecules, and biofuels. Although efflux pumps improve stress tolerance, pump overexpression can result in a substantial fitness cost to the cells. We hypothesized that the ideal pump expression level would involve a rate-dependent trade-off between the benefit of pumps and the cost of their expression. To test this, we evaluated the benefit of the AcrAB-TolC pump under different rates of stress introduction, including a step, a fast ramp, and a gradual ramp. Using two chemically diverse stresses, the antibiotic chloramphenicol and the jet biofuel precursor pinene, we assessed the benefit provided by the pumps. A mathematical model describing these effects predicted the benefit as a function of the rate of stress introduction. Our findings demonstrate that as the rate of introduction is lowered, stress response mechanisms provide a disproportionate benefit to pump-containing strains, allowing cells to survive beyond the original inhibitory concentrations.

IMPORTANCE  Efflux pumps are ubiquitous in nature and provide stress tolerance in the cells of species ranging from bacteria to mammals. Understanding how pumps provide tolerance has far-reaching implications for diverse fields, from medicine to biotechnology. Here, we investigated how the rate of stressor appearance impacts tolerance. We focused on two distinct substrates of AcrAB-TolC efflux pumps, the antibiotic chloramphenicol and the biofuel precursor pinene. Interestingly, tolerance is highly dependent on the rate of stress introduction. Therefore, it is important to consider not only the total quantity of a stressor but also the rate at which it is applied. The implications of this work are significant because environments are rarely static; antibiotic concentrations change during dosing, and metabolic engineering processes change with time.

KEYWORDS  antibiotics, biofuels, dynamic environment, efflux pumps, stress tolerance

Under realistic conditions, the environments bacteria are exposed to are seldom as constant as those in the laboratory. For example, in clinical applications, antibiotic concentrations at the site of infection will depend on in vivo drug absorption and elimination (1). In metabolic engineering, the synthesis of a product such as a biofuel can depend heavily on the cell cycle or stage of the production process and thus change dramatically with time (2). Studying how bacteria respond to dynamic, stressful environments is fundamental to a wide range of fields. Although recent literature has begun to explore the effect of fluctuating environments on bacterial fitness, the focus...
has primarily remained on step changes, such as switching suddenly from a nonstressful to a stressful environment (3–7). Other studies have focused on the long-term effects of changing environments, including the impact of spatial gradients on mutations and the response of a general stress response pathway to environmental change (8–10). In contrast, in this study, we investigated how varying the rate at which stress is applied over short, key periods of time affects fitness.

To survive in stressful environments, cells utilize numerous stress response mechanisms, including efflux pumps, inactivating enzymes, and regulation of outer membrane protein channels (11–13). However, despite the substantial growth advantage these mechanisms can provide under stress, they can also be costly and thus place an extraneous burden on the cell (14, 15). Therefore, expression of stress response genes may introduce negative fitness effects (16). Understanding how cells balance these cost-benefit trade-offs will provide insight into how cells respond and cope with stressful environments.

As a case study, we focused on a well-known multidrug resistance pump, AcrAB-TolC of *Escherichia coli*. Multidrug resistance pumps have been studied extensively for the ability to export a wide variety of compounds, including antibiotics, biofuels, signaling molecules, dyes, and detergents (17, 18). The pumps maintain low intracellular concentrations of stressors through active efflux via the proton motive force (19–21). These membrane transporters are found across prokaryotic and eukaryotic species (22). For instance, in eukaryotic cells, efflux pumps present a significant hurdle for cancer therapy as they provide resistance to anticancer drugs (23). In prokaryotic cells, efflux pumps increase the antibiotic dose required for the treatment of infections and also play a role in quorum sensing and biofilm formation (18, 24). Along with their clinical relevance, efflux pumps offer potential as a metabolic engineering tool. For instance, efflux pumps are able to improve the fitness and solvent tolerance of cells with engineered biofuel production pathways (25–28). Thus, efflux pumps are a significant stress tolerance mechanism that operates on a diverse range of substrates.

In this work, we investigated how the trade-off between the benefit of the pumps and the cost of pump expression depends on the rate of stress introduction. By analogy, consider a bilge pump on a boat. If water leaks slowly into the boat, the pump can keep up and the boat will stay afloat. However, if the same volume of water appears rapidly, the boat may sink. We asked whether stress tolerance has a similar rate-dependent effect. To study this, we evaluated the benefit of the AcrAB-TolC pump under time-varying stress environments. We assessed the performance of cells with and without pumps when the stressors were presented in different forms—a step, a fast ramp, and a gradual ramp. Our overall goal was to quantitatively determine the trade-off between stress tolerance and the growth advantage for cells with pumps. To achieve this, we cocultured cells with and without AcrAB-TolC efflux pumps. The relative fractions of cells with and without the pumps changed with time and depended on the rate of stress introduction. We validated our results by using two structurally distinct pump substrates, the antibiotic chloramphenicol and the jet biofuel precursor pinene. We developed and experimentally validated a mathematical model that captures the system’s behavior. Using this model to evaluate the cost-benefit landscape of pump expression, we found that lower rates of stress introduction exaggerate the benefit of the pumps. This work demonstrates that the rate at which stress is applied can have an important impact on bacterial fitness.

RESULTS

We began by quantifying the benefit and cost of expressing efflux pumps in an environment with a constant, unchanging level of stress. We initially used chloramphenicol as a stressor because it is often considered for the treatment of bacterial infections (29, 30). It is a bacteriostatic agent that works by inhibiting protein synthesis (31). Chloramphenicol is a known substrate of the AcrAB-TolC pump; the pump conveys a 5-fold increase in the MIC (32). To measure the benefit of pumps, we initially grew cells with and without *acrB* in different constant levels of chloramphenicol. Since
the AcrB protein is the active pumping unit and produces efflux driven by the proton motive force, deleting acrB renders the entire AcrAB-TolC efflux pump nonfunctional (19). We conducted experiments with wild-type E. coli and with the same strain with an acrB deletion and confirmed that the efflux pump provides protection against chloramphenicol (Fig. 1A). We were able to recover chloramphenicol tolerance by complementing ΔacrB mutant cells with a plasmid containing an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible version of the acrAB operon, acrAB-sfgfp. Even without induction, the basal expression was sufficient to restore wild-type levels of chloramphenicol tolerance. Therefore, the AcrAB-TolC efflux pump system does impose a significant fitness cost when overexpressed.

Next, we asked whether there is a cost associated with expressing these pumps. Although it is known that overexpression of membrane proteins can be costly to cells (33–35), the mechanisms behind the fitness cost of efflux pumps are not entirely clear (35). One potential mechanism is a change in the intracellular pH that impacts cellular metabolic pathways (36). When inducing the acrAB-sfgfp mutant strain with IPTG, we found that at high induction levels there was a severe reduction in growth, indicative of the harmful effects of overexpression (Fig. 1B). As a result, we conducted subsequent experiments without IPTG induction to balance the benefit of chloramphenicol tolerance against the cost of the pumps. Furthermore, we concluded that the AcrAB-TolC efflux pump system does impose a significant fitness cost when overexpressed.

To determine whether the benefit and cost of efflux pumps change in dynamic stress environments, we competed strains with and without pumps against each other and recorded the relative proportions of the species in the cocultured population over time under different antibiotic treatment conditions. In clinical settings, bacteria that contain efflux pumps are able to outcompete those without them and are found at a higher frequency in clinical isolates (37), motivating our use of a competition assay. As the strains are required to compete for limited resources, this assay can identify subtle differences in growth among strains because more fit strains become overrepresented in the population (6, 26).
We began by competing strains with and without efflux pumps in a constant environment where we added antibiotics at $t = 0$ h. First, we conducted a control experiment with two $\Delta acrB$ mutant strains, one harboring a plasmid encoding superfolder green fluorescent protein (sfGFP) and a second with a plasmid encoding red fluorescent protein (RFP) (Fig. 1C). We first measured the optical density (OD) of the cocultured competing strains (see Fig. S1A in the supplemental material). The fluorescent reporters allowed us to quantify the fraction of each cell present in the coculture over time by flow cytometry (Fig. S1B). Consequently, we were able to quantify the relative proportions of the two competing strains by using the fraction of sorted cells containing $rpf$ or $sfgfp$ to estimate the fraction of the total population harboring each plasmid (Fig. S1C and D). We recorded the cumulative cell density and the proportions of the two competing strains in the coculture as a function of time.

As expected, the $sfgfp$ and $rpf$ strains performed similarly under all levels of antibiotics since the only difference between the strains was the color of the fluorescent reporter. Next, we competed a $\Delta acrB$ mutant strain complemented with $acrAB$-$sfgfp$ against the same strain with $rpf$ alone. We found that in the absence of antibiotics, the strain without pumps outperformed the strain with pumps (Fig. 1C). Because efflux pumps are costly and unnecessary under conditions without antibiotics, the strain with no pumps was able to dominate. In contrast, under conditions with low doses of chloramphenicol, the efflux pump-containing strain dominated. Beyond a certain concentration of antibiotic, neither strain was able to survive. These results are consistent with our earlier findings that the benefit of the pumps exists only for certain antibiotic doses (Fig. 1A).

To explore the effect of antibiotic addition and the benefit of pumps, we developed a mathematical model by using a system of coupled ordinary differential equations to describe the competition between the species. The model is based on the bacterial growth model of Van Impe et al., which builds upon the Monod equation for growth kinetics (38–40). The state variables describe the population size of each of the species and a substrate that is consumed by both species. The growth rate of each population depends upon the available substrate and also the concentration of the antibiotic in the environment. The model parameters were estimated by minimizing the sum of squared residuals between the model and experimental data for the growth and toxicity curves for the single species (Fig. S2 and Table S1). The model shows good agreement with the trends in our experimental findings, both in the overall growth of the two species and in the approximate proportion of each species in the culture.

To visualize the relative effect of efflux pumps, we plotted the data from the $sfgfp$ strain alongside those from the $acrAB$-$sfgfp$ strain (Fig. 1D). These data are extracted from the coculture experiments shown in Fig. 1C, where the $sfgfp$ strain is competed against the $rpf$ strain (top) and the $acrAB$-$sfgfp$ strain is competed against the $rpf$ strain (bottom). This comparison allows us to directly highlight the growth differences across environments and strains without and with efflux pumps. The model captures these trade-offs, demonstrating its predictive power in estimating where strains outcompete each other under competitive growth conditions.

Next, we asked how differences in the rate of antibiotic addition affected the cost and benefit trade-offs for efflux pump expression. We tested dynamic environments where antibiotics were applied at different rates during the exponential growth phase. We kept the cumulative amount of antibiotic added constant but varied the ramp rate (Fig. 2A to C). We first considered a step increase in antibiotics at $t = 3$ h (Fig. 2A and D). Under these conditions, the cells grew rapidly prior to the addition of antibiotics, with the $sfgfp$ strain outperforming the $acrAB$-$sfgfp$ strain prior to $t = 3$ h, making it difficult for the $acrAB$-$sfgfp$ strain to recover after the antibiotic was added, even under conditions where the pumps offer an advantage.

When we decreased the rate of chloramphenicol addition, the $acrAB$-$sfgfp$ strain was able to outperform the $sfgfp$ strain under a broader range of chloramphenicol concentrations. First, we spaced the addition of chloramphenicol out over the range of $t = 2$ to $4$ h (Fig. 2B and E). As predicted by the mathematical model, at intermediate
chloramphenicol concentrations, we observed a modest benefit of the pumps. For the lowest antibiotic addition rate, we added chloramphenicol from $t_{1/2} = 0.5$ to 5.5 h (Fig. 2C and F). In this case, we found a more dramatic increase in the benefit of the pumps. In particular, we observed a substantial benefit in fitness for efflux pump-containing strains that exists well above the MIC of 1 $\mu$g/ml (Fig. 1A and B). This finding emphasizes the importance of the rate at which stresses are introduced.

Building on the success of our model predictions, we next used the model to quantify the benefits and costs of efflux pump expression as a function of the total amount of antibiotic added and the rate at which it is introduced. To quantify the growth advantage provided by the efflux pumps, we calculated the “benefit ratio” provided by the pumps, which we defined as the ratio of the biomass of the acrAB-sfgfp strain to the biomass of the sfgfp strain after 8 h (41). As a result, a benefit ratio of $>1$ means that strains with efflux pumps are able to outcompete cells without efflux pumps, while a value of $<1$ means that pump expression hinders growth. Using our model, we calculated the benefit ratio across a range of chloramphenicol introduction rates and total antibiotic amounts (Fig. 3A). At very low concentrations of chloramphenicol, pumps are unnecessary and there is a cost to their expression, so the benefit ratio is $<1$, regardless of the rate of introduction. At very high concentrations, neither strain can grow, so the benefit ratio is $\sim 1$ for all introduction rates. Meanwhile, at intermediate chloramphenicol concentrations, we observed dramatic rate-dependent differences between the strains. When the stress appears slowly, the strains with the pumps are at a significant advantage. In fact, this phenomenon can result under conditions where bacteria are able to survive antibiotic doses well beyond those they can tolerate with rapid drug introduction. This benefit is likely due to the ability of bacteria to maintain low intracellular levels of antibiotics by using efflux pumps when undergoing slow antibiotic introduction. Therefore, the rate at which an antibiotic or stressor is added will have a critical impact on bacterial survival.

To verify the model predictions, we calculated the benefit ratio from the experimentally measured data from Fig. 2D to F by evaluating the ratio of acrAB-sfgfp to sfgfp strain biomasses under the same antibiotic treatment profiles. When the rate of

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**FIG 2** The rate of chloramphenicol addition affects survival. (A to C) Three different rates of chloramphenicol introduction, a step (A), a fast ramp (B), and a gradual ramp (C), are shown. The thick solid line shows the values used in the mathematical model; the thin solid line shows the experimental treatment used to approximate the constant introduction rate. The total amounts of chloramphenicol added in panels A to C are equal. (D to F) Competitive growth under different rates of chloramphenicol addition. The growth of the acrAB-sfgfp strain is compared to the growth of the sfgfp strain in the competition experiments (dots), and model predictions (solid lines) for different chloramphenicol introduction rates as shown in panels A to C, respectively, are shown. As in Fig. 1D, these data were extracted from competition experiment data. Note that dead cells can still cloud the solution; therefore, nonzero ODs do not necessarily imply that cells are alive. Data points show mean values and standard deviations of three biological replicates.
introduction is a step, cells with efflux pumps have a negligible benefit (Fig. 3B); as the introduction rate is lowered, the benefit of the pumps slightly increases at intermediate chloramphenicol concentrations (Fig. 3C) and lowering the rate provides an even greater benefit (Fig. 3D). We note that the model was fitted to raw data from toxicity curves and growth measurements obtained without antibiotics (Fig. S2). Without further fitting, the model is able to predict trends in the benefit of the efflux pumps given different rates of stress introduction. Statistical analysis suggests that the model agrees well with the data on the basis of an F test assessing goodness of fit (Table S2). Additionally, we performed experiments where the initial biomass was an order of magnitude lower than under the original conditions (Fig. S3). The data show good qualitative agreement with the model predictions, where slow antibiotic introduction results in a greater benefit of pumps. These results indicate that our findings are not specific to one set of initial conditions.

We next asked whether our findings on the rate-dependent benefit of efflux pumps would generalize to other stressors. To do this, we conducted experiments with a structurally and functionally dissimilar efflux pump substrate. Pinene is a jet biofuel precursor that can be synthesized by *E. coli*; however, pinene is also toxic to the cells (42). The AcrAB-TolC efflux pump is known to increase tolerance to pinene and other solvents (26, 43). We first measured the benefit of the pumps and, as expected, observed an increase in pinene tolerance in strains with the efflux pump (Fig. 4A). We next measured the cost of pump expression in the presence of pinene by using the IPTG-inducible *acrAB-sfgfp* strain (Fig. 4B). As *acrAB-sfgfp* is induced, there is an impact on cell growth. However, low levels of induction do convey a slight benefit compared to basal levels (Fig. S4); therefore, we conducted the subsequent experiments with

![FIG 3 Model predictions and experiments measuring the benefit of pumps. (A) Contour plot of the benefit ratio of efflux pumps. Model predictions for biomass, *N*, of an *acrB*-containing strain in relation to that of a Δ*acrB* mutant control strain after 8 h are used to predict the benefit ratio landscape. The plot shows results for different maximum levels of chloramphenicol and different rates of chloramphenicol addition. (B to D) Experimental results showing the benefit of efflux pumps compared to model predictions. Data are ratios of the biomasses of the *acrAB-sfgfp* and *sfgfp* strains after 8 h. The rates of antibiotic introduction are shown in Fig. 2A to C, respectively, and denoted by the white dashed lines on the contour plot in panel A. Error bars show the standard deviations of three biological replicates.](image-url)
H9262 IPTG, as this induction level best mirrors the wild type in the presence of pinene (Fig. 4A). The cost-benefit characteristics of pinene closely mirror the trade-offs that we observed for chloramphenicol in a constant environment.

To accurately capture the effect of pinene, we modified our mathematical model to include a term that allows for cell lysis. Chloramphenicol is bacteriostatic, so OD measurements remain roughly constant after the cells have died (44). In contrast, we observed decreases in OD following pinene treatment (Fig. S5). To accommodate the bactericidal effect of pinene, we adjusted our model to include a term describing this effect. We simulated the trade-off landscape for different rates of pinene addition (Fig. 4C) and observed a general trend where, as with chloramphenicol, the benefit ratio is highest at intermediate levels of pinene stress when the rate of introduction is low. However, the peak for pinene is taller, as the efflux pumps convey an even larger benefit under pinene stress.

We next used the model to select pinene rates that show low, moderate, and high benefit ratios and conducted competition experiments under these conditions (Fig. S6). Extracting these data, the experimental and computational results demonstrate that a dramatic benefit is conveyed at low introduction rates (Fig. 4D to F). Statistical analysis suggests a reasonable goodness of fit between the theoretical model and pinene experimental data (Table S2). We observed that cells with pumps can survive significantly higher levels of pinene when it is added slowly than they can when it is added all at once.

**DISCUSSION**

In this study, we focused on the rate-dependent nature of the benefit of efflux pumps, which is significant given the role pumps play across diverse fields. Our work here extends to both understanding antibiotic tolerance and potential applications in biosynthetic processes. By studying two unique substrates of the AcrAB-TolC pump, we
were able to validate that with slow introduction of stress, pumps provide a disproportionate benefit that is not unique to the individual substrate. Understanding complex strategies bacteria employ to tolerate stress can provide insight into the development of therapeutic techniques and can enable us to exploit these effects in biochemical engineering. By determining conditions where efflux pump-containing strains lack a competitive edge, we can identify domains where antibiotic tolerance is reduced. For metabolic engineering applications, this optimization can help characterize and improve yields of biosynthetic compounds such as biofuels (45, 46).

Under realistic conditions, cells are rarely exposed to environments as constant as those in laboratory experiments. Since the environment can have a large impact on how bacteria respond to stress, it is important to study the behavior of cells under time-varying conditions. These ideas have been explored previously in the context of extended exposure to stress and through temporal and spatial gradients. In spatially distinct environments, studies have demonstrated that prolonged exposure to a stressful spatial barrier can be overcome by cells adapting to the stress through tolerance and then resistant mutants (9). Similarly, graded increases in antibiotic concentrations across several days can lead to mutations (10). Thus, even a subtle benefit in fitness on a short-term scale can result in mutant daughter cells in stressful environments. Additionally, on a shorter time scale, stress response pathways have been shown to depend on the rate of environmental change. For example, Bacillus subtilis turns on stress-specific or general stress response pathways, depending on the rate at which stress is applied (8). By studying time-varying stress, we can better understand how stress response mechanisms operate in realistic environments.

In this work, we have demonstrated that the benefit of efflux pumps depends heavily on the rate of stress introduction. We found that strains exposed to stress at lower rates were able to tolerate cumulative concentrations well beyond what they could survive if the stress appeared suddenly. We also confirmed this through mathematical modeling; fits to data where the stressor was added all at once allowed us to accurately predict the benefit that pumps confer under different stress introduction rates. We found that efflux pumps provide a disproportionate benefit when the rate of stress introduction is low.

MATERIALS AND METHODS

Strains and plasmids. We used E. coli strains BW25113 and BW25113 ΔacrB. Wild-type strain BW25113 is the parent strain of the Keio collection (47). BW25113 ΔacrB was derived from Keio collection strain JW0451 (BW25113 ΔacrB::kan), where we removed the kanamycin resistance marker in accordance with the pCP20 protocol in reference 48.

We used plasmids pBBASK-rfp, pBBAsk-sfgfp, and pBBAsk-acrAB-sfgfp in experiments. Plasmid pBBAsk-rfp is from the BglBricks library (49, 50). The pBBASK vector contains a medium-copy-number (p15A) origin of replication, a P promoters, and a kanamycin resistance marker. pBBAsk-sfgfp was constructed by using the pBBASK vector and sfgfp from pBBsfk-sfgfp (51). Plasmid pBBASK-acrAB-sfgfp is a transcriptional fusion of acrAB and sfgfp. We constructed it by using the pBBAsk vector and sfgfp from pBBsfk-sfgfp (51). Plasmid pBBASK-acrAB-sfgfp contains the ribosome binding site of sfgfp from pBBsfk-sfgfp (51) in the cloning process. For all constructs, we used the Gibson assembly method and verified results by sequencing (52). The primers for all constructs are listed in Table S3 in the supplemental material. Plasmids were transformed into E. coli BW25113 ΔacrB and isolated on Luria broth (LB) plates with kanamycin (30 μg/ml).

Bacterial growth conditions. For all experiments, overnight cultures were inoculated from a single colony in 5 ml of LB with 30 μg/ml kanamycin, where necessary. Overnight cultures were then grown at 37°C with orbital shaking at 200 rpm. Following this, precultures were prepared by diluting the overnight culture 1:50 in LB with 30 μg/ml kanamycin, where necessary. The precultures were grown at 37°C with orbital shaking at 200 rpm for 2 h and then diluted back to an OD at 700 nm (OD700) of approximately 0.2. We used OD700 to minimize overlap with the RFP emission spectrum (53, 54).

For toxicity curves of the individual species and single-species growth parameters, 800-μl volumes of these cultures were aliquoted into 24-well plates and chloramphenicol, IPTG, or pinene was added as described below.

For the competition experiments, cocultures were created by mixing 400 μl each of the two competing strains, the acrAB-sfgfp and rfp strains or the sfgfp and rfp strains, after individually diluting the strains back to an OD700 of 0.2 as described above. As a result, there was a total of 800 μl per well of a 24-well plate with a final OD700 of 0.2. For the competition experiments starting at the lower OD in Fig. 5, the cocultures were created by mixing 400 μl each of the two competing strains after they were diluted back to an OD700 of 0.01.
Toxicity experiments. To determine the toxicity of chloramphenicol, we added a final concentration of 0.01, 0.02, 0.5, 1, 2, 5, or 10 µg/ml to each culture. To evaluate the benefit of pump expression, P_{pump} expression was induced with 0, 1, 10, or 100 µM IPTG. The samples were sealed with evaporation-limiting membranes (Thermo Scientific AB-0580) and grown in 24-well plates at 37°C with orbital shaking at 200 rpm. OD_{600} readings were taken before incubation (t = 0 h) and after antibiotic exposure (t = 24 h). All experiments were performed in triplicate by using biological replicates.

Mirroring the chloramphenicol toxicity experiments, pinene (α-pinene; Sigma-Aldrich product no. P45680) was added to each culture to a final concentration of 0, 0.1, 0.2, 0.5, 1, or 2% (vol/vol). To evaluate the benefit of pump expression, P_{pump} expression was induced with 0, 1, 5, 10, 50, or 100 µM IPTG. OD_{600} readings were taken before incubation (t = 0 h) and after the end of the exponential growth phase (t = 8 h).

Competition experiments. The cocultures were treated with increasing concentrations of chloramphenicol or pinene as shown in Fig. 2A to C. The OD_{600} was measured at intervals, every hour for chloramphenicol and every other hour for pinene, through the exponential growth phase. In addition, after each OD_{600} reading, 15-µl samples of each culture were diluted 1:10 in phosphate-buffered saline and measured with a Guava easyCyte HT sampling flow cytometer. The excitation and emission wavelengths were 485 and 510 nm for sfGFP and 555 and 584 nm for RFP fluorescence channels (55, 56).

Flow cytometry data were collected as FCS 3.0 files and analyzed with custom Matlab scripts. To avoid cross talk between the red and green channels, control experiments with single-color strains were performed to identify a threshold for classifying a cell as containing sfGFP or RFP during postprocessing. The same thresholds were applied for all experiments.

Mathematical model. To fit the growth of single strains under different environmental conditions, we used a single-species model for predicting biomass N (equation 1) and substrate availability S (equation 2) based on the cell growth model of Van Impe et al. (25, 40, 57). This model incorporates environmental conditions such as a substrate-limiting term based on the physiological environment. For the version presented here, we include a term describing the effect of a stressor, E (38).

The single-species model is defined as follows:

\[
\frac{dN}{dt} = \alpha N_{\text{max}} \left( \frac{S}{K_s + S} \right) \left( \frac{1}{1 + \frac{E}{R}} \right) N(t) \quad (1)
\]

\[
\frac{dS}{dt} = -1 \mu N_{\text{max}} \left( \frac{S}{K_s + S} \right) N(t) \quad (2)
\]

The maximum growth rate is \( N_{\text{max}} \), the growth yield provided by the substrate is \( \gamma \), and the half-saturation constant is \( K_s \). \( \alpha \) is a normalizing term that converts biomass to OD. The parameters for these models were selected by using a least-squares regression minimizing the sum of the residuals for the best fits to the growth curves and the toxicity curves. The coefficients from the models were fitted simultaneously. The values for \( N_{\text{max}}, \gamma, \) and \( K_s \) were selected on the basis of the growth curves of individual strains (Fig. S2A and B). Parameter values are listed in Table S4. Additionally, we added a stressor term to adjust the growth on the basis of the effect of a given stressor concentration \( E \) at time \( t \), where

\[
E_{\text{fast ramp}} = \begin{cases} 0 & t < 3 \\ 1 & t \geq 3 \end{cases} \quad (3)
\]

\[
E_{\text{gradual ramp}} = \frac{t - 1.5}{3} \quad 1.5 \leq t < 4.5 \\
1 \quad t \geq 4.5 \quad (4)
\]

\[
E_{\text{gradual ramp}} = \begin{cases} 0 & t < 0 \\ t/6 & 0 \leq t < 6 \\ 1 & t \geq 6 \end{cases} \quad (5)
\]

The Hill coefficient \( n \) and repression coefficient \( R \) were fitted to the species’ toxicity curve (Fig. S2C) as follows:

\[
V_i(t) = \frac{1}{1 + \frac{E}{R}} \quad (6)
\]

The single-species model was extended to a multispecies model based upon reference 39 that models the growth of two species, \( N_1 \) (equation 7) and \( N_2 \) (equation 8). We extended the multispecies model in a fashion similar to the adaption of the Monod model above to include the limiting term of substrate availability \( S \) (equation 9). We used two different multispecies models, one for bacteriostatic stressors such as chloramphenicol, which stop cells from growing, and one for bactericidal stressors such as pinene, which cause cell lysis (58).

The multispecies bacteriostatic model is defined as follows:
In addition, it has been used to assess the fit of models for bacterial since the effects of nonlinearity have been shown to be negligible when comparing models of the same F-test statistics. There is growing support for the F test over other alternatives for nonlinear regression and average errors for the model sets. In addition, we evaluated the goodness of fit by calculating the sum of squared residuals to estimate the relative precision of the model, along with the maximum to the growth and toxicity curve data, see Table S1.

For the multispecies bacteriostatic model, the growth yield provided by the substrate and the half-saturation constant were fitted by using the growth curves of a coculture of the two strains with equal initial biomasses. The maximum growth rates of the individual species were derived from the individual growth curves, and the coefficients for the antibiotic terms were fitted to individual species’ toxicity curves. For additional information on the accuracy of model fits to the growth and toxicity curve data, see Table S1.

The multispecies bactericidal model is defined as follows:

\[
\frac{dN_1}{dt} = 2\alpha \mu_{\text{max},1} \left( \frac{S}{K_s + S} \right) V_1(t) N_1(t) - \frac{1}{2\alpha} \left[ 1 - V_1(t) \right] N_1(t) \\
\frac{dN_2}{dt} = 2\alpha \mu_{\text{max},2} \left( \frac{S}{K_s + S} \right) V_2(t) N_2(t) - \frac{1}{2\alpha} \left[ 1 - V_2(t) \right] N_2(t) \\
\frac{dS}{dt} = -\frac{1}{\gamma} \left( \frac{S}{K_s + S} \right) \left[ \mu_{\text{max},1} N_1(t) + \mu_{\text{max},2} N_2(t) \right]
\]  

The parameters of the bactericidal multispecies model were fitted as described above. We calculated the sum of squared residuals to estimate the relative precision of the model, along with the maximum and average errors for the model sets. In addition, we evaluated the goodness of fit by calculating the F-test statistics. There is growing support for the F-test over other alternatives for nonlinear regression since the effects of nonlinearity have been shown to be negligible when comparing models of the same type (i.e., the same order) (59). In addition, it has been used to assess the fit of models for bacterial growth curves, including logistic and Gompertz models (60).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00525-17.

SUPPLEMENTAL FILE 1, PDF file, 2.8 MB.

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